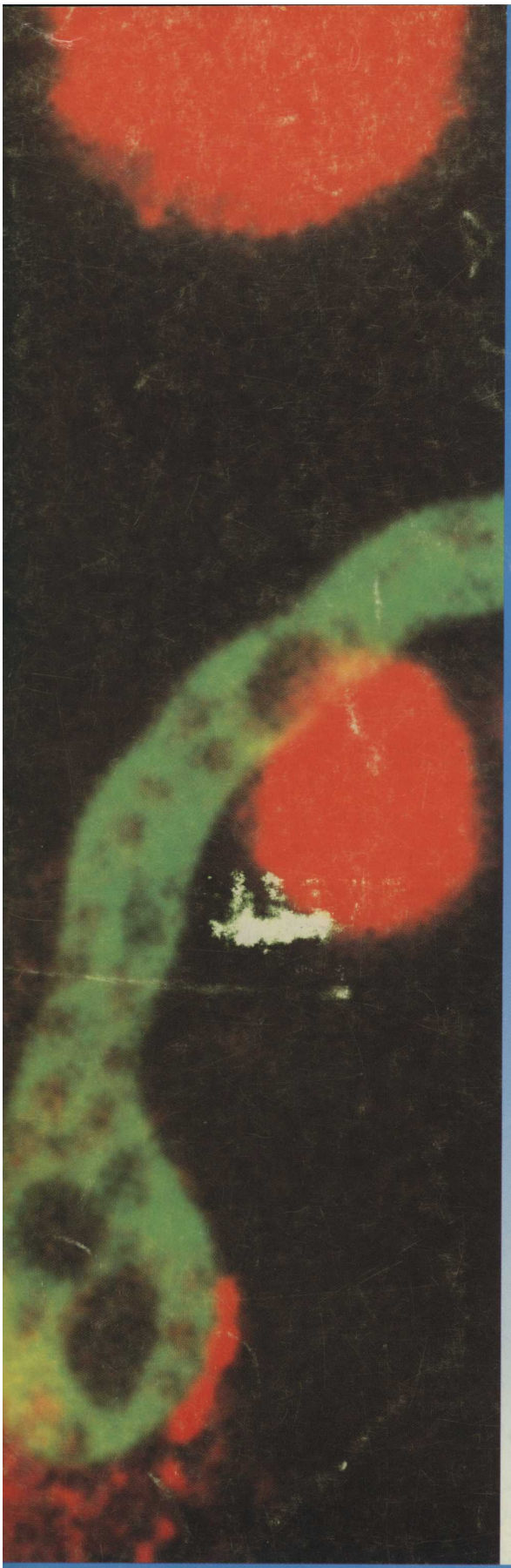
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Aims and Scope

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Cover illustration:
The cover picture shows the transgenic plant pathogenic oomycete *Phytophthora palmivora* producing the green fluorescent protein (GFP). GFP localization shows part of a germinated cyst with an appressorium. From FEMS Microbiol. Lett. 178 (1999) 71–80.

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Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*

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Abstract

During the past 15 years, the methylotrophic yeast *Pichia pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins. The increasing popularity of this particular expression system can be attributed to several factors, most importantly: (1) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most well-characterized experimental systems in modern biology; (2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; (3) the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing; and (4) the availability of the expression system as a commercially available kit. In this paper, we review the *P. pastoris* expression system: how it was developed, how it works, and what proteins have been produced. We also describe new promoters and auxotrophic marker/host strain combinations which extend the usefulness of the system. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Foreign gene expression; Heterologous protein production; Methylotrophic yeast; *Pichia pastoris*; Alcohol oxidase 1 gene promoter; Protein secretion

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1. Introduction

1.1. Pichia pastoris as an experimental organism

Thirty years ago, Koichi Ogata first described the ability of certain yeast species to utilize methanol as a sole source of carbon and energy [1]. The methylotrophs attracted immediate attention as potential sources of single-cell protein (SCP) to be marketed primarily as high-protein animal feed. During the 1970s, Phillips Petroleum Company developed media and protocols for growing *Pichia pastoris* on methanol in continuous culture at high cell densities (>130 g l^{-1} dry cell weight, Fig. 1) [2]. Unfortunately, the oil crisis of the 1970s caused a dramatic increase in the cost of methane. Concomitantly, the price of soybeans, the major alternative source of animal feed, fell. As a result, the economics of SCP production from methanol were never favorable.

In the following decade, Phillips Petroleum contracted with the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA) to develop *P. pastoris* as an organism for heterologous protein expression. Researchers at SIBIA isolated the gene and promoter for alcohol oxidase, and generated vectors, strains, and corresponding protocols for the molecular genetic manipulation of *P. pastoris*. The combination of the fermentation methods developed for the SCP process and the alcohol oxidase promoter's strong, regulated expression effected surprisingly high levels of foreign protein expression. In 1993, Phillips Petroleum sold its *P. pastoris* expression system patent position to Research Corporation Technologies (Tucson, AZ), the current patent holder. In addition, Phillips Petroleum licensed Invitrogen Corporation (Carlsbad, CA) to sell components of the system, an arrangement that continues under Research Corporation Technologies.

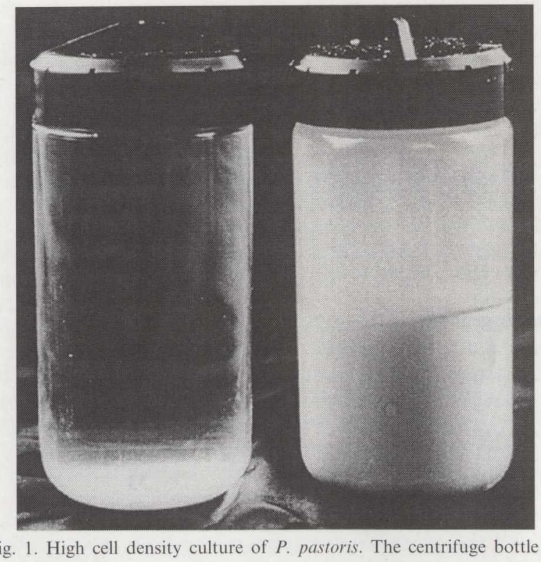


Fig. 1. High cell density culture of *P. pastoris*. The centrifuge bottle on the left shows a *P. pastoris* culture grown in a flask to a density of 1 OD_{600} unit. The bottle on the right contains a sample of the strain grown in a fermenter to a density of 130 g l^{-1} dry cell weight (~ 500 OD_{600} units).

1.2. Methanol metabolism

The conceptual basis for the *P. pastoris* expression system stems from the observation that some of the enzymes required for methanol metabolism are present at substantial levels only when cells are grown on methanol [3,4]. Biochemical studies showed that methanol utilization requires a novel metabolic pathway involving several unique enzymes [3]. The enzyme alcohol oxidase (AOX) catalyzes the first step in the methanol utilization pathway, the oxidation of methanol to formaldehyde and hydrogen peroxide (Fig. 2). AOX is sequestered within the peroxisome along with catalase, which degrades hydrogen peroxide to oxygen and water. A portion of the formaldehyde generated by AOX leaves the peroxisome and is further oxidized to formate and carbon dioxide by two cytoplasmic dehydrogenases, reactions that are a source of energy for cells growing on methanol.

The remaining formaldehyde is assimilated to form cellular constituents by a cyclic pathway that starts with the condensation of formaldehyde with xylulose 5-monophosphate, a reaction catalyzed by a third peroxisomal enzyme dihydroxyacetone synthase (DHAS). The products of this reaction, glyceraldehyde 3-phosphate and dihydroxyacetone, leave the peroxisome and enter a cytoplasmic pathway that regenerates xylulose 5-monophosphate and, for every three cycles, one net molecule of glyceraldehyde 3-phosphate. Two of the methanol pathway enzymes, AOX and DHAS, are present at high levels in cells grown on methanol but are not detectable in cells grown on most other carbon sources (e.g., glucose, glycerol, or ethanol). In cells fed methanol at growth-limiting rates in fermenter cultures, AOX levels are dramatically induced, constituting $>30\%$ of total soluble protein [5,6].

1.3. AOX1 promoter

There are two genes that encode alcohol oxidase in *P. pastoris*: *AOX1* and *AOX2*; *AOX1* is responsible for a vast majority of alcohol oxidase activity in the cell [7–9].

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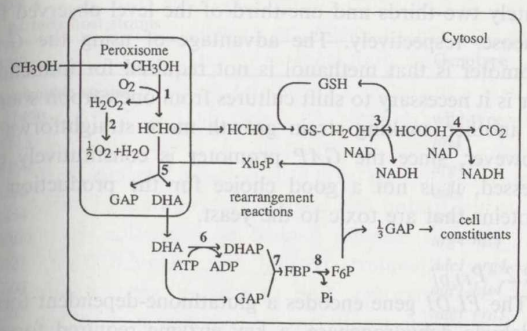


Fig. 2. The methanol pathway in *P. pastoris*. 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase.

Expression of the *AOX1* gene is controlled at the level of transcription [7–9]. In methanol-grown cells, ~5% of poly(A)$^+$ RNA is from *AOX1*; however, in cells grown on most other carbon sources, *AOX1* message is undetectable [10]. The regulation of the *AOX1* gene appears to involve two mechanisms: a repression/derepression mechanism plus an induction mechanism, similar to the regulation of the *Saccharomyces cerevisiae GAL1* gene. Unlike *GAL1* regulation, the absence of a repressing carbon source, such as glucose in the medium, does not result in substantial transcription of *AOX1*. The presence of methanol is essential to induce high levels of transcription [7].

1.4. Molecular genetic manipulation

Techniques required for the molecular genetic manipulation of *P. pastoris*, such as DNA-mediated transformation, gene targeting, gene replacement, and cloning by functional complementation, are similar to those described for *S. cerevisiae*. *P. pastoris* can be transformed by electroporation, a spheroplast generation method, or whole cell methods such as those involving lithium chloride and polyethylene glycol$_{1000}$ [11–14]. As in *S. cerevisiae*, *P. pastoris* exhibits a propensity for homologous recombination between genomic and artificially introduced DNAs. Cleavage of a *P. pastoris* vector within a sequence shared by the host genome stimulates homologous recombination events that efficiently target integration of the vector to that genomic locus [15]. Gene replacements occur at lower frequencies than those observed in *S. cerevisiae* and appear to require longer terminal flanking sequences to efficiently direct integration [14].

P. pastoris is a homothallic ascomycetous yeast that can also be manipulated by classical genetic methods [10,16]. Unlike homothallic strains of *S. cerevisiae*, which are diploid, *P. pastoris* remains haploid unless forced to mate. Strains with complementary markers can be mated by subjecting them to a nitrogen-limited medium. After 1 day on this medium, cells are shifted to a standard minimal medium supplemented with nutrients designed to select for complementing diploid cells (not self-mated or non-mated parental cells). The resulting diploids are stable as long as they are not subjected to nutritional stress. To obtain spore products, diploids are returned to the nitrogen-limited medium, which stimulates them to proceed through meiosis and sporulation. Spore products are handled by random spore techniques rather than micromanipulation, since *P. pastoris* asci are small and difficult to dissect. Yet most standard classical genetic manipulations, including mutant isolation, complementation analysis, backcrossing, strain construction, and spore analysis, can be accomplished.

2. Construction of expression strains

Expression of any foreign gene in *P. pastoris* requires three basic steps: (1) the insertion of the gene into an expression vector; (2) introduction of the expression vector into the *P. pastoris* genome; and (3) examination of potential expression strains for the foreign gene product. A variety of *P. pastoris* expression vectors and host strains are available. A generalized diagram of an expression vector and a list of possible vector components are shown in Fig. 3 and Table 1, respectively. More detailed information on vectors and strains can be found elsewhere [17,18]. In addition, the DNA sequence of many vectors can be found at the Invitrogen website (www.invitrogen.com). Table 2 shows a list of commonly used *P. pastoris* host strains.

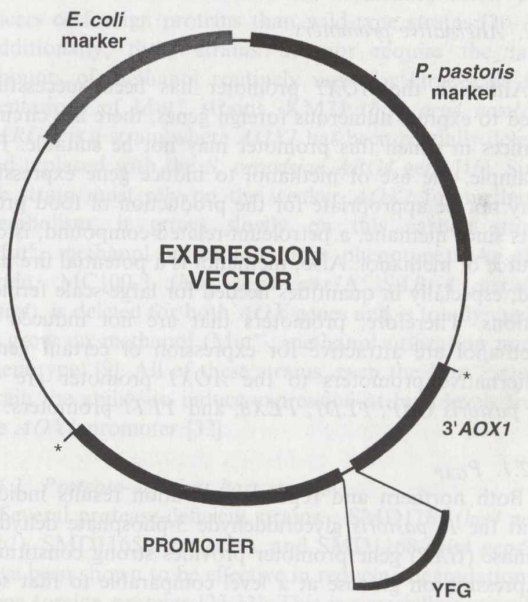


Fig. 3. General diagram of a *P. pastoris* expression vector. YFG, 'Your Favorite Gene;' *, sites for cassette amplification.

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Table 1
Relevant components of vectors used for protein expression in *P. pastoris*

Secretion signals	none, PHO1, α-MF, SUC2, PHA-E
Marker genes	ADE1, ARG4, G418, HIS4, URA3, Zeo^r
Promoters	AOX1, GAP, FLD1, PEX8, YPT1

See text for explanation of different elements.

2.1. Expression vectors

All expression vectors have been designed as *Escherichia coli*/*P. pastoris* shuttle vectors, containing an origin of replication for plasmid maintenance in *E. coli* and markers functional in one or both organisms. Most expression vectors have an expression cassette composed of a 0.9-kb fragment from *AOX1* composed of the 5′ promoter sequences and a second short *AOX1*-derived fragment with sequences required for transcription termination [19]. Between the promoter and terminator sequences is a site or multiple cloning site (MCS) for insertion of the foreign coding sequence. In the native *AOX1* gene, the alcohol oxidase open reading frame (ORF) is preceded by an unusually long 5′ untranslated region (116 nt) [8]. Generally, the best expression results are obtained when the first ATG of the heterologous coding sequence is inserted as close as possible to the position of the *AOX1* ATG. This position coincides with the first restriction site in most MCSs. In addition, for secretion of foreign proteins, vectors are available where in-frame fusions of foreign proteins and the secretion signals of *P. pastoris* acid phosphatase (*PHO1*) or *S. cerevisiae* α-mating factor (α-MF) can be generated.

2.2. Alternative promoters

Although the *AOX1* promoter has been successfully used to express numerous foreign genes, there are circumstances in which this promoter may not be suitable. For example, the use of methanol to induce gene expression may not be appropriate for the production of food products since methane, a petroleum-related compound, is one source of methanol. Also, methanol is a potential fire hazard, especially in quantities needed for large-scale fermentations. Therefore, promoters that are not induced by methanol are attractive for expression of certain genes. Alternative promoters to the *AOX1* promoter are the *P. pastoris GAP*, *FLD1*, *PEX8*, and *YPT1* promoters.

2.2.1. P_{GAP}

Both northern and reporter activation results indicate that the *P. pastoris* glyceraldehyde 3-phosphate dehydrogenase (*GAP*) gene promoter provides strong constitutive expression on glucose at a level comparable to that seen with the *AOX1* promoter [20]. *GAP* promoter activity levels in glycerol- and methanol-grown cells are approximately two-thirds and one-third of the level observed for glucose, respectively. The advantage of using the *GAP* promoter is that methanol is not required for induction, nor is it necessary to shift cultures from one carbon source to another, making strain growth more straightforward. However, since the *GAP* promoter is constitutively expressed, it is not a good choice for the production of proteins that are toxic to the yeast.

2.2.2. P_{FLD1}

The *FLD1* gene encodes a glutathione-dependent formaldehyde dehydrogenase, a key enzyme required for the metabolism of certain methylated amines as nitrogen sources and methanol as a carbon source [21]. The *FLD1* promoter can be induced with either methanol as a sole carbon source (and ammonium sulfate as a nitrogen source) or methylamine as a sole nitrogen source (and glucose as a carbon source). After induction with either methanol or methylamine, P_{FLD1} is able to express levels of a β-lactamase reporter gene similar to those obtained with methanol induction from the *AOX1* promoter. The *FLD1* promoter offers the flexibility to induce high levels of expression using either methanol or methylamine, an inexpensive nontoxic nitrogen source.

2.2.3. P_{PEX8}, P_{YPT1}

For some applications, the *AOX1*, *GAP*, and *FLD1* promoters may be too strong, expressing genes at too high a level. There is evidence that, for certain foreign genes, the high level of expression from P_{AOX1} may overwhelm the post-translational machinery of the cell, causing a significant proportion of foreign protein to be misfolded, unprocessed, or mislocalized [22,23]. For these and other applications, moderately expressing promoters are desirable. Toward this end, the *P. pastoris PEX8* and *YPT1* promoters may be of use. The *PEX8* gene encodes a peroxisomal matrix protein that is essential for peroxisome biogenesis [24]. It is expressed at a low but significant level on glucose and is induced modestly when cells are shifted to methanol. The *YPT1* gene encodes a GTPase involved in secretion, and its promoter provides a low but constitutive level of expression in media containing either glucose, methanol, or mannitol as carbon sources [25].

2.3. Selectable markers

Although classical and molecular genetic techniques are generally well-developed for *P. pastoris*, few selectable marker genes have been described for the molecular genetic manipulation of the yeast. Existing markers are limited to the biosynthetic pathway genes *HIS4* from either *P. pastoris* or *S. cerevisiae*, *ARG4* from *S. cerevisiae*, and the *Sh ble* gene from *Streptoalloteichus hindustanus* which confers resistance to the bleomycin-related drug zeocin [11,26,27]. The stable expression of human type III collagen illustrates the need for multiple selectable markers in

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Table 2
P. pastoris host strains

Strain	Genotype	Reference
Auxotrophic strains		
Y-11430	wild-type	NRRL[a]
GS115	*his4*	[11]
GS190	*arg4*	[16]
JC220	*ade1*	[16]
JC254	*ura3*	[16]
GS200	*arg4 his4*	[11]
JC227	*ade1 arg4*	[29]
JC304	*ade1 his4*	[29]
JC305	*ade1 ura3*	[29]
JC306	*arg4 ura3*	[29]
JC307	*his4 ura3*	[29]
JC300	*ade1 arg4 his4*	[29]
JC301	*ade1 his4 ura3*	[29]
JC302	*ade1 arg4 ura3*	[29]
JC303	*arg4 his4 ura3*	[29]
JC308	*ade1 arg4 his4 ura3*	[29]
Protease-deficient strains		
KM71	*Δaox1::SARG4 his4 arg4*	[7]
MC100-3	*Δaox1::SARG4Δaox2::Phis4 his4 arg4*	[9]
SMD1168	*Δpep4::URA3 his4 ura3*	[38]
SMD1165	*prb1 his4*	[38]
SMD1163	*pep4 prb1 his4*	[38]
SMD1168 kex1::SUC2	*Δpep4::URA3 Δkex1::SUC2 his4 ura3*	[34]

[a]Northern Regional Research Laboratories, Peoria, IL.

P. pastoris [28]. The production of collagen requires the coexpression of prolyl 4-hydroxylase, a central enzyme in the synthesis and assembly of trimeric collagen. Since prolyl 4-hydroxylase is an $\alpha_2\beta_2$ tetramer, the β subunit of which is protein disulfide isomerase (PDI), three markers – Arg, His, and zeocin resistance – were necessary to coexpress all three polypeptides in the same *P. pastoris* strain.

Recently, a new set of biosynthetic markers has been isolated and characterized: the *P. pastoris ADE1* (PR-amidoimidazolesuccinocarboxamide synthase), *ARG4* (argininosuccinate lyase), and *URA3* (orotidine 5′-phosphate decarboxylase) genes [29]. Each of these selectable markers has been incorporated into expression vectors. In addition, a series of host strains containing all possible combinations of *ade1*, *arg4*, *his4*, and *ura3* auxotrophies has been generated (Table 2).

2.4. Host strains

All *P. pastoris* expression strains are derived from NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, IL). Most have one or more auxotrophic mutations which allow for selection of expression vectors containing the appropriate selectable marker gene upon transformation. Prior to transformation, all of these strains grow on complex media but require supplementation with the appropriate nutrient(s) for growth on minimal media.

2.4.1. Methanol utilization phenotype

Most *P. pastoris* host strains grow on methanol at the wild-type rate (Mut$^+$, methanol utilization plus phenotype). However, two other types of host strains are available which vary with regard to their ability to utilize methanol because of deletions in one or both *AOX* genes. Strains with *AOX* mutations are sometimes better producers of foreign proteins than wild-type strains [30–32]. Additionally, these strains do not require the large amounts of methanol routinely used for large-scale fermentations of Mut$^+$ strains. KM71 (*his4 arg4 aox1Δ::SARG4*) is a strain where *AOX1* has been partially deleted and replaced with the *S. cerevisiae ARG4* gene [15]. Since the strain must rely on the weaker *AOX2* for methanol metabolism, it grows slowly on this carbon source (Mut^s, methanol utilization slow phenotype). Another strain, MC100-3 (*his4 arg4 aox1Δ::SARG4 aox2Δ::Phis4*), is deleted for both *AOX* genes and is totally unable to grow on methanol (Mut$^-$, methanol utilization minus phenotype) [9]. All of these strains, even the Mut$^-$ strain, retain the ability to induce expression at high levels from the *AOX1* promoter [32].

2.4.2. Protease-deficient host strains

Several protease-deficient strains – SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 prb1*), and SMD1168 (*his4 pep4*) – have been shown to be effective in reducing degradation of some foreign proteins [23,33]. This is especially noticeable in fermenter cultures, because the combination of high cell

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density and lysis of a small percentage of cells results in a relatively high concentration of these vacuolar proteases. An additional protease-deficient strain SMD1168 *Δpep4::URA3 Δkex1::SUC2 his4 ura3* was recently developed to inhibit proteolysis of murine and human endostatin. Kex1 protease can cleave carboxy-terminal lysines and arginines. Therefore, the deletion strain was generated to inhibit carboxy-terminal proteolysis. After 40 h of fermentation, purification of intact endostatin was achieved [34].

Unfortunately, these protease-deficient cells are not as vigorous as wild-type strains with respect to *PEP4*. In addition to lower viability, they possess a slower growth rate and are more difficult to transform. Therefore, the use of protease-deficient strains is only recommended in situations where other measures to reduce proteolysis have yielded unsatisfactory results.

2.5. Integration of expression vectors into the P. pastoris genome

Expression vectors are integrated into the *P. pastoris* genome to maximize the stability of expression strains. This can be done in two ways. The simplest way is to restrict the vector at a unique site in either the marker gene (e.g., *HIS4*) or the *AOX1* promoter fragment and then to transform it into the appropriate auxotrophic mutant. The free DNA termini stimulate homologous recombination events that result in single crossover-type integration events into these loci at high frequencies (50–80% of His^+ transformants). The remaining transformants have undergone gene conversion events in which only the marker gene from the vector has integrated into the mutant host locus without other vector sequences.

Alternatively, certain *P. pastoris* expression vectors can be digested in such a way that the expression cassette and marker gene are released, flanked by 5′ and 3′ *AOX1* sequences. Approximately 10–20% of transformation events are the result of a gene replacement event in which the *AOX1* gene is deleted and replaced by the expression cassette and marker gene. This disruption of the *AOX1* gene forces these strains to rely on the transcriptionally weaker *AOX2* gene for growth on methanol [31], and, as a result, these strains have a Mut^s phenotype. These gene replacement strains are easily identified among transformed colonies by replica-plating them to methanol and selecting those with reduced ability to grow on methanol. As mentioned previously, the potential advantage of Mut^s strains is that they utilize less methanol and sometimes express higher levels of foreign protein than wild-type (Mut^+) strains, especially in shake-flask cultures [15].

2.6. Generating multicopy strains

Optimization of protein expression often, but not always, includes the isolation of multicopy expression strains. A strain that contains multiple integrated copies of an expression cassette can sometimes yield more heterologous protein than single-copy strains [22,35].

Three approaches lead reliably to multicopy expression strains in *P. pastoris*. As shown in Fig. 4, the first approach involves constructing a vector with multiple head-to-tail copies of an expression cassette [23]. The key to generating this construction is a vector which has an expression cassette flanked by restriction sites which have complementary termini (e.g., *Bam*HI-*Bgl*II, *Sal*I-*Xho*I combinations). The process of repeated cleavage and reinsertion results in the generation of a series of vectors that contain increasing numbers of expression cassettes. A particular advantage to this approach, especially in the production of human pharmaceuticals, is that the precise number of expression cassettes is known and can be recovered for direct verification by DNA sequencing.

A second method utilizes expression vectors that contain the *P. pastoris HIS4* and the bacterial *Tn903kan^r* genes. The bacterial kanamycin resistance gene also confers resistance to the related eukaryotic antibiotic G418 [36]. The level of G418 resistance can be roughly correlated to vector copy number. *P. pastoris* must first be transformed to His^+ prototrophy; then multicopy transformants are screened by replica-plating to plates containing G418. This method results in a subset of colonies enriched for those containing multiple expression vector copies. However, the vector copy number varies greatly; thus, a significant number (50–100) of transformants must be subjected to further analysis of copy number and expression level. By this approach, strains carrying up to 30 copies of an expression cassette have been isolated [35].

A third approach to constructing multicopy strains involves the use of a vector with the bacterial *Sh ble* gene which confers resistance to the antibiotic zeocin [27]. Unlike G418 selection, strains transformed with expression cassettes containing the zeocin marker can be selected directly by resistance to the drug. Additionally, populations of transformants can be enriched for multicopy expression cassette strains simply by plating on increased concentrations of zeocin in the selection plates. Also, because the *Sh ble* gene can serve as a selectable marker in both bacteria and yeast, these expression vectors are compact and convenient to use. However, as with the G418 selection, most transformants resistant to high levels of zeocin do not contain multiple vector copies, and numerous transformants must be screened for ones that do.

2.7. High cell density growth in fermenter cultures

P. pastoris is a poor fermenter, a major advantage relative to *S. cerevisiae*. In high cell density cultures, ethanol (the product of *S. cerevisiae* fermentation) rapidly builds to toxic levels which limit further growth and foreign protein production. With its preference for respiratory growth, *P. pastoris* can be cultured at extremely high den-

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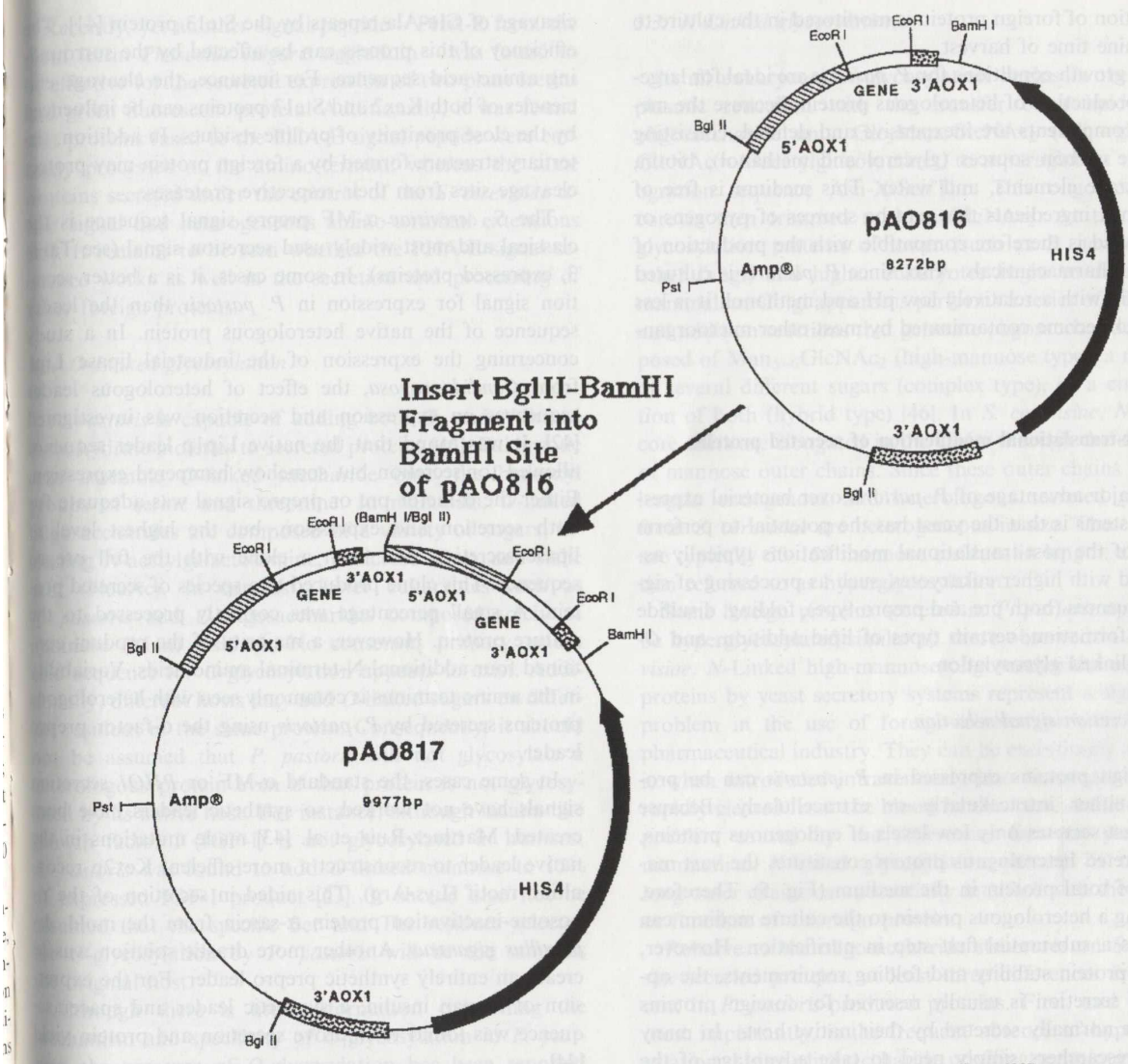


Fig. 4. Scheme for construction of vectors with multiple copies of a foreign gene expression cassette (from [22]).

sities (500 OD_{600} U ml^{-1}) in the controlled environment of the fermenter with little risk of 'pickling' itself. Fermentation growth is especially important for secreted proteins, as the concentration of product in the medium is roughly proportional to the concentration of cells in culture. Another positive aspect of growing *P. pastoris* in fermenter cultures is that the level of transcription initiated from the *AOX1* promoter can be 3–5 times greater in cells fed methanol at growth-limiting rates compared to cells grown in excess methanol. Thus, even for intracellularly expressed proteins, product yields are significantly higher from fermenter cultured cells. Also, methanol metabolism utilizes oxygen at a high rate, and expression of foreign genes is negatively affected by oxygen limitation. Only in the controlled environment of a fermenter is it feasible to monitor and adjust oxygen levels in the culture medium.

A hallmark of the *P. pastoris* system is the ease with which expression strains scale-up from shake-flask to high-density fermenter cultures. Although some foreign proteins have expressed well in shake-flask cultures, expression levels are typically low compared to fermenter cultures. Considerable effort has gone into the optimization of heterologous protein expression techniques, and detailed fed-batch and continuous culture protocols are available [23,37–39]. In general, strains are grown initially in a defined medium containing glycerol as its carbon source. During this time, biomass accumulates but heterologous gene expression is fully repressed. Upon depletion of glycerol, a transition phase is initiated in which additional glycerol is fed to the culture at a growth-limiting rate. Finally, methanol or a mixture of glycerol and methanol is fed to the culture to induce expression. The con-

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centration of foreign protein is monitored in the culture to determine time of harvest.

The growth conditions for *P. pastoris* are ideal for large-scale production of heterologous protein, because the medium components are inexpensive and defined, consisting of pure carbon sources (glycerol and methanol), biotin, salts, trace elements, and water. This medium is free of undefined ingredients that can be sources of pyrogens or toxins and is therefore compatible with the production of human pharmaceuticals. Also, since *P. pastoris* is cultured in media with a relatively low pH and methanol, it is less likely to become contaminated by most other microorganisms.

3. Post-translational modification of secreted proteins

A major advantage of *P. pastoris* over bacterial expression systems is that the yeast has the potential to perform many of the post-translational modifications typically associated with higher eukaryotes, such as processing of signal sequences (both pre and prepro type), folding, disulfide bridge formation, certain types of lipid addition, and *O*- and *N*-linked glycosylation.

3.1. Secretion signal selection

Foreign proteins expressed in *P. pastoris* can be produced either intracellularly or extracellularly. Because this yeast secretes only low levels of endogenous proteins, the secreted heterologous protein constitutes the vast majority of total protein in the medium (Fig. 5). Therefore, directing a heterologous protein to the culture medium can serve as a substantial first step in purification. However, due to protein stability and folding requirements, the option of secretion is usually reserved for foreign proteins that are normally secreted by their native hosts. In many cases, researchers simply need to take advantage of the pre-made expression cassettes available from Invitrogen. Using selected *P. pastoris* vectors, researchers can clone a foreign gene in frame with sequences encoding either the native signal, the *S. cerevisiae* α-factor prepro peptide, or the *P. pastoris* acid phosphatase (*PHO1*) signal.

Although several different secretion signal sequences, including the native secretion signal present on heterologous proteins, have been used successfully, results have been variable. The *S. cerevisiae* α-factor prepro peptide has been used with the most success. This signal sequence consists of a 19-amino acid signal (pre) sequence followed by a 66-residue (pro) sequence containing three consensus *N*-linked glycosylation sites and a dibasic Kex2 endopeptidase processing site [40]. The processing of this signal sequence involves three steps. The first is the removal of the pre signal by signal peptidase in the endoplasmic reticulum. Second, Kex2 endopeptidase cleaves between Arg-Lys of the pro leader sequence. This is rapidly followed by cleavage of Glu-Ala repeats by the Ste13 protein [41]. The efficiency of this process can be affected by the surrounding amino acid sequence. For instance, the cleavage efficiencies of both Kex2 and Ste13 proteins can be influenced by the close proximity of proline residues. In addition, the tertiary structure formed by a foreign protein may protect cleavage sites from their respective proteases.

The *S. cerevisiae* α-MF prepro signal sequence is the classical and most widely used secretion signal (see Table 3, expressed proteins). In some cases, it is a better secretion signal for expression in *P. pastoris* than the leader sequence of the native heterologous protein. In a study concerning the expression of the industrial lipase Lip1 from *Candida rugosa*, the effect of heterologous leader sequences on expression and secretion was investigated [42]. It was found that the native Lip1p leader sequence allowed for secretion but somehow hampered expression. Either the α-factor pre or prepro signal was adequate for both secretion and expression, but the highest level of lipase secretion was from a clone with the full prepro sequence. This clone produced two species of secreted protein. A small percentage was correctly processed to the mature protein. However, a majority of the product contained four additional N-terminal amino acids. Variability in the amino terminus is commonly seen with heterologous proteins secreted by *P. pastoris* using the α-factor prepro leader.

In some cases, the standard α-MF or *PHO1* secretion signals have not worked, so synthetic leaders have been created. Martinez-Ruiz et al. [43] made mutations in the native leader to reconstruct a more efficient Kex2p recognition motif (Lys-Arg). This aided in secretion of the ribosome-inactivation protein α-sarcin from the mold *Aspergillus giganteus*. Another more drastic solution was to create an entirely synthetic prepro leader. For the expression of human insulin, a synthetic leader and spacer sequence was found to improve secretion and protein yield [44].

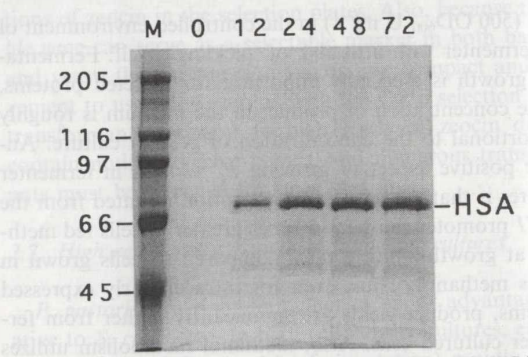


Fig. 5. Secreted expression of human serum albumin. 7.5% SDS-PAGE of 25-μl sample of culture supernatant from a *P. pastoris* strain (GS-HSA #4141) expressing human serum albumin. Cells were induced in BMMY (buffered methanol-complex medium) for 0, 12, 24, 48, and 72 h. Lane M contains molecular mass markers (kDa).

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Recently, yet another signal peptide – PHA-E from the plant lectin *Phaseolus vulgaris* agglutinin – was found to be effective for the secreted expression of two plant lectins and green fluorescent protein. Additionally, it was found that proteins fused to the PHA-E signal peptide were correctly processed at the amino-termini, whereas the same proteins secreted under the control of the *S. cerevisiae* α-MF signal had heterogeneous amino-terminal extensions [45]. It remains to be seen whether the PHA-E signal sequence works as well in the secretion and processing of other foreign proteins.

3.2. O-Linked glycosylation

P. pastoris is capable of adding both *O*- and *N*-linked carbohydrate moieties to secreted proteins [46]. Eukaryotic cells assemble *O*-linked saccharide onto the hydroxyl groups of serine and threonine. In mammals, *O*-linked oligosaccharides are composed of a variety of sugars, including *N*-acetylgalactosamine, galactose (Gal), and sialic acid (NeuAc). In contrast, lower eukaryotes such as *P. pastoris* add *O*-oligosaccharides composed solely of mannose (Man) residues. No consensus primary amino acid sequence for *O*-glycosylation appears to exist. Additionally, different hosts may add *O*-linked sugars on different residues in the same protein. Consequently, it should not be assumed that *P. pastoris* will not glycosylate a heterologous protein even if that protein is not glycosylated by its native host. For instance, although insulin-like growth factor I (IGF-I) is not glycosylated in humans, *P. pastoris* was found to add *O*-linked mannose to 15% of expressed IGF-I product [23]. It should also not be assumed that the specific Ser and Thr residues selected for *O*-glycosylation by *P. pastoris* will be the same as the original host.

Although there is little information concerning the mechanism and specificity of *O*-glycosylation in *P. pastoris*, the presence of *O*-glycosylation has been reported in some heterologous proteins, such as the *Aspergillus awamori* glucoamylase catalytic domain [47], human IGF-1 [23], barley α-amylases 1 and 2 [48], and human single-chain urokinase-type plasminogen activator [49].

Duman et al. [50] used a variety of chromatographic procedures [phenol/sulfuric acid colorimetric assay, Dionex high-pH anion-exchange chromatography (HPAEC)] and exoglycosidases (jack bean α-mannosidase, *Aspergillus saitoi* α-1,2-mannosidase, *Xanthomonas manihotis* α-1,2/1,3-mannosidase) to study endogenous cellular proteins and recombinant human plasminogen produced in *P. pastoris*. The study revealed the presence of *O*-linked α-1,2-mannans containing dimeric, trimeric, tetrameric, and pentameric oligosaccharides. No α-1,3 linkages were detected. Also, the majority of oligosaccharides was equally distributed between α-1,2-linked dimers and trimers [50].

3.3. N-Linked glycosylation

In all eukaryotes, *N*-glycosylation begins in the endoplasmic reticulum with the transfer of a lipid-linked oligosaccharide unit, $Glc_3Man_9GlcNAc_2$ (Glc = glucose; GlcNAc = *N*-acetylglucosamine), to asparagine at the recognition sequence Asn-X-Ser/Thr. This oligosaccharide core is then trimmed to $Man_8GlcNAc_2$. At this point, glycosylation patterns of lower (such as *P. pastoris* and other fungi) and higher eukaryotes begin to differ. The mammalian Golgi apparatus performs a series of trimming and addition reactions that generate oligosaccharides composed of $Man_{5-6}GlcNAc_2$ (high-mannose type), a mixture of several different sugars (complex type), or a combination of both (hybrid type) [46]. In *S. cerevisiae*, *N*-linked core units are elongated in the Golgi through the addition of mannose outer chains. Since these outer chains vary in length, endogenous and heterologous secreted proteins from *S. cerevisiae* are heterogeneous in size. These chains are typically 50–150 mannose residues in length, a condition referred to as hyperglycosylation.

Some foreign proteins secreted in *P. pastoris* appear to be hyperglycosylated similar to those observed in *S. cerevisiae*. *N*-Linked high-mannose oligosaccharides added to proteins by yeast secretory systems represent a significant problem in the use of foreign-secreted proteins by the pharmaceutical industry. They can be exceedingly antigenic when introduced intravenously into mammals and are rapidly cleared from the blood by the liver. An additional problem caused by the differences between yeast and mammalian *N*-linked glycosylation patterns is that the long outer chains can potentially interfere with the folding or function of a foreign protein.

Relative to the oligosaccharide structures on *S. cerevisiae*-secreted proteins, at least three differences are apparent in *P. pastoris*-produced proteins. First, and perhaps most importantly, is the frequent absence of hyperglycosylation. Using oligosaccharide profiling techniques, it has been shown that the typical outer chain on *P. pastoris*-secreted proteins is $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$ [51]. Another difference is the presence of α-1,6-linked mannose on core-related structures reported in *P. pastoris*-secreted invertase [52], and the kringle-2 domain of tissue-type plasminogen activator [53] and other proteins [54]. Finally, *P. pastoris* oligosaccharides appear not to have any terminal α-1,3-linked mannosylation [51,55]. These linkages make many yeast-produced recombinant proteins unsuitable for human pharmaceutical uses [56].

4. Conclusions

The *P. pastoris* expression system has gained acceptance as an important host organism for the production of foreign proteins as illustrated by the fact that a number of proteins synthesized in *P. pastoris* are being tested for use

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Table 3
Heterologous proteins expressed in *P. pastoris*

Protein	Comments: mode, amount, signal sequence	Reference
Bacteria		
Bacillus licheniformis α-amylase	S, 2.5 g l^{-1}, SUC2	[51,60]
Bacillus stearothermophilus D-alanine carboxypeptidase	S, 100 mg l^{-1}, native	[61]
Bordetella pertussis pertussis pertactin (P69)	I, 3 g l^{-1}	[62]
Clostridium botulinum neurotoxin (BoNT) serotype A and B	I, 78 mg l^{-1}	[63]
Clostridium botulinum neurotoxin heavy chain fragment, serotype B	I, 390 μg g^{-1}	[64]
Clostridium botulinum neurotoxin serotype A binding domain	I, 2.4 mg total	[65]
Clostridium tetani tetanus toxin fragment C	I, 12 g l^{-1}	[66]
Escherichia coli acid phosphatase/phytase (appA2)	S, 28.9 U mg^{-1}	[67]
Escherichia coli β-galactosidase	I, 2.0×10^3 U mg^{-1}	[7]
Escherichia coli β-lactamase	I	[20]
Leishmania major cathepsin B-like protease	S, α-MF	[68]
Staphylococcus aureus staphylokinase	S, 50 mg l^{-1}, α-MF	[69]
Streptococcus equisimilis streptokinase	I, 77 mg l^{-1}	[70]
Streptomyces subtilisin inhibitor	S	[71]
Streptomyces viridosporus T7A peroxidase, endoglucanase	S, 2.47 g l^{-1} total protein, α-MF	[72]
Toxoplasma gondii SAG1 antigen	S, 12 mg l^{-1}, α-MF	[73]
Vibrio cholerae accessory cholera enterotoxin (Acc)	S, 7 mg l^{-1}, α-MF	[74]
Fungi		
Alternaria Alt 1 allergen	S, α-MF	[75]
Aspergillus awamori glucoamylase	S, 400 mg l^{-1}, native	[76]
Aspergillus awamori glucoamylase catalytic domain	S, 400 mg l^{-1}, PHO1	[47]
Aspergillus fumigatus catalase L	S, 2.3 g l^{-1}, PHO1	[77]
Aspergillus fumigatus dipeptidyl peptidase IV (DPP IV)	S, PHO1	[78]
Aspergillus fumigatus dipeptidyl peptidase V (DPP V)	S, 0.15 mg l^{-1}, PHO1	[79]
Aspergillus giganteus α-sarcin ribotoxin	S, 1 mg l^{-1}, synthetic native, PHO1	[43]
Aspergillus niger phytase (phyA)	S, 65 U ml^{-1}, α-MF	[80]
Candida guilliermondii xylose reductase gene (xylI)	I, 0.65 U mg^{-1}; S, 0.18 U mg^{-1}, α-MF	[81]
Candida rugosa lipase 1 (CRL)	S, 150 U ml^{-1}, α-MF	[42]
Fusarium solani pectate lyase (pelC)	S, 1 mg l^{-1}, PHO1	[82]
Fusarium solani pectate lyase (pelD)	S, native	[83]
Geotrichum candidum lipase isoenzymes	S, 60 mg l^{-1}, α-MF	[84]
Phytophthora cryptogea β-cryptogein	S, 45 mg l^{-1}, PHO1	[85]
Rhizopus oryzae lipase	S, 60 mg l^{-1}, α-MF	[86]
Saccharomyces cerevisiae invertase	S, 2.5 g l^{-1}, native	[30]
Saccharomyces cerevisiae Ktr1p	S, 400 mg l^{-1}, PHO1	[87]
Saccharomyces cerevisiae (α-1,2-mannosyltransferase)	S, 40 mg l^{-1}, PHO1	[87]
Schizophyllum commune vitamin B2-aldehyde-forming enzyme	S, 120 mg l^{-1}, α-MF	[88]
Trametes versicolor (white rot fungus) laccase (lccI)	S, native and α-MF	[89]
Trichoderma harzianum β-(1–6)-glucanase	S, 9.3 mg l^{-1}	[90]
Protists		
Chondrus crispus red alga hexose oxidase	I	[91]
Gracilariopsis lemaneiformis red alga α-1,4-glucan lyase (GLq1)	I	[92]
Plasmodium falciparum merozoite surface protein 1 (MSP-1)	S, 24 mg l^{-1}, α-MF	[93]
Plasmodium vivax apical membrane antigen I (AMA-1)	S, 50 mg l^{-1}, PHO1	[94]
Reticulomyxa filosa (giant freshwater ameba) α2, β2 tubulin isoforms	I, 400 μg g^{-1}	[95]
Trypanosoma cruzi acid α-mannosidase	S, 11.5 μg l^{-1}, native	[96]
Plants		
Allium sativum (garlic) alliin lyase	I, 2.167 U g^{-1}	[97]
Arabidopsis thaliana NADH:nitrate reductase	I, 18 μg g^{-1}	[98,99]
Barley (*Hordeum vulgare*) sucrose fructan 6-fructosyl transferase	S, α-MF	[100]
Barley α-amylase 1	S, 50 mg l^{-1}, native	[48]
Barley α-amylase 2	S, 1 mg l^{-1}, native	[48]
Barley aleurone tissue α-glucosidase	S, α-MF	[101]
Coffee bean α-galactosidase	S, 400 mg l^{-1}, α-MF	[102]
Cynara cardunculus (cardoon) cyprosin	S, 1 mg l^{-1}, native	[103]
Cynodon dactylon (Bermuda grass) Cyn d 1	S, 1.5 g l^{-1}, PHO1	[104,105]
Galanthus nivalis agglutinin	S, PHA-E	[45]
Hevea brasiliensis hydroxynitrile lyase	I, 22 g l^{-1}	[106]
Hevea brasiliensis Hev b 7 patatin-like allergen	S, 10 mg l^{-1}, α-MF	[107,108]
Maize cytokinin oxidase	S, native	[109]
Oat phytochrome A, phA	I, 30 μg g^{-1}	[110,111]
Oat phytochrome A, phyA65 apoprotein	I, 20 μg g^{-1}	[112]

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Table 3 (*continued*)

Protein	Comments: mode, amount, signal sequence	Reference
Olea europaea (olive tree) aeroallergen Ole e 1	S, 60 mg l^{-1}, α-MF	[113]
Pepper endo-β-1,4-glucanase cCel1	S, α-MF	[114]
Pepper endo-β-1,4-glucanase cCel2	S, native	[114]
Persea americana (avocado) prs a 1 major allergen	S, 50 mg l^{-1}, α-MF	[115]
Phaseolus vulgaris agglutinin (phytohemagglutinin)	S, native	[45]
Potato phytochrome B	I, 25 μg g^{-1}	[116]
Ragweed allergen Amb a 6	S, 1 mg l^{-1}, α-MF	[117]
Soybean root nodule acid phosphatase	S, 10 mg l^{-1}, α-MF	[118]
Spinach glycolate oxidase	I, 250 U g^{-1}	[119,120]
Spinach phosphoribulokinase	I, 0.5 mg g^{-1}	[121]
Timothy grass group I allergen	S, α-MF	[122]
Tomato *Lycopersicon esculentum* Mill. LeMir (*L. esculentum* miraculin)	S, PHO1	[123]
Wheat lipid transfer protein	S, 720 mg l^{-1}, PHO1	[124]
Invertebrates		
Achacina fulica Ferussac (giant African snail) achacin	S, 0.2 mg l^{-1}, native	[125]
Aplysia californica (marine invertebrate) ADP ribosyl cyclase	S, 300 mg l^{-1}, α-MF	[126]
Aequorea victoria (jellyfish) green fluorescent protein	I, S, PHA-E	[45,127]
Boophilus microplus (cattle tick) Bm86	I, S*, 1.5 g l^{-1}, SUC2	[128–131]
Cockroach allergen, Bla g 4	S, 50 mg l^{-1}	[132]
Drosophila melanogaster angiotensin I-converting enzyme	S, 160 mg l^{-1}, α-MF	[133]
Firefly luciferase	I (peroxisome)	[134]
GAVAC™ vaccine against cattle tick	S, 2.0 g l^{-1}	[135]
Haementeria ghilanii (South American leech) ghilanten	S, 10 mg l^{-1}, α-MF	[136]
Hirudo medicinalis (leech) hirudin	S, 1.5 g l^{-1}, α-MF	[137]
Honey bee odorant-binding protein (ASP2)	S, 150 mg l^{-1}, native	[138]
Nippostrongylus brasiliensis (parasitic nematode) non-neuronal secreted acetylcholine sterase	S, 27 mg l^{-1}, α-MF	[139]
Spider dragline silk protein	I, 663 mg l^{-1}	[140]
Tick anticoagulant peptide	S, 1.7 g l^{-1}	[141]
Vertebrates (non-human)		
Bovine enterokinase catalytic domain	S, 6.3 mg l^{-1}, α-MF	[142]
Bovine follicle-stimulating hormone β-subunit	S, 4 μg ml^{-1}, α-MF	[143]
Bovine IFN-omega 1	S, 4 mg l^{-1}, SUC2	[144]
Bovine lysozyme c2	S, 550 mg l^{-1}, native	[145]
Bovine opsin	S*, 0.3 mg l^{-1}, PHO1	[146]
Bovine pancreatic trypsin inhibitor (aprotinin)	S, 930 mg l^{-1}, α-MF	[147]
Bovine β-casein	I, 1 g l^{-1}	[148]
Bovine β-lactoglobulin	S, >1 g l^{-1}, α-MF	[149–151]
Bovine tissue-type plasminogen activator (tPA)	S, 1.1 mg l^{-1}, α-MF	[152]
Brushtail possum TNFα	S, α-MF	[153]
Bungarus fasciatus (snake) venom gland acetylcholinesterase	S, 2 mg l^{-1}, native	[154]
Chicken liver α-*N*-acetylgalactosaminidase	S, 11.6 mg l^{-1}, α-MF, PHO1	[155]
Electrophorus electricus acetylcholinesterase AChE type T	S, native	[156]
Hen lysozyme	S, 20 mg l^{-1}, α-MF	[157]
Mammalian lipocalin allergen Bos d2	S, mg amounts, native	[158]
Mouse 5HT5A 5-tryptamine receptor	S*, 40 pmol mg^{-1}, α-MF	[159]
Mouse epidermal growth factor	S, 450 mg l^{-1}, α-MF	[35]
Mouse gelatinase B	S, 10 mg l^{-1}, α-MF	[160]
Mouse lysosomal acid α-mannosidase	S, native	[161]
Mouse major urinary protein complex (MUP)	S, 270 mg l^{-1}, native	[162]
Mouse Mdr3 P-glycoprotein	I (membrane-bound), 6 μg mg^{-1}	[163–165]
Mouse single-chain Fv fragments (sFv)	S, 250 mg l^{-1}, α-MF, PHO1	[166]
Murine endostatin	S, 200 mg l^{-1}, α-MF	[34]
Murine Golgi mannosidase IA	S, PHO1	[167]
Murine macrophage inflammatory protein-2 (MIP-2)	S, 40 mg l^{-1}, α-MF	[168]
Ovine follicle-stimulating hormone (oFSH)	S, 22 mg l^{-1}, α-MF	[169]
Porcine follicle-stimulating hormone	S, 10 mg l^{-1}, PHO1	[170]
Porcine inhibitor of carbonic anhydrase (transferrin family)	S, 5 mg l^{-1}, α-MF	[171]
Porcine leukocyte 12-lipoxygenase	I	[172]
Rabbit intestinal peptide transporter (PEPT1)	I	[173]
Rabbit intestinal peptide transporter (PEPT2)	I	[174]
Rabbit monoclonal single-chain Fv specific for recombinant human leukemia inhibitory factor	S, 100 mg l^{-1}, α-MF	[175]

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Table 3 (*continued*)

Protein	Comments: mode, amount, signal sequence	Reference
Rabbit plasma cholesteryl ester transfer protein	S, PHO1	[176]
Rabbit testicular angiotensin-converting enzyme	S, PHO1, native	[177]
Rat acetylcholinesterase	S, 1 mg l^{-1}, native	[154]
Rat brain acetylcholinesterase T subunit	S, 100 U l^{-1} α-MF	[178]
Rat complement regulator, crry	S, α-MF	[179]
Rat Golgi sialoglycoprotein MG160	S, 10 mg l^{-1}, α-MF	[180]
Rat high-mobility group 1 (HMG 1)	S, 50 mg l^{-1}, α-MF	[181]
Rat liver mitochondrial carnitine palmitoyl transferases I and II (CPTI and II)	I (mitochondria)	[182,183]
Rat NO synthase reductase domain	I, 25 mg l^{-1}	[184]
Rat peroxisomal multifunctional enzyme (perMFE-II)	I	[185]
Rat procathepsin B	S, 100 mg l^{-1}, α-MF	[186,187]
Sea raven type II antifreeze protein (SRAFP)	S, 30 mg l^{-1}, α-MF	[188,189]
Shark 17α-hydroxylase/C17,20-lyase	I	[190]
Syrian golden hamster prion protein PrP^c	I, <0.1 mg l^{-1}	[191]
Humans		
α(1,3/4)-Fucosyltransferase	S, 30 mg l^{-1}, α-MF	[128]
α-1,2-Mannosidase 1B w/o TM domain	S, α-MF	[192]
α-*N*-Acetylgalactosaminidase (α-NAGAL)	S, 11.6 mg l^{-1}, α-MF	[193]
α1-Antitrypsin (α1-AT)	S, inulinase signal sequence	[194]
β2-Adrenergic receptor	S*, 25 nmol g^{-1}, α-MF	[159]
μ-Opioid receptor	S*, α-MF	[195]
ADAR1, ADAR2, ds-RNA-specific adenosine deaminases	I, 1 mg l^{-1}	[196]
Alzheimer's disease amyloid precursor protein α, β, and γ-secretase products	S, PHO1	[197]
Alzheimer's disease amyloid precursor protein, 2 domains	S, 24 mg l^{-1}, 0.1 mg l^{-1}, α-MF	[198]
Amyloid precursor-like protein 2 (APLP2)	S, 40 mg l^{-1}, α-MF	[199]
Amyloid precursor protein (APP)	S, 24 mg l^{-1}, PHO1	[200,201]
Amyloid precursor proteins, rAPP695, rAPP770	S, 4.5+1 mg l^{-1}, native	[202]
Bile salt-stimulated lipase	S, 300 mg l^{-1}, native, INV	[203]
Bivalent diabody against carcinoembryonic antigen (CEA), T-cell coreceptor CD2	S, 1 mg l^{-1}, α-MF	[204]
c-Kit receptor kinase domain	I, 0.2 mg l^{-1}	[205,206]
Carcinoembryonic antigen	S, 20 mg l^{-1}, α-MF	[207]
Caspase-3	I, 1 μg g^{-1}	[208]
Cathepsin K	S, 38 mg l^{-1}, α-MF	[209,210]
Cathepsin L propeptide	S, 10 mg l^{-1}, α-MF	[211,212]
Cathepsin V	S, α-MF	[213]
CD38	S, 455 mg l^{-1}, α-MF	[214]
CD40 ligand soluble form	S, 255 mg l^{-1}	[215]
Chimeric B7-2 antibody fusion protein	S, 15 mg l^{-1}, α-MF	[216]
Chorionic gonadotropin α subunit, β subunit, and αβ heterodimer	S, 24 mg l^{-1} (α), 3 mg l^{-1} (β), 16 mg l^{-1} (αβ), α-MF	[217]
Cromer blood group antigen decay-accelerating factor	S, α-MF	[218]
Cytomegalovirus ppUL44 antigen	I, 0.1 mg ml^{-1}	[219]
Decay-accelerating factor DAF (CD55)-Echovirus-7 receptor	S, 6 mg l^{-1}, α-MF	[220]
Double-stranded RNA-specific editase I (hREDI)	I, 1 mg l^{-1}	[221]
Endostatin	S, 20 mg l^{-1}, α-MF	[34,222]
Fas ligand	S, 100 mg l^{-1}, α-MF	[223]
Fibrinogen, 143–411, 143–427	S, 100 mg l^{-1}, 75 mg l^{-1}, α-MF	[224]
Fibroblast collagenase (proMMP-1)	S, 2.3 mg l^{-1}, α-MF	[225]
Fibrinogen-420 αEC domain	S, α-MF	[226]
Gastric cathepsin E	S, 0.6 mg l^{-1}, native	[227]
Heart muscle carnitine palmitoyltransferase I (M-CPTI)	I (mitochondria)	[228]
Insulin	S, synthetic signal	[44]
Insulin-like growth factor-1 (IGF-1)	S, 600 mg l^{-1}, α-MF	[23]
Interferon-γ receptor cytoplasmic domain	I	[229]
Interleukin-17 (hIL-17)	S, 0.35 mg l^{-1}, α-MF	[230]
Intracellular proteinase inhibitor (PI-6)	I, 50 mg l^{-1}	[231]
Kunitz-type protease inhibitor domain of protease nexin-2/amyloid β-protein precursor	S, 1.0 g l^{-1}, α-MF	[232]
Leukemia inhibitory factor (LIF)	S, 17 mg l^{-1}, α-MF	[233]
Lymphocyte surface antigen CD38	S, 400 mg l^{-1}, PHO1	[234]
Lysosomal α-mannosidase	S, 83 μg l^{-1}, native	[235]
Mast cell tryptase	S, 6.5 mg l^{-1}, α-MF	[236,237]

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Table 3 (*continued*)

Protein	Comments: mode, amount, signal sequence	Reference
MHC class II heterodimers (soluble form/HLA-DR2)	S, 400 μg l^{-1}, α-MF	[238]
Monoclonal single-chain Fv	S, 50 mg l^{-1}, α-MF	[239]
Monocyte chemoattractant protein-1 (MCP-1)	S, 100 mg l^{-1}, native and α-MF	[240]
Monocyte chemotactic protein 3 (hMCP-3)	S, 1 mg l^{-1}, PHO1	[241]
Neural cell adhesion molecule (NCAM)	S, 50 mg l^{-1}, PHO1	[242]
NonO nucleic acid binding protein	I (endoplasmic reticulum)	[243]
Pancreatic α-amylase	S, 20 mg l^{-1}, α-MF	[244]
Pancreatic triglyceride lipase	S, 75 ml l^{-1}, PHO1	[245]
Papain nitrile hydratase	S, 5 mg l^{-1}, α-MF	[246]
Placental alkaline phosphatase (PLAP)	S, 2 mg l^{-1}, PHO1	[247]
Placental protein-14 (PP-14)	S, α-MF	[248]
Plasminogen kringles 1–4	S, 17 mg l^{-1}, PHO1	[50]
Plasminogen kringles 1–4, angiostatin protein	S, 10% total protein, PHO1	[249]
Procarboxypeptidase A2	S, 180 mg l^{-1}, α-MF	[250]
Procathepsin B	S, 20 mg l^{-1}, α-MF	[251]
Procolipase	S, 30 mg l^{-1}, native	[252]
Protein kinase C interacting protein 1 (PKCI-1)	I, 0.25 mg l^{-1}	[253]
Proteinase 3, Wegener's antigen	S, 670 mg l^{-1}, α-MF	[254]
Proteinase inhibitor 8	I, 15% total protein	[255]
scFv (against ovarian carcinoma)-biotin mimetic peptide	S	[256,257]
scFv (against squamous carcinoma)	S, 50 mg l^{-1}, α-MF	[239]
Serum albumin	S, 3 g l^{-1}, native	[58,258-260]
Serum transferrin N-lobe	S, 240 mg l^{-1}, α-MF	[261-263]
Sex steroid binding protein	S, 4 mg l^{-1}, α-MF	[264]
Single-chain urokinase-type plasminogen activator	S, 5 mg l^{-1}, pre *Mucor pusillus* rennin signal	[49]
Thrombomodulin	S	[33]
Tissue factor extracellular domain	S, 10 mg l^{-1}, PHO1	[265]
Tissue kallikrein	S, 30 mg l^{-1}, α-MF	[266,267]
Tissue-type plasminogen activator kringle 2 domain	S, 170 mg l^{-1}, α-MF	[9,26,53,268–270]
Transforming growth factor β receptor extracellular domain	S, 10 mg l^{-1}, α-MF	[271]
Tumor necrosis factor α (TNF)	I, 10 g l^{-1}	[272,273]
Type 1 plasminogen activator inhibitor (PAI-1)	S, 3 mg l^{-1}, α-MF	[274]
Type III collagen (with prolyl 4-hydroxylase)	I, 15 mg l^{-1}	[28]
Urokinase-type plasminogen activator-annexin V chimeras	S, 600 IU ml^{-1}, pre *Mucor pusillus* rennin signal	[275]
Vascular endothelial growth factor (VEGF165)	S, 40 mg l^{-1}, PHO1	[276]
Viruses		
A/VICTORIA/3/75 influenza virus neuraminidase head domain	S, 3 mg ml^{-1}, α-MF	[277,278]
Bovine herpes virus-1 glycoprotein D	S, 20 mg l^{-1}, α-MF	[279,280]
Dengue virus type 1 structural gene recombinant E protein	S, PHO1, prM virus signal sequence	[281]
Hepatitis B virus surface antigen	I, 400 mg l^{-1}	[31,282]
Hepatitis B virus surface antigen-HIV gp41 epitope chimera	I	[283]
Hepatitis E virus ORF3	I	[284]
Human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein, gp120 (ENV)	S, 20 mg l^{-1}, α-MF	[285]
Polyomavirus large T antigen	I, 0.5 mg l^{-1}	[286]
Reovirus lambda 1 core protein	I, 0.8 mg l^{-1}	[287]
Reovirus sigma 1 protein	I	[288]
Vaccinia virus complement control protein	S, 3 mg l^{-1}, α-MF	[289]

I = intracellular (with subcellular location), S = secreted, S* = secreted to plasma membrane. Amounts are highest reported for particular protein. Signal sequences: α-MF (*S. cerevisiae* α-mating factor); PHO1 (*P. pastoris* acid phosphatase); SUC2 (*S. cerevisiae* invertase).

as human pharmaceuticals in clinical trials. IGF-1 in a treatment for amyotrophic lateral sclerosis [57] and human serum albumin (HSA) in a serum replacement product [58] have passed through clinical trials and are awaiting final approval. The angiogenesis inhibitors endostatin and angiostatin are in or rapidly approaching clinical trials [59]. Another protein, hepatitis B surface antigen, is currently on the market as a subunit vaccine against the hepatitis B virus in South America. A complete list of heterologous proteins expressed successfully in *P. pastoris* is shown in Table 3.

Yet, despite the success of the *P. pastoris* system, opportunities exist to develop a larger range of proteins that can be expressed in the system. The new alternative promoters and marker/host strain combinations make possible the expression of heterooligomeric proteins and essential cofactors. Still little is known about *AOX1* promoter regulation at the molecular level. Such studies could lead to

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modified *AOX* promoters with increased transcriptional strength or to the identification and overexpression of factors that limit transcription of P_{AOX1}.

Studies are also needed to address problems associated with the secretion of mammalian proteins from *P. pastoris*. A better understanding of secretion signals, glycosylation, and endogenous *P. pastoris* proteases would be extremely helpful in developing and improving the *P. pastoris* heterologous expression system.

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Regulation of methanol utilisation pathway genes in yeasts

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Abstract

Methylotrophic yeasts such as *Candida boidinii*, *Hansenula polymorpha*, *Pichia methanolica* and *Pichia pastoris* are an emerging group of eukaryotic hosts for recombinant protein production with an ever increasing number of applications during the last 30 years. Their applications are linked to the use of strong methanol-inducible promoters derived from genes of the methanol utilisation pathway. These promoters are tightly regulated, highly repressed in presence of non-limiting concentrations of glucose in the medium and strongly induced if methanol is used as carbon source. Several factors involved in this tight control and their regulatory effects have been described so far. This review summarises available data about the regulation of promoters from methanol utilisation pathway genes. Furthermore, the role of *cis* and *trans* acting factors (e.g. transcription factors, glucose processing enzymes) in the expression of methanol utilisation pathway genes is reviewed both in the context of the native cell environment as well as in heterologous hosts.

Background

The yeast *Saccharomyces cerevisiae* was used by mankind for millennia, mainly in brewing and baking. Because of its unchallenged use for thousands of years, this species, also known as baker's or brewer's yeast, became a synonym for yeast. However, in research and industrial biotechnology more and more yeast species with outstanding characteristics have been investigated, starting in the 1970's to 80's [1-7]. Among them a limited number of yeast species capable of growing on methanol as sole carbon and energy source have been characterised and their application in biotechnology was established for single cell protein production [8] and later for recombinant protein production [9,10]. These so-called methylotrophic yeasts belong to the four genera *Hansenula*, *Pichia*, *Candida* and *Torulopsis* [11-13] including their most prominent representatives *Pichia pastoris*, *Hansenula polymorpha* (*Pichia angusta*), *Candida boidinii* and *Pichia methanolica* [14,15].

The uses of methylotrophic yeasts reported so far have relied on their ability to grow on methanol or at least on the unique regulatory features of their pathways for methanol utilisation. These efforts have been concentrated in three areas:

a) Since the initial reactions of methanol utilisation are compartmentalised within peroxisomes, these organelles are strongly induced upon shifting the cells to methanol as sole carbon source. Therefore these yeasts are frequently used model organisms to study peroxisome biogenesis and function [16-19]. However, methylotrophic yeasts as model organisms in cell biology are not restricted to peroxisome research. *Pichia pastoris* is perfectly suited to study the early secretion pathway because it exhibits a dis-

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crete transitional endoplasmic reticulum (ER) and coherent Golgi stacks [20,21].

b) Methylotrophic yeasts or enzymes thereof have been developed for bioconversions or as recognition elements in biosensor applications, mainly for the oxidation or detection of alcohols [22-26].

c) The major application of methylotrophic yeasts is recombinant protein production. As reviewed extensively [27-32], promoter elements derived from genes involved in methanol utilisation, especially the alcohol oxidase promoters, have been utilised to express several hundreds of different proteins in methylotrophic yeasts so far.

Like *Saccharomyces cerevisiae*, methylotrophic yeasts combine the advantages of microorganisms (i.e. simple cultivation on inexpensive growth media, well established genetic techniques and tremendous experience on industrial cultivation and scale-up) with the ability to perform typical eukaryotic protein processing and other posttranslational modifications such as glycosylation. Many more advantages became relevant for pharmaceutical proteins. The products are free of endotoxins and also of oncogenic and viral DNA. Due to their use in food industry for thousands of years some yeasts possess a GRAS status (Generally Regarded As Safe).

The industrial relevance of methylotrophic yeasts is still dramatically increasing due to some technologically interesting features. In contrast to *S. cerevisiae* they belong to the group of Crabtree negative yeasts, thus ethanol production occurs at very low level under fully aerobic conditions. This enables these yeasts to grow to very high cell densities in bioreactor cultures and consequently results in high product yields [6,33]. Secondly, there is a tendency of non-conventional yeasts (including *P. pastoris* and *H. polymorpha*) for secretion of proteins, even with higher molecular mass [2] while in *S. cerevisiae* retention of proteins in the periplasmatic space occurs even for peptides of low molecular weight. The high secretion capacity of methylotrophic yeasts in combination with well-established high cell-density fermentation techniques resulted in secretion of heterologous proteins up to levels of several grams per litre (e.g. phytase [34,35], gelatin [36-40]).

Although hyper-mannosylation occurs less frequently compared to *S. cerevisiae*, the major difference in respect to glycosylation is the lack of hyper-immunogenic terminal α-1,3-linked mannoses in N-linked glycans in proteins produced by *P.pastoris* and *H.polymorpha* [41-45]. The potential to replace cell culture systems as the preferred production system for therapeutic proteins drives research to engineer methylotrophic yeasts for homogenised and humanised N-linked protein glycosylation. This was already successful in terms of trimming complex Golgi-derived glycan structures to ER-derived core-glycans. Core-glycosylation is conserved among higher and lower eukaryotes [46-48]. Very recently, in an interesting engineering approach the multi-step pathways to synthesise galactose and sialic acid as well as the respective pathways to add these sugars to glycans was introduced into *P. pastoris*, providing a battery of individual strains which add specific and homogenous human-like glycan structures to recombinant proteins [45,49-53]. This breakthrough has opened the door to increasing applications of this methylotrophic yeast for the production of biopharmaceuticals such as therapeutic antibodies, a market so far dominated by *E. coli*, *S. cerevisiae* and mammalian cell lines [53-56].

Review

Introduction

The success of methylotrophic yeasts in the production of recombinant proteins is highly linked to the very strong and tightly regulated promoters of some genes of the methanol utilisation pathway (MUT pathway). Many of these genes and their respective promoter regions have been isolated (Table 1). Especially the promoter of the gene coding the first enzyme of the pathway in *Pichia pastoris*, the alcohol oxidase I (*AOX1*), and the corresponding promoters from other methylotrophic yeasts are most commonly applied for recombinant protein production in these hosts [10,29,57]. *H. polymorpha* where the *FMD* (formate dehydrogenase) promoter is most frequently employed is an exception [58,59]. These promoters enabled the production of intracellular as well as secreted proteins up to a concentration of several grams per litre (e.g. [34,35,60]).

The following chapters review the regulation of these genes and the application of their corresponding promoters for the production of recombinant proteins in methylotrophic and other yeasts.

The methanol utilisation pathway (MUT pathway)

Methylotrophic yeasts share a unique MUT pathway whose expression is tightly regulated at the level of transcription [61-64]. Since a part of the MUT pathway takes place in the peroxisomes, these organelles proliferate massively upon methanol induction. After induction they can account for up to 80% of the cytoplasmic space [65]. In the first step of methanol utilisation, methanol is oxidised to formaldehyde and hydrogen peroxide by alcohol oxidases (*AOX*, EC 1.1.3.13). The toxic H_2O_2 is broken down to oxygen and water by the action of catalase (*CAT*, EC 1.11.1.6). Both enzymes are sequestered in peroxisomes (see Figure 1) [32,66,67]. Formaldehyde is either oxidised by two subsequent dehydrogenase reactions (dissimilation pathway) or assimilated in the cell metabolism by

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Table 1: Methanol utilisation pathway genes in methylotrophic yeasts.

Organism[a]	Gene/Promoter	Genbank Acc. No.[b]	Reference[b]
Candida boidinii S2 [175]	*AOD1*	M81702	[63]
	FLD1	AB085186	[71]
	FDH1	AF004096	[70]
	CTA1	AB064338	[108]
	DAS1	AF086822	[68]
Hansenula polymorpha (*Pichia angusta*) CBS4732 (ATCC 34438)	*MOX*	X02425	[84]
	FLD	AF364077	[76]
	FMD	A06214	[104, 176]
	DAS	X02424, A11168	[69, 177]
	CAT	X56501	[178]
Pichia pastoris NRRL Y-11430 (CBS7435)	*AOX1*	U96967	[62]
	AOX2	U96968, X79871	[62]
	FLD1	AF066054	[77]
	*ZZA1**	S62281	[166]
Pichia methanolica IAM12901	*MOD1* (*AUG1*)	AF141329	[82], [101]
	MOD2 (*AUG2*)	AF141330, AB223040	[101]
	FLD	AB112935	[78]

[a]Strains used to isolate and characterise genes and promoters.
[b]The Genbank accession numbers (Acc. No.) for the entire gene or the promoter and the respective reference are given. *(from H. Phaff ID 72–1033)

condensation with xylulose 5-phosphate (Xu_5P). The latter peroxisomal condensation reaction is catalysed by a special transketolase called dihydroxyacetone synthase (*DAS*, 2.2.1.3) [32,64,66,68,69]. *DAS* converts Xu_5P and formaldehyde into the C_3-compounds dihydroxyacetone (DHA) and glyceraldehyde 3-phosphate (GAP), which are further metabolised in the cytosol (Figure 1).

In the dissimilation pathway, formaldehyde spontaneously reacts with glutathione to *S*-hydroxymethylglutathione which then is oxidised in two consecutive reactions to carbon dioxide by the action of a glutathione (GSH)- and NAD^+-dependent formaldehyde dehydrogenase (*FLD*, EC 1.2.1.1) and a NAD^+-dependent formate dehydrogenase (*FDH* 1.2.1.2), both located in the cytosol. NADH, generated in both dehydrogenase reactions, is considered to be used in energy production for growth on methanol [32,70]. Beside the role in energy production, the dissimilation pathway enzymes play a role in the detoxification of formaldehyde and formate, respectively [70,71]. A third enzyme (or enzymatic activity) involved in this pathway, *S*-formylglutatione hydrolase (*FGH*, EC 3.1.2.12) hydrolyses S-formylglutathione to formate and glutathione [71-74]. It participates in the detoxification of formaldehyde and regenerates glutathione [71,72]. While a separate enzyme was found in *C. boidinii*, *Kloeckera* sp. 2201 (a strain of *C. boidinii*) and *P. pastoris*, in *H. polymorpha FGH* activity was found to be associated with the *FDH* [75]. The methanol dissimilation pathway also plays a major role in the metabolism of methylated nitrogen sources such as choline or methylamine where formaldehyde is a by-product. In the latter, detoxification of formaldehyde is the main task of the methanol dissimilation pathway [71,76,77]. Knockout studies on *FLD* and *FDH* strains of *C. boidinii* and *P. methanolica* demonstrated that the *FLD* knockout phenotype is more severe than *FDH* knockout, which was explained by the higher toxicity of formaldehyde compared to formate [71,78].

In general, expression of the methanol pathway genes is repressed by glucose and ethanol and strongly induced by methanol. Ethanol and glucose act via two distinct mechanism to repress the MUT pathway [79-81]. In induced cells the key enzymes alcohol oxidase, dihydroxyacetone synthase and formate dehydrogenase can account for up to 30 % (*AOX*) or 20 % (*DAS*, *FDH*) of the total soluble protein in induced cells [10,14,15]. Due to their role in nitrogen metabolism the regulation of the genes of the dissimilation pathway is slightly different. *AOX* and *DAS* genes are repressed by glucose while methanol is necessary for induction. Induction of *FLD* and *FDH* expression was also accomplished by methylated nitrogen sources such as methylamine or choline, even in the presence of glucose [70,71].

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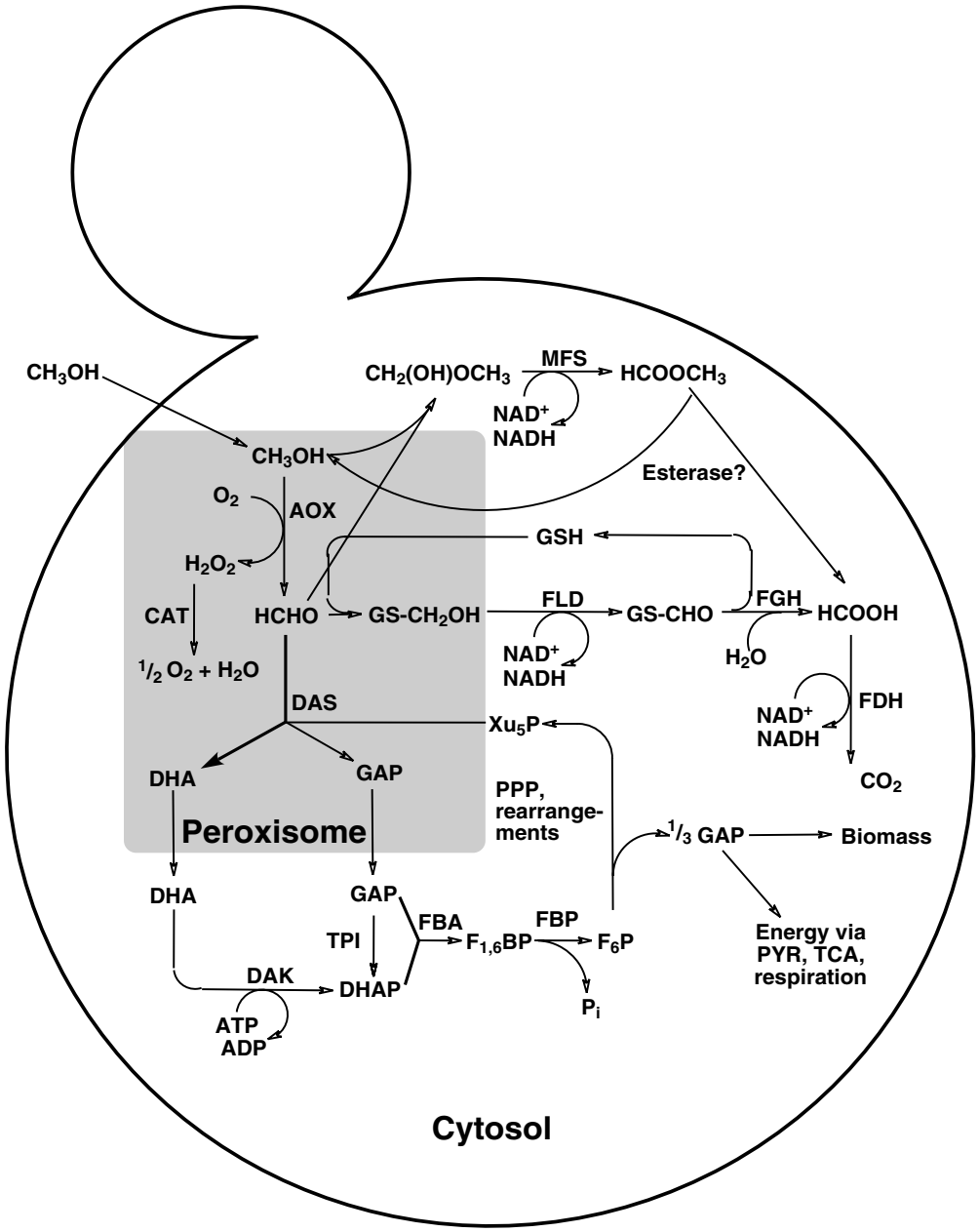


Figure 1
Methanol utilisation pathway in methylotrophic yeasts: The main pathways and the respective enzymes working in the methanol metabolism in methylotrophic yeasts are shown. AOX: alcohol oxidase (EC 1.1.3.13), CAT: catalase (EC 1.11.1.6), FLD: formaldehyde dehydrogenase (EC 1.2.1.1), FGH: S-formylglutathione hydrolase (EC 3.1.2.12), FDH: formate dehydrogenase (EC 1.2.1.2), DAS: dihydroxyacetone synthase (EC 2.2.1.3), TPI: triosephosphate isomerase (EC 5.3.1.1), DAK: dihydroxyacetone kinase (EC 2.7.1.29), FBA: fructose 1,6-bisphosphate aldolase (EC 4.1.21.13), FBP: fructose 1,6-bisphosphatase (EC 3.1.3.11), MFS: methylformate synthase; DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, $F_{1,6}BP$: fructose 1,6-bisphosphate, F_6P: fructose 6-phosphate, P_i: phosphate, Xu_5P: xylulose 5-phosphate, GSH: glutathione, PYR: pyruvate; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle

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Alcohol oxidases – the entry into the pathway

The number of genes coding for alcohol oxidases varies among methylotrophic yeasts. While in *Candida boidinii* and *Hansenula polymorpha* only one gene codes for an alcohol oxidase (*AOD1* and *MOX*, respectively), two genes are found in *Pichia pastoris* and *Pichia methanolica* (*AOX1* and *AOX2*, and *MOD1* (*AUG1*) and *MOD2* (*AUG2*), respectively) [62,63,82-85]. Furthermore, not only is the number of alcohol oxidase genes diverse among the methylotrophic yeasts, but also their regulation in the respective yeasts varies significantly.

The regulation of the *AOX* genes in *C. boidinii* and *H. polymorpha* is very similar: the *HpMOX* and the *CbAOD1* promoter are repressed during growth on glucose or ethanol as sole carbon source, derepressed in the presence of glycerol and strongly induced by methanol in absence of glucose. The level of derepression on glycerol differs between these two yeasts. While in *C. boidinii* the *AOD1* derepression on glycerol results in only ~20% activity compared to the methanol-induced level, the *H. polymorpha MOX* activity under these conditions is almost ~80% of the methanol-induced level (Table 2). Expression of *AOD1* or *MOX* was also repressed when both glucose and methanol were present in the medium (glucose in non-limiting concentrations) while a glycerol and methanol mixture resulted in alcohol oxidase activity which is comparable to the activity in media containing methanol as sole carbon source [57,86-90]. Several other carbon sources (e.g. xylose, ribose and sorbitol) are also able to derepress *MOX* expression in *H. polymorpha*. The expression level on these carbon sources is lower than with glycerol, i.e. only ~50 % (xylose), ~25 % (ribose) or ~10 % (sorbitol) of the fully induced level obtained with methanol [91].

Pichia pastoris is different: at non-growth-limiting conditions both alcohol oxidase genes, *AOX1* and *AOX2* are repressed by glucose, ethanol and also by glycerol [61,62,83]. The mRNA level of *AOX1* upon derepression was ~1–2% of the induced level [92]. The regulation pattern of the *AOX2* gene was identical to the *AOX1* gene [93] but *AOX1* contributes to the vast majority of the expressed alcohol oxidase [62]. Thus knockout of the *AOX1* gene results in a slow growth phenotype on methanol, called Mut^S (methanol utilisation slow), while growth of the *AOX2* knockout strain on methanol is comparable to the wild type (Mut^+). Double knockout strains are unable to grow on methanol (Mut^-). The specific maximum growth rates of the 3 phenotypes are 0.14, 0.04 an 0 h^{-1}, respectively [33]. Although glycerol does not lead to derepression of *AOX1* expression other so-called non-repressing carbon sources like e.g. sorbitol, mannitol, trehalose and alanine do so, but published data do not allow to estimate the strength of the derepression effect [94,95].

Pichia methanolica, formerly described as *Pichia pinus* MH4 [96], was one of the latest methylotrophic yeasts developed as an expression host [82]. As earlier described for *P. pastoris*, Raymond *et al*. [82] initially described *MOD1* (*AUG1*) as the major alcohol oxidase gene and *MOD2* (*AUG2*) as minor one. Mod1p (Aug1p) was the only alcohol oxidase detected in a methanol-fed bioreactor culture. Also *P. methanolica mod1Δ* (*aug1Δ*) strains displayed a slow growth phenotype on methanol while *mod2Δ* (*aug2Δ*) strains showed wild type growth on methanol. The double knockout strain was unable to grow on methanol [82]. Thus, like with *P. pastoris*, the *MOD1* promoter serves as a major tool for the expression of heterologous proteins in *P. methanolica*. However, the situation is not that simple. In 1999, Nakagawa *et al*. observed the formation of 9 Mod1p and Mod2p alcohol oxidase hybrid octamers in *P. methanolica* [97]. The fraction of Mod2p in the oligomers was raised with increasing concentrations of methanol (up to 4%) [97]. Induction of *MOD2* expres-

Table 2: Repression and derepression effect of carbon sources on *AOX* genes of methylotrophic yeasts.

		Glucose, EtOH	Glycerol	Methanol	Glycerol+MeOH
P. pastoris [61, 62, 83, 92, 93]	*AOX1*	Repression	Repression	Induction	Repression
	AOX2	Repression	Repression	Induction	Repression
H. polymorpha [76, 88–91]	*MOX*	Repression	Derepr. (~60–70%)	Induction	Induction (~100%)
	FMD	Repression	Derepr. (~60%)	Induction	
C. boidinii [57, 68, 70, 71, 86, 87]	*AOD1*	Repression	Derepr. (~3–30%)	Induction	Induction (~90%)
	FLD	No activity	Derepr. (~20%)	Induction	Induction (~70%)
	FDH	Repression	No activity	Induction	Induction (~30%)
	DAS	Repression	Derepr. (~2%)	Induction	Induction (~70%)
P. methanolica [78, 82, 100, 101]	*MOD1*	Repression	Derepr. (~60–70%)	Induction	Induction (~100%)
	MOD2	Repression	No activity	Induction	Induction (~100%)
	FLD	No activity	Derepr. (~20%)	Induction	Induction (~70%)

Effect and level of derepression compared to the methanol induced level are shown (Induction by methanol for each gene denotes 100%). The data was extracted from the literature given by the citations and are taken from experiments were ammonia was used as nitrogen source.

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sion by high methanol concentrations is in accordance with earlier findings of K_M values of MOD homooctamers where MOD-A (corresponding to a Mod1p homooctamer) has a 10-times higher affinity towards methanol (K_M(MOD-A) = 0.56 mM, K_M(MOD-B) = 5.6 mM) than MOD-B [98]. It also turned out that the growth phenotypes described by Raymond *et al*. in 1998 [82] was characteristic only for low methanol concentration. As mentioned above, at 1 % methanol [82], the *mod1Δ* strain had the slow growth phenotype whereas the *mod2Δ* strain showed wild type growth. At a concentration of 2 % methanol, both strains grew slowly, although still better than the *mod1Δ* strain at 0.25% methanol. The growth deficiency of the *mod1Δ* variant was still more severe than the one of the *mod2Δ* strain. At 3% methanol both strains grew like the wild type. Under these conditions each of the genes could compensate the lack of the other, thereby restoring wild-type growth rate and biomass yield [99]. In contrast to *AOX1* in *Pichia pastoris*, *MOD1* is also expressed by *P. methanolica* when glycerol is used as carbon source, just like the *H. polymorpha MOX* [100,101]. Isolation of ~1.5–1.6 kb long 5' regions of *MOD1* and *MOD2* genes as promoter regions and coupling to acid phosphatase as enzyme reporter confirmed previous results from *MOD* isozyme activity measurements: both genes are repressed as long as glucose is present, *MOD1* is expressed when glycerol is the carbon source. Methanol strongly induced the expression of both, at low concentration mainly *MOD1*, at higher concentrations both genes. *MOD2* had a regulation pattern similar to the *P. pastoris AOX1* promoter. It is repressed by glucose and methanol is necessary for induction. No expression is found in glycerol-containing media but, in contrast to *P. pastoris AOX1*, addition of glycerol to methanol medium did not repress *MOD2* promoter driven expression [101]. Additional regulation of the *MOD1*/*MOD2* promoters by elevated oxygen concentrations was observed. At low methanol concentrations (0.1%) *MOD1*-driven expression could be boosted by a higher shaking speed where no effect was found for the *MOD2* promoter. At higher concentrations of methanol (0.3% to 1%) the reverse was true, *MOD2*-driven expression was increased massively while expression driven by the *MOD1* promoter was unaffected [101]. However, these studies were done in shake flasks with no control of methanol and oxygen concentrations, and the obtained results should have been confirmed with well controlled bioreactor experiments.

The exact physiological role of the *AOX2* gene in *P. pastoris* is not known. For *P. methanolica* it is assumed to be an advantage under high methanol concentrations. The same was demonstrated in *C. boidinii*. An *aod1Δ* strain which expressed the *PmMOD2* gene (under the control of the *C. boidinii AOD1* promoter) exhibited a shorter lag-phase when grown on 3 % methanol, compared to the strain expressing the *Pm*Mod1p or both, *Pm*Mod1p and *Pm*Mod2p. The system using two different subunits with different K_M values to compose catalytically active heteromultimers is a belting evolutionary trick for adapting the affinity of the functional alcohol oxidase octamer to the methanol concentration in the medium. The adaptation of subunit composition of the octamer (and therefore the K_M) to increasing methanol concentrations was mainly regulated by higher expression of the Mod2p subunit under these conditions, as shown by zymogram pattern analyses and reporter studies [99-101]. For *P. pastoris* no studies on the physiological function of the *AOX2* gene are published which leaves the mystery about its physiological role unanswered.

Formaldehyde dehydrogenase

Like the alcohol oxidase also the formaldehyde dehydrogenase genes were isolated from all four prominent methylotrophic yeasts. The strength of their promoters, which is comparable to the *AOX* promoters makes them attractive alternatives for recombinant protein production [76,77].

Interestingly, there is an intron at the very 5' end of the gene in *FLD* genes of *C. boidinii*, *P. pastoris* and *P. methanolica*. The size of the intron differs between these 3 yeasts but all are located at the same position, 18 nucleotides downstream of the start codon ATG. No intron is present in the *H. polymorpha FLD* gene. The sequences of the 5' and 3' splice junctions and of the branch points are quite similar if not identical to the respective splice site consensus sequences of *S. cerevisiae* and *S. pombe* but also of higher eukaryotes. After splicing, the 3 genes of *C. boidinii*, *P. pastoris* and *P. methanolica* have the same size as the *H. polymorpha FLD* gene which results in proteins of 380 amino acids length [71,76-78].

Like the other enzymes from the MUT pathway, expression of formaldehyde dehydrogenases is mainly regulated at the transcriptional level [71,78]. In contrast to the alcohol oxidase genes, *FLD* expression is not subject to glucose repression since methylamine or choline are able to induce gene expression even in presence of glucose (as long as ammonia is absent) [71,77,78]. Methanol, formaldehyde, methylamine or choline induced *C. boidinii FLD1* expression [71]. In *P. methanolica* and *H. polymorpha* the degree of derepression in media lacking glucose differed and was highly dependent on the carbon source. *P. methanolica FLD1* expression was induced by glycerol, xylose and trehalose although at a much lower level compared to methanol [78]. In the case of *H. polymorpha* xylose, ribose, sorbitol and glycerol were reported to induce *FLD* expression [91]. Addition of methanol further increased the *PmFLD* expression level in presence of glycerol, xylose and trehalose. Glucose does not impede *FLD*

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expression. Thus, lack of expression is just due to a lack of induction and not due to strong repression. If both glucose and methanol were present in the medium the *FLD* activity level was comparable to the level found with glycerol as sole carbon source. For all these studies ammonia was used as nitrogen source [78]. Interestingly, in *C. boidinii* almost no activity was found with glycerol as carbon source when ammonia was the nitrogen source [71]. If methylamine was used as sole nitrogen source, *FLD* expression was induced in cells even during growth in glucose-containing media and further increased in methanol or glycerol media [78]. Increasing methanol concentrations increased *FLD1* expression [78], an effect which can also be assumed for *H. polymorpha* where increasing dilution rates in chemostat cultures (which usually means higher steady-state concentrations of methanol) resulted in higher *FLD* activities in the cells [102].

In a *P. pastoris aox1 aox2* double mutant (KM7121) decreased *FLD* activities were found which suggested a predominant regulation by methanol but also a regulatory contribution by formaldehyde [77]. The same effect was described for *FLD* expression in a *P. methanolica mod1*Δ/*mod2*Δ double knockout strain [78]. *PpFLD1* activities or *PpFLD1*-promoter driven *lacZ* expression when glucose was the carbon source and methylamine was the nitrogen source reported by Shen *et al*. was not found by Resina *et al*. when expressing *Rhizopus oryzae* lipase under the control of the *FLD1* promoter [103]. Also no lipase activity was found using glycerol and methylamine as carbon and nitrogen source, respectively. But sorbitol was a non-repressing carbon source since the combination of sorbitol and methylamine resulted in relatively high lipolytic activities [103]. The discrepancies between these two reports can not be clarified since both publications did not show activities in dependence of time nor did they describe exact enough when the cultures were harvested and analysed or if there were residual carbon or nitrogen sources present in the medium. Nonetheless, although some discrepancies appear in reports in the case of *P. pastoris FLD* activity two distinct regulatory systems by the carbon and the nitrogen source are evident. E.g. glucose is not able to repress *FLD* expression when methylamine is the sole nitrogen source and the same is true for ammonia when methanol is used as carbon source. In this respect the regulation in *P. pastoris* needs to be clarified. Thus, we conclude that no repression acts on the *FLD* promoter. No expression is just due to lack of induction. Once either an inductive carbon or nitrogen source is present, both might independently induce expression of the *FLD* gene.

Formate dehydrogenase

Formate dehydrogenases are frequently used for NADH regeneration in redox biotransformations due to the cheap substrate formate and the gaseous product CO_2, thereby enabling NADH regeneration without disturbing by-product formation. The most popular *FDH* is derived from *Candida boidinii* [70]. Despite their frequent application, the *FDH* genes are not as well characterised as the two genes described before. So far the *FDH* genes including the respective promoter were only isolated from *C. boidinii* [70] and *H. polymorpha* [104,105]. In the case of *P. pastoris* only a DNA fragment coding for the open reading frame was described [106]. In *C. boidinii*, formate dehydrogenase was not induced by glucose and glycerol. Methanol as sole carbon source or methylamine as sole nitrogen source were inducers of *CbFDH1* expression. Thus, there is some similarity in regulation to the *FLD* genes. Formate also induced *CbFDH1* expression, even in the presence of glucose and glycerol. This suggests that formate is a much more powerful inducer than methanol since methanol was just able to induce formate dehydrogenase synthesis in the presence of glycerol but not if glucose was the second carbon source. However, although formate was able to induce synthesis of *Cb*Fdh1p, even in presence of glucose, the induction was much weaker compared to methanol alone or a glycerol/formate mixture as carbon source [70,87]. In *H. polymorpha*, synthesis of formate dehydrogenase is partly derepressed by glycerol. Derepression by xylose, sorbitol and ribose was below 10 % of optimal expression, if present at all [91].

Dihydroxyacetone synthase

Dihydroxyacetone synthases (*DAS*) and their promoters were isolated from *C. boidinii* [68], *H. polymorpha* [69] and only a *DAS* promoter region was described in *P. pastoris* [92]. Where studied, the *DAS* genes were found to be regulated similarly and as tightly as the respective alcohol oxidase genes. Again, regulation was shown to occur mainly at the transcriptional level [68,69,92]. In general, *DAS* activity was repressed by glucose and induced by methanol. In *C. boidinii* no *DAS* activity was found in media with glycerol as sole carbon source and ammonia as nitrogen source, although at varying extent among the available literature. But addition of methanol resulted in an induction of *DAS* activity up to ~60–70 % of the level obtained by methanol as sole carbon source. Methylamine and choline as sole nitrogen sources also induced *DAS* expression, even in presence of glycerol. Both nitrogen sources failed to induce expression when glucose was the carbon source [68]. Using *S. cerevisiae* acid phosphatase (*ScPHO5*) as reporter enzyme, the *C. boidinii DAS1* promoter was shown to enable higher expression levels than the *AOD1* promoter [87]. This result is in agreement with earlier findings in *P. pastoris* where higher levels of β-galactosidase activities were found with a *DAS* promoter than with the *AOX1* promoter, at least when *lacZ* was employed as a reporter gene [92].

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Catalase

Due to its very important role in disarming the toxic hydrogen peroxide generated by the alcohol oxidase during oxidation of methanol to formaldehyde, catalase (*CAT*) synthesis is also induced when cells are growing on methanol as carbon source. In order to be prepared for the toxic H_2O_2, catalase expression precedes the expression of *AOX* and the other genes involved in MUT pathway upon glucose depletion in batch cultures [91,107].

Catalase expression was shown to be controlled mainly on the transcriptional level [86], although some regulation at the posttranslational level, namely import into peroxisomes, has been reported [108].

Other enzymes involved in methanol utilisation

Several other but less prominent enzymes from methylotrophic yeasts involved in methanol utilisation have been isolated and described. An S-formylglutathione hydrolase gene (*FGH*) was found in *C. boidinii* [73]. The presence of a separate *FGH* enzyme was also reported for *P. pastoris* and *Kloeckera* sp. 2201 (a strain of *C. boidinii*) [72], while in *H. polymorpha FGH* activity was reported to be associated with the formate dehydrogenase [75]. The *C. boidinii FGH* has a typical PTS1-type (peroxisomal targeting signal 1) signal sequence at his C-terminus, in contrast to the *P. pastoris* enzyme. Its localisation in the cell was confirmed via a GFP-fusion to be bimodal. The regulation of expression was similar to *FDH* and *FLD*. Methanol and formaldehyde were able to induce expression and *FGH* is not subject to glucose repression. A *C. boidinii FGH* knockout strain was unable to grow on methanol as sole carbon source in batch cultures, and it also displayed growth defects on methylamine and choline as nitrogen sources when grown in glucose-containing media. Since the *fghΔ* strain was able to grow under methanol-limited chemostat conditions, the authors concluded that *FGH* is not essential but important for optimal growth on methanol. Due to the lower growth rate and growth yield under these conditions, a role in detoxification is assumed for this enzyme [73].

An antioxidant system of *C. boidinii* cells has been described which belongs to the Pmp20 or peroxiredoxin family [109,110]. Expression of this peroxisomal membrane-bound enzyme is highly induced by methanol and absolutely necessary for growth on this carbon source. The *C. boidinii PMP20* knockout strain did not grow on methanol. Thus the effect of this disruption is more severe than in the catalase knockout strain which is still able to grow on methanol as sole carbon source [109]. Expression of acid phosphatase driven by the *PMP20* promoter was comparable to the *FDH*-promoter driven expression [87].

Methylformate (MF) was found to accumulate during growth of *C. boidinii* on methanol as carbon source. The enzymatic synthesis of MF was dependent on NAD^+ but not dependent on the glutathione-pathway for methanol oxidation [111]. Later on, the enzyme responsible for MF synthesis was described as Zn^{2+}-containing alcohol dehydrogenase (*ADH*) [112]. At least three *ADH*-gene products were found to catalyse MF synthesis. From the three genes isolated, *CbADH1* encodes for the major MFS (methylformate synthase) activity in the cell. An *adh1Δ* strain displayed a prolonged lag phase when grown on methanol, which is even more severe in an *adh1Δ adh2Δ* double knockout strain. Thus, the authors concluded that *MFS* activity plays a major role in formaldehyde detoxification in *C. boidinii* cells growing on methanol as carbon source [113].

Chemostat studies with methylotrophic yeasts

A large portion of the information on the regulation of MUT pathway enzymes, mainly in *H.polymorpha* (CBS4732) and *C. boidinii* (*Kloeckera* sp. 2201), has been published since the late 1970s. For an overview see Table 3.

Studies about methanol utilisation enzymes of *H. polymorpha* in a methanol-limited chemostat revealed a clear dependence of specific enzyme activities on the dilution rate. Interestingly, while the specific activities of *FLD* and *FDH* were increasing with increasing dilution rates, or being constant like *CAT* activity, the *AOX* activity decreased significantly [102]. The situation is quite similar in *Kloeckera* sp. 2201 (*C. boidinii*) [107]. Glucose-limited chemostat studies on *H. polymorpha* revealed a remarkable level of derepression of *MOX* activity at dilution rates below 0.4 h^{-1}. In *Kloeckera* sp. 2201 *AOX* induction started at lower dilution rates compared to *H. polymorpha*. No induction was detectable above D = 0.2 h^{-1}. Below this dilution rate *AOX* activity slowly increased with decreasing dilution rates [114], but always stayed far below the activity determined in a methanol-limited chemostat at the same dilution rates [107]. Almost no alcohol oxidase activity was found in ethanol-limited chemostat cultures at a low dilution rate of 0.1 h^{-1}. At this rate significant activity was found in methanol or glycerol-limited chemostat cultures [114], which suggests a much higher repression effect of ethanol compared to glucose. In this context it has to be mentioned that ethanol production was already reported several times to reduce *AOX* promoter driven protein expression [115]. A remarkable result from glucose-limited chemostat cultures was found for *FLD* activities. Both strains exhibited significant derepression of *FLD* activity at D < 0.4 h^{-1} with an additional boost in the derepression level below D = 0.1 h^{-1} [114]. At these low dilution rates *FLD* activity reached the level found in methanol-limited chemostat cultures in *H. poly-*

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Table 3: Chemostat studies of methylotrophic yeasts [90, 102, 107, 114, 118, 119].

		AOX	*CAT*	*FLD*	*FDH*
Methanol-limited chemostat	*C. b.*	Decreasing with increasing dilution rate	Relatively constant over the D range tested	Increasing with increasing dilution rate	Increasing with increasing dilution rate
	H. p.	Decreasing with increasing dilution rate	Increasing with increasing dilution rate	Increasing with increasing dilution rate	Increasing with increasing dilution rate
Glucose-limited chemostat	*C. b.*	Low level of derepression at D's < 0.2 h^{-1}	Increasing with increasing dilution rate	High level of derepression at D's < 0.4 h^{-1}	Low level of derepression at D's < 0.1 h^{-1}
	H. p.	High level of derepression at D's < 0.4 h^{-1}	Increasing with increasing dilution rate	High level of derepression at D's < 0.4 h^{-1}	Low level of derepression at D's < 0.2 h^{-1}
Glucose/methanol fed chemostat	*C. b.*	Decreasing with increasing D	Increasing with increasing D up to 0.3 h^{-1}	Activity peak at D ~0.12 h^{-1}	Almost constant at D < 0.3 h^{-1}
	H. p.	Decreasing with increasing D	Increasing with increasing D up to 0.3 h^{-1}	Increasing with increasing D up to 0.3 h^{-1}	Increasing with increasing D up to 0.3 h^{-1}
Sorbitol-limited chemostat	*H. p.*	Decreasing with increasing dilution rate	Decreasing with increasing dilution rate	Decreasing with increasing dilution rate	Decreasing with increasing dilution rate
Sorbitol/methanol fed chemostat	*H. p.*	Decreasing with increasing dilution rate	Increasing with increasing dilution rate	Increasing with increasing D above 0.3 h^{-1}	Activity peak at D = 0.37 h^{-1}
Glycerol/methanol fed chemostat	*H. p.*	High specific enzyme activity at D = 0.1 h^{-1}	High specific enzyme activity at D = 0.1 h^{-1}	High specific enzyme activity at D = 0.1 h^{-1}	High specific enzyme activity at D = 0.1 h^{-1}
Ethanol/methanol fed chemostat	*H. p.*	No activity found at D = 0.1 h^{-1}	No activity found at D = 0.1 h^{-1}	No activity found at D = 0.1 h^{-1}	No activity found at D = 0.1 h^{-1}

The general trend of specific enzyme activity with varying dilution rates (D) are given. *C. b.*: *Candida boidinii* (*Kloeckera* sp. 2201), *H. p.*: *Hansenula polymorpha* CBS4732

morpha [102] and 50 % of the *FLD* activity level in *Kloeckera* sp. 2201 at the same growth rate [107].

In chemostat cultivations with a carbon source mixture of glucose and methanol, both substrates were utilised simultaneously to completion by *H. polymorpha* and *Kloeckera* sp. 2201 (*C. boidinii*) at a dilution rate of 0.14 to 0.15 h^{-1}, independent of the glucose/methanol ratio of the carbon source. Specific enzyme activities of *AOX*, *CAT*, *FLD* and *FDH* rose with increasing proportions of methanol up to ~20 % (*H. polymorpha*) or 40 % (*Kloeckera* sp. 2201) in the mixture. No further increase in specific enzyme activities was found with higher proportions of methanol in the mixture [116]. On the contrary, the lower the methanol proportion of the carbon mixture, the higher the dilution rate up to which complete methanol utilisation took place [117]. The biomass produced was found to be the sum of the theoretically possible biomass produced from the individual substrates [116]. Thus, both yeast species were able to utilise methanol efficiently in presence of glucose also at dilution rates higher than μ_{max} when grown on methanol as sole carbon source. Methanol induction only became possible once a significant level of derepression of *AOX* expression occurred. Specific activity of *AOX* in the chemostat culture with mixed substrates was ~50% of the activity of methanol-limited cultures at the same dilution rate. Similar results were found for the specific activity profiles of *CAT*, *FLD* and *FDH* but, in contrast to *AOX*, activities increased with increasing dilution rates up to ~0.3 h^{-1} [118]. Increasing specific enzyme activities of *AOX*, *CAT*, *FLD* and *FDH* with decreasing dilution rates were reported for chemostat cultures with sorbitol as sole carbon source. Mixed feed cultures with sorbitol and methanol as a carbon source resulted in a strongly increasing activity of these enzymes starting already at high dilution rates. The specific enzyme activities during mixed feed were comparable to the specific enzyme activities with methanol as sole carbon source at the same dilution rate. A remarkable feature observed with this combination of carbon sources was a peak of formate dehydrogenase activity at a dilution rate of 0.37 h^{-1} where methanol was only partially utilised [119]. Shift from carbon-limited chemostat cultures with glucose/methanol as a carbon source to nitrogen-limited conditions resulted in severe repression of methanol utilisation genes. Nitrogen limitation, rather than elevated glucose concentrations, were responsible for the repression of MUT pathway genes [120].

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Recently, such a detailed chemostat study was published for *P. pastoris*. Identical to *Kloeckera sp*. 2201, *AOX* activity increased with decreasing dilution rates in glycerol-limited chemostat cultures, but this enzyme activity was also just in the range of 1–5 % of the AOX activity found in methanol-limited chemostat cultures at the same dilution rate [121]. The increasing *AOX* activity with decreasing dilution rates on methanol as sole carbon source was almost identical to the trend found in *H. polymorpha* by van Dijken *et al*. [102].

From all these studies it got evident that the degree of sensitivity to carbon catabolite repression by glucose varies between the methylotrophic yeasts studied. Despite the general regulation by methanol, the data also suggest some specific regulation of the individual genes of the MUT pathway since all of them behave differently under varying environmental conditions (e.g. dilution rate, carbon source composition). Varying degrees of derepression or induction for the individual genes as well as for the different host strains under the conditions tested are a remarkable result from these studies. This diversity should be kept in mind when designing a general model for the regulation of the MUT pathway in methylotrophic yeasts.

Although salient differences exist between chemostat cultivation and batch cultivation, results from continuous cultivation under nutrient-limiting conditions for repression/derepression and induction kinetics are in agreement with promoter activities of MUT genes reported for batch-cultures. In contrast to *H. polymorpha*, where a single strain (CBS4732 = ATCC 34438) was used in almost all batch and chemostat studies published, *Kloeckera sp*. 2201 is definitively a different *C. boidinii* strain as the one used for most of the other studies (*C. boidinii* S2). But also in this case, the MUT pathway gene expression data from batch studies and from the chemostat cultivations were in a very good agreement. Unfortunately, *H. polymorpha*, chemostat studies under glycerol-limited conditions were performed only at a dilution rate of 0.1 h^{-1}. Since *AOX* expression on glycerol as sole carbon source is the most remarkable difference between the four methylotrophic yeasts studied, comprehensive glycerol-limited chemostat studies of these yeasts will provide interesting insights on the diverse modes of MUT gene regulation.

cis-*acting regulatory elements*

In addition to gene deletions, batch- and chemostat studies of methylotrophic yeasts molecular deletions within several methanol-inducible promoter sequences from *Pichia pastoris*, *Hansenula polymorpha* (*Pichia angusta*) and *Candida boidinii* were studied. Thereby *cis*-acting elements were identified and some insight into gene regulation was obtained.

HpMOX *promoter*

H. polymorpha MOX promoter deletions allowed some insight into the regulation of MOX expression [88]. Promoter variants with a spectrum of reporter activity from almost no to 100 % of the wild type were found under derepressing conditions using glycerol as carbon source or under conditions of methanol induction. Three out of a series of regulatory promoter sequence stretches were characterised in more detail (Figure 2): two UAS (upstream activation sequence) and one URS (upstream repressing sequence). First, it should be mentioned, that no significant expression of the reporter protein was found with any of the promoter variants under conditions if glucose was present in the medium. Binding of proteins to the *MOX* promoter within the UAS1/2 and the URS1 region was found by footprinting but neither the attached proteins nor the respective genes were identified. Since the genome sequence of *H. polymorpha* was not known at that time, identification of such proteins should get easier in the near future. The UAS1 binding site was narrowed by the footprinting technique. Its sequence showed a high similarity to a sequence stretch within the co-regulated *CAT* promoter. The UAS2 and URS1 were also identified but no sequence similarity to other co-regulated promoters was described for the latter regulatory promoter elements [88]. Later on, Pereira *et al*. identified a region which is responsible for the repression of the *MOX* promoter in the stationary phase of *H. polymorpha* cultures upon depletion of glucose [122]. The region, called *MOX-B*, is located between the sequences identified by Gödecke *et al*. [88] and the putative TATA box of the *MOX* promoter. Deletion of this region caused a 40-fold increase of reporter enzyme activity in the stationary phase upon glucose depletion, compared to the wild-type promoter at the same conditions. However, this is still not more than 40% of the reporter activity under derepressing conditions in glycerol containing media. The mutant promoter led to the synthesis of ~130 % of reporter enzyme under derepressing conditions [122].

Astonishingly, two *MOX* mRNA species were present during derepression of *MOX* in a *H. polymorpha MOX* multicopy strain. The longer *MOX* transcript (with a minor transcription start at position -425; termed l-*MOX*) was transcribed only in glucose-grown cells. However, no *MOX* activity was observed. Upon induction by methanol, l-*MOX* became down-regulated and transcription of the other *MOX* transcript, originating from the major transcription start point (TSP) at position -25 was highly up-regulated. Only after expression from the -25 TSP, *MOX* activity became apparent. Presence of the l-*MOX* transcript under glucose growth conditions and its repression upon induction were confirmed in the respective *H. polymorpha* wild-type strain [89]. Two transcripts were also observed in *C. boidinii*, although under inducing condi-

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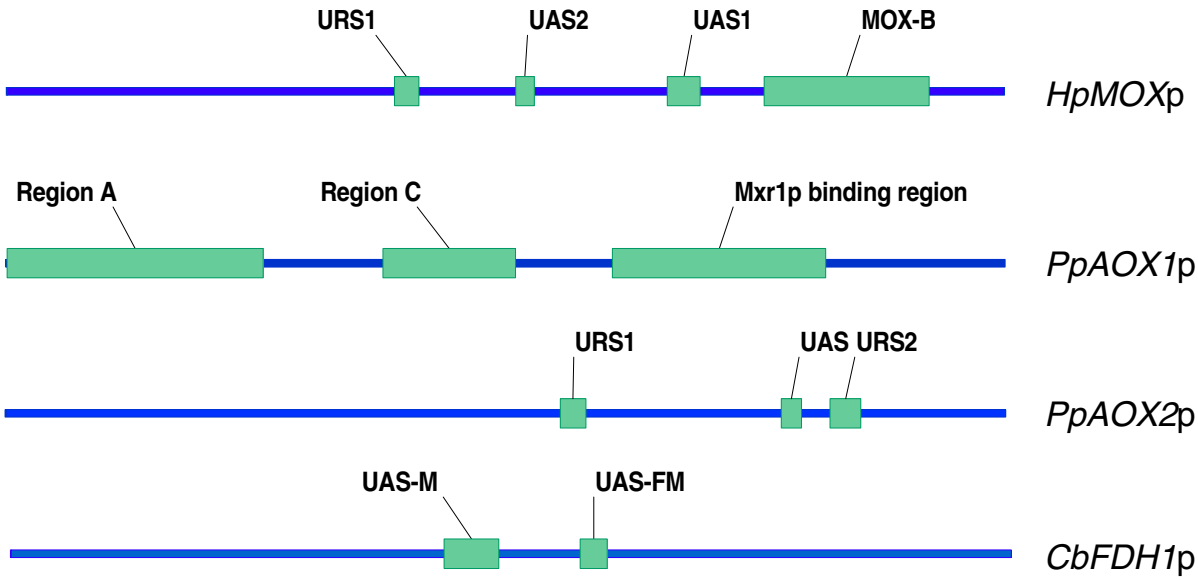


Figure 2
Regions within methanol utilisation pathway gene promoters identified as *cis*-acting elements: The regions indicated in this figure were identified either by deletion from the original promoter and proving there activity in a different promoter environment, by footprinting and/or by bandshift assays. *HpMOX*p: *H. polymorpha MOX* promoter [88, 122, 123], *PpAOX1*p: *P. pastoris AOX1* promoter [125, 126], *PpAOX2*p: *P. pastoris AOX2* promoter [93], *CbFDH1*p: *C. boidinii FDH1* promoter [128]; URS1, URS2: upstream repressing sequences, UAS, UAS1, UAS2: upstream activation sequences, UAS-M, UAS-FM: upstream activation sequence for methanol and for methanol and formate, respectively, Region A: region upstream of -690, Region C: region between -540 and -400.

tions [63]. Derepression and induction of *MOX* transcription was associated with the repression of l-*MOX*. Upon shift from repressing to derepressing conditions, l-*MOX* transcription was transiently induced followed by a complete inhibition. Therefore a regulatory mechanism involving this TSP shift was postulated [89,123]. Interestingly, it was shown that inhibition of respiration by antimycin A also inhibited this TSP shift [89]. It is thought that respiration is needed for reoxidation of NADH generated during methanol oxidation. Therefore a crosstalk between respiration and methanol metabolism to coordinate gene expression of these two pathways makes intuitively sense. This aspect is further supported by the finding that retrograde signalling from *S. cerevisiae* mitochondria influences peroxisomal gene regulation [124].

PpAOX2 *promoter*

Deletion analyses of the *P. pastoris AOX2* promoter revealed two URS sequences (URS1 and URS2) and an UAS region. Shortening the promoter and adding 1–3 UAS elements to promoter fragments resulted in a strong increase in reporter protein production in the presence of methanol. In shake flask cultures the production of the reporter protein HSA (human serum albumin) could be enhanced up to 120 fold (from 1 to 120 µg ml^{-1}) with some of the promoter variants compared to the wild type promoter. The expression was also enhanced 50–60 times just by introduction of a point mutation at position -255 (C to T) or by a duplication mutation between position -233 and -215. Both mutations are positioned within the URS2 element which is located near the TATA box (-204) of the promoter. HSA production levels were also increased in glucose-containing medium, although at a very low level. However, glucose repression could not be overcome in any *AOX2* promoter construct tested since methanol never induced HSA production in cultures with a mixture of glucose/methanol as carbon source. The minimal HSA levels found in glucose containing media are most probably due to the long incubation time of 72 h, thereby a result of production under derepressing conditions upon glucose starvation. By sequence comparison of this UAS with promoter regions of the *PpAOX1*, *CbAOD1* and the *HpDAS* genes, homologous sequence stretches were identified but their functions still remain to be elucidated [93]. Although a remarkable increase in *AOX2* promoter strength was reached, it has to be mentioned that *AOX2* promoter driven protein expression is usually very low compared to the strong *AOX1* promoter. By engineer-

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ing a weaker promoter, it might be relatively easy to reach increased levels compared to a very strong promoter where optimisation hardly ever results in better variants compared to naturally evolved strong promoters.

PpAOX1 *promoter*

In the *AOX1* promoter a region of 243 bp (-415 to – 172) was identified, which is involved in induction by methanol. It has been shown that a homolog of the *S. cerevisiae* transcription factor Adr1p, termed Mxr1p (methanol expression regulator 1) binds to this region [125]. This is consistent with an earlier finding: deletion of ~152 bp within the aforementioned 243 bp resulted in a loss of ~80 % promoter activity under inducing conditions [126,127]. Two further protein binding sites were identified in the promoter region using bandshift assays, one upstream of position -690, and the second between -540 and -400 (note: numbers are counted starting 5' of the ATG start codon of the *AOX1* gene). A binding event upstream of the -690 position was detectable in medium containing methanol as sole carbon source, but no bandshift was found as long as ethanol was present in the medium. Under both conditions a protein was found to bind between positions -540 and -400, when methanol was the sole carbon source or with a mixture of methanol and ethanol in the medium. Since the two proteins, which are still unknown, do not compete for the two fragments, there is some evidence that they are of different nature [126].

CbFDH1 *promoter*

Two *cis*-acting elements were identified by deletion analysis of the *C. boidinii FDH1* promoter region. Deletion of the first had a strong impact on methanol induction without affecting induction of reporter enzyme (*S. cerevisiae* acid phosphatase *ScPHO5*) expression by formate. This region was termed UAS-M. The second region was involved in both, methanol and formate induction and was therefore named UAS-FM. Induction was carried out with methanol as sole carbon source while formate induction was done with glucose as second carbon source present in the medium. In order to determine the effect of these elements, several promoter variants of the *FDH1* and the constitutive *ACT1* (actin) promoter bearing one or more extra copies of the UAS-FM sequence stretch were created. Addition of two extra copies of this element to the *FDH1* promoter at different positions resulted in a ~2.5-fold increase while addition of four copies resulted in an almost 5-fold increase of reporter enzyme activity upon either methanol or formate induction. The effect was also tested on the constitutive *CbACT1* promoter. Interestingly, addition of several copies of UAS-FM to the *ACT1* promoter resulted not only in an inducible promoter, but the effect was even more pronounced than in the *FDH1* promoter. Addition of two copies resulted in a ~4–5-fold increase of promoter activity while an *ACT1* variant with four copies of the UAS-FM element displayed an 8–10-fold increase of reporter enzyme activity. While the best *FDH1*-derived promoter was almost 5-times stronger than the wild type promoter, the best *ACT1*-derived variant reached 60 % of the *FDH1* wild-type strength if induced by methanol. Remarkably, if induced by formate the activity due to the best *ACT1* promoter variant was ~180% of the *FDH1* and ~200 % of the *ACT1* wild-type promoter. Although an induction module was added to the *ACT1* promoter, no additional glucose repression was found since its activity on glucose was only slightly affected by these modifications [128]. Part of the UAS-FM sequence fits to the consensus sequence of 5 methanol-inducible promoters as defined by Ohi *et al*. [93] and re-defined by Yurimoto *et al*. [87]. These increases in promoter strength are quite remarkable. Unfortunately no further reports were published where these promoters were used for recombinant gene expression.

HpMOX *and* HpFMD *promoter*

A rather interesting approach to engineer promoters of the methylotrophic yeast *H. polymorpha* was published by Suckow *et al*. [129,130]. Sequences similar to palindromic sequences of leucine zipper transcription factor (bZip, e.g. Gcn4p, [131]) binding sites were mutated to get a palindromic or a pseudo-palindromic binding site. By introducing palindromic bZip-protein recognition sites by mutagenesis of cryptic binding sites within the methanol-inducible *MOX* and *FMD* promoters and the heat-inducible *TPS1* promoter in *H. polymorpha*, both an increase in promoter strength was achieved as well as a change in the regulation pattern of these promoters. One engineered *FMD* promoter resulted in an enhanced production of the reporter enzymes β-galactosidase (3.5-fold) and phytase (~1.5-fold) in medium with glycerol as carbon source while a second variant resulted in elevated production rates of the two reporter enzymes (5 and 2-fold, respectively) in glucose-containing media [129,130]. This is a very remarkable result, because enhancing the activity of an already very strong promoter just by introduction of new (pseudo-) palindromic sites (which might act as transcription factor binding sites) might be a very interesting approach in general.

Trans-*acting factors and signalling*

H. polymorpha homologs of *S. cerevisiae* Snf1p and Tup1p, two major players in glucose repression [132], were isolated and disrupted. Although disruption of these proteins had a severe impact on growth of *S. cerevisiae*, no major effect was observed in *H. polymorpha* when grown either on glucose or methanol as carbon source. In contrast to the *TUP1* knockout where no apparent phenotype could be observed, transcription of the *MOX* and *DAS* genes was reduced under methanol inducing conditions

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in the *snf1Δ* strain. Interestingly, although the mRNA levels of these two genes were significantly reduced, the growth of the *snf1Δ* was not distinguishable from the wild type strain, indicating that reduction of the *MOX* and *DAS* transcription does not result in a slowdown or bottleneck in methanol utilisation [133].

Expression of *lacZ* under the control of the *H. polymorpha MOX* promoter in *S. cerevisiae* was repressed by glucose. Derepression occurred upon glucose depletion and the observed derepression effect was dependent on the transcription factor Adr1p [122]. Adr1p is a major regulator of the glucose-repressed *ADH2* gene coding for alcohol dehydrogenase 2 and some peroxisomal proteins in *S. cerevisiae* [134,135]. In contrast to Adr1p, Mig1p (a main repressing DNA-binding protein in *S. cerevisiae* [132]) was not involved in the repression/derepression process of the *MOX* promoter in *S. cerevisiae* [123]. At least in *H. polymorpha* the *MOX* promoter was shown to be organised into nucleosomes [136], and a conformational change in chromatin structure takes place upon derepression [123]. As for several other genes, chromatin remodelling took place during derepression of the *ADH2* gene in *S. cerevisiae* and this event was presumably a prerequisite for TATA-box accessibility to the transcription machinery and therefore efficient transcription. Adr1p is thought to be involved in this remodelling by triggering chromatin destabilisation thereby enabling transcription upon glucose depletion [137,138].

Two homologs of the *S. cerevisiae SWI/SNF* complex, involved in chromatin remodelling, were isolated and disrupted in *H. polymorpha*. The *H. polymorpha* homologs of the *S. cerevisiae* Snf2p and Swi1p play a role in the regulation of the MUT pathway. Growth of the *H. polymorpha snf2Δ* and *swi1Δ* strains was fully impaired on methanol while they exhibited only a slightly slower growth rate on glucose and sucrose. But in both cases the cultures ultimately reached the same final optical density. On glycerol or oleate as sole carbon source, the strains exhibited an even more pronounced slow-growth phenotype, and the final optical density was also lower. The expression of the native *MOX* and the reporter β-lactamase, both regulated by the *MOX* promoter, were drastically reduced in these mutants (more than 200 fold in the *snf2Δ* and more than 50-fold in the *swi1Δ* strain). Down-regulation of *MOX* might cause the growth defect on methanol [139].

Therefore, a strong evidence exists for an involvement of chromatin remodelling by the *SWI/SNF* complex in derepression of the *MOX* and most probably also of the *DAS* promoter in *H. polymorpha*. In *S. cerevisiae* the *SWI/SNF* complex was estimated to control ~6 % of all genes, with the control presumably being exerted at the level of individual promoters rather than over chromosomal domains. Therefore, recruitment of the *SWI/SNF* complex by DNA-binding regulatory proteins [140] might be a major function of *MOX* binding regulatory proteins in *H. polymorpha* [88,122].

From all the reports published on the regulation of the *HpMOX* promoter, it seems clear that the TSP shift and chromatin remodelling play a major role for the derepression this promoter. Whether these two mechanisms are acting together or just acting in parallel remains to be studied. However, because similar studies are absent for the other methylotrophic yeasts, extrapolation of the results to these yeasts is not possible.

Recently, a key regulator of the MUT pathway and other peroxisomal genes, known as Mxr1p, was identified in *Pichia pastoris* [125]. Binding of this factor to the *AOX1* promoter sequences was already described in the chapter about *cis* elements. A mutation or deletion in the *MXR1* gene caused the loss or at least a reduction of transcription of MUT pathway genes and other peroxisomal genes. This was accompanied by phenotypical effects such as a complete loss of the ability to grow on methanol or oleate as carbon source but also a reduction of the growth rates on glucose, glycerol and ethanol [125]. In addition, the deletion caused a complete loss of *AOX1* promoter driven expression as shown by western analysis and reporter assays using β-lactamase as reporter enzyme. Also the transcription of *DAS* was almost completely abolished. The effects on the other MUT pathway enzymes were not as severe as for *AOX1* and *DAS*, but still significant. Specific enzyme activities of *FLD*, *CAT* and *FDH* were reduced to ~7 % (*FDH*) or ~20–30% (*FLD* and *CAT*) [125]. In contrast to Adr1p in *S. cerevisiae*, which was regulated on the transcriptional level and expressed only if glucose was absent in the medium [132], Mxr1p was expressed constitutively at a very low level. Its regulation was performed via its subcellular localisation: Mxr1p was mainly in the cytosol as long as glucose was present in the medium and mainly in the nucleus in media containing methanol or oleate as carbon source. Regulation by nuclear localisation is also known for Mig1p in *S. cerevisiae*, however, due to the repressor function of *Sc*Mig1p, its localisation pattern is the opposite of the *Pp*Mxr1p localisation pattern [132].

Interestingly, cytochrome c (*cyt c*), isolated from *P. pastoris* or *S. cerevisiae*, was found to bind *in vitro* specifically to the *AOX2* UAS found by Ohi *et al*. [93]. *Cyt c* isolated from rabbit heart was not able to bind to this sequence. Mutations within the UAS which were described to abolish the ability to activate transcription *in vivo* also abolished *cyt c* binding *in vitro* [141]. Besides its role in respiration by transferring electrons from complex III to complex IV of the respiratory chain, *cyt c* is also involved in apoptotic

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pathways after its release from mitochondria (e.g. [142]). Interestingly, employing *AOX1* promoter based expression, significant fractions of apoptotic cells in deep well plate cultures grown at high glucose or glycerol concentrations led to reduced expression of active enzymes in *Pichia pastoris* [143]. As already discussed in the chapter about *cis*-acting elements, crosstalk of mitochondria and peroxisomes is quite interesting in the context of the high oxygen demand of alcohol oxidase for the oxidation of methanol. As demonstrated and widely discussed in the case of apoptosis, retrograde signalling to the nucleus might also be intuitively logic in the coordination of respiration and peroxisomal function in yeast. Whether cytochrome c is a part of the signalling cascade from mitochondria to the nucleus (where all peroxisomal proteins and most mitochondrial proteins are encoded) or if there is a regulatory role of *cyt c* directly in the nucleus in *P. pastoris* by binding to e.g. the *AOX2* promoter remains to be elucidated.

A single strand binding (SSB) protein was identified in *P. pastoris* cells [144]. The protein which binds to the near upstream region of the *AOX1* promoter between -288 and the major transcription start point at -115 was identified as zeta crystalline (*ZTA1*). Interestingly, the SSB activity of recombinant *Pp*Zta1p (produced in *E. coli*) *in vitro* is completely abolished in presence of NADPH while NADH does not have any influence. Although not proven experimentally, the authors speculate on a regulatory function of this protein under oxidative stress conditions as described for the bovine or the *Arabidopsis thaliana ZTA1* homolog [144].

Effects of glucose signalling, transport and phosphorylation on gene regulation of MUT pathway gene promoters

Studying glucokinase (GK) and hexokinase (HK) mutants of *H. polymorpha* derived from EMS (ethane methyl sulfonate) mutagenesis revealed the necessity of glucose or fructose phosphorylation for repression by these carbon sources. A hexokinase mutant did not perform fructose repression of *AOX* and *CAT* activities (HK is the only fructose-phosphorylating enzyme) [145,146]. However, since both enzymes (GK and HK) are able to phosphorylate glucose, only the double knockout of GK and HK releases glucose repression. Once no phosphorylation of the substrate occured no repression of the two MUT pathway enzymes studied took place [145-147]. A similar effect was found in *S. cerevisiae* where substrate phosphorylation by either one of the both hexokinases and the glucoskinase was necessary for the repression effect [148,149].

Another *H. polymorpha* mutant insensitive to glucose repression was found to have a mutation in a protein homologous to glucose transporters. The mutant earlier found upon UV mutagenesis (*gcr1-2*) [150] could further be assigned to a gene encoding for a glucose transporter homolog (*GCR1*) [151]. Interestingly, the release of glucose repression phenotype was less severe in the deletion knockout strain compared to the mutated strain. In addition, the mutated strain had no apparent growth phenotype on fructose as carbon source and *AOX* activity was still repressed by fructose. On the contrary, the deletion knockout strain was severely hampered for growth on fructose and repression of *AOX* activity by fructose was released in this strain. This difference was explained by the fact that in the mutated gene, only the transport of glucose was impaired while the function relating to both glucose and fructose was inhibited in the knockout deletion strain [151].

In *H. polymorpha* two glucose uptake systems, one high-affinity and one low-affinity glucose transporter, were described [152]. While the high-affinity transporter was found to be expressed selectively at glucose-derepressing conditions (0.1 % glucose) and when methanol and ethanol are used as carbon source, the low-affinity transporter was found to be acting at high glucose concentrations. The K_m values for both transporters were estimated to be ~0.05 mM and 1.75 mM, respectively. These numbers were similar to the respective values of the glucose uptake systems found in *P. pinus* MH4 (*P. methanolica*) [152]. Since a *gcr1-2* mutant strain exhibited a more severe growth defect on low glucose concentrations than at high glucose concentrations, the *GCR1* gene was thought to encode for a high-affinity glucose transporter [151]. It is noteworthy, that in *K. lactis* the glucose transport system was found to have one major low-affinity glucose transporter, encoded by *RAG1* and one high-affinity transporter, encoded by *HGT1* [153]. The unique hexokinase (encoded by *RAG5*), which is the only glucose phosphorylating enzyme in *K. lactis*, was responsible for expression of the transporter genes as well as for glucose repression [154].

Taken together, although part of the work was performed using a different wild type backgrounds, this data depicts a situation similar to that found in *S. cerevisiae* and *K. lactis*. Glucose (or e.g. fructose) uptake and phosphorylation of the substrate by kinases (gluko- or hexokinases) are prerequisites for repression of the MUT pathway. If no substrate sensing or uptake or phosphorylation takes place, the MUT pathway is released from repression.

Glucose repression mutants and autophagy

Several mutants of the methylotrophic yeasts *C. boidinii* [80], *H. polymorpha* [79,150,155], *P. methanolica* (*P. pinus* MH4) [156,157] and *P. pastoris* [158,159] have been isolated which released the glucose repression of MUT path-

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way genes. The mutants have high activities of MUT pathway enzymes when grown on glucose or both glucose and methanol as carbon source. When growing on a mixture of glucose and methanol as carbon source, they usually utilise both substrates in parallel, in contrast to the wild type strains which possess a diauxic growth when grown on glucose and methanol mixtures in batch cultures [79,80,150,151]. Mutants with released glucose repression still were repressed by ethanol [79,80,151,157]. Vallini *et al*. isolated several *H. polymorpha* Mut$^-$ mutants grouping together in 12 complementation groups. Mutants in only three complementation groups were found to possess very low alcohol oxidase activities due to low promoter strength, not only in methanol, but also in glycerol and methylamine containing medium [160].

Mutants with released glucose repression still showed glucose-induced autophagy of peroxisomes when shifted from methanol to glucose containing medium which reveals again a distinct mechanism for autophagy induction [79,80,151]. Catabolite inactivation of alcohol oxidase and peroxisomes upon shift from methanol to the repressive carbon source glucose was found to be triggered by a short pulse of intracellular cAMP in *H. polymorpha* cells [161]. A cAMP pulse prior to degradation of *AOX* activity was also found in *P. pastoris* when shifted to ethanol-containing media [162]. The mode of autophagy was reported to depend on the intracellular ATP level [163]. The α-subunit of phosphofructokinase was involved in triggering the signal for autophagy in *P. pastoris*. The enzyme, but not the enzymatic activity, was found to be responsible for the onset of peroxisome degradation, indicating a direct role of phosphofructokinase enzyme in signal transduction or different functional domains on the peptide [164].

Promoters of MUT pathway genes in heterologous hosts

So far, the *P. pastoris AOX1*, *DAS* and *H. polymorpha MOX* promoters were studied in *S. cerevisiae* where they promoted expression of the reporter enzyme β-galactosidase (*lacZ* of *E. coli*) [92,122]. With a few exceptions in the details, the regulation profiles of these promoters in *S. cerevisiae* were similar to their natural hosts: glucose repressed gene expression, during carbon starvation conditions expression was slightly derepressed, and glycerol as carbon source induced expression, even in the case of the *P. pastoris* promoters. β-galactosidase levels expressed under the control of *AOX1* and *DAS* regulatory regions in *S. cerevisiae* grown on glycerol were comparable to those obtained with the strong *S. cerevisiae CYC1* and *GAL2* promoters on raffinose and galactose, respectively [92]. In *S. cerevisiae* expression driven by the *MOX* promoter was also induced by ethanol, methanol or oleic acid. The involvement of Adr1p in derepression/induction of the *MOX* promoter was a very important finding in this context [122].

Two genes coding for the alcohol oxidase isoenzymes, *ZZA1* and *ZZA2*, are known from a *Pichia pastoris* strain isolated at the UC Davis campus (H. Phaff, strain ID 72–1033). The promoter of *ZZA1* gene was employed to drive expression of α-amylase in *S. cerevisiae*. In *S. cerevisiae* α-amylase expression from this promoter, which shares 66% identity to the *AOX1* promoter from *P. pastoris* NRRL Y-11430, was repressed by glucose but induced by glycerol, sucrose and ethanol, which is similar to the *AOX1* regulation in *S. cerevisiae* [165,166]. Unfortunately, no regulation was described for this promoter in *P. pastoris* therefore no information is available for the regulation of the *ZZA1* promoter in its own host. Although the regulation pattern is similar in *S. cerevisiae*, no conclusion could be drawn on the similarities or differences between the *ZZA1* and the *AOX1* promoter in *P. pastoris*.

As mentioned before, the regulation patterns of the *AOX1* and the *MOX* genes are significantly different in their natural hosts due to the derepression of *MOX* when glycerol is present. Using the *AOX1* promoter region in *H. polymorpha* revealed that the this promoter was not repressed by glycerol in the heterologous host [167,168]. For example, expression of the Kunitz protease inhibitor domain of the human β-amyloid precursor protein (KPI) [167] was detected using a glycerol containing medium. Both mRNA as well as protein were found in significant amounts whereas none was detected in a *P. pastoris* expression strain under the same conditions [167]. Similar results were found for the expression of the *S. cerevisiae* invertase (*SUC2*) gene under control of the *P. pastoris AOX1* promoter in *H. polymorpha* [168]. Thus, the heterologous *AOX1* promoter is regulated like the homologous *MOX* promoter in *H. polymorpha*. Therefore, the *AOX1* promoter is well recognised in the heterologous host and repression by glycerol is a host specific effect. Interestingly, the glucose-repressed and maltose-induced *H. polymorpha* maltase gene promoter is also subjected to glucose repression and maltose induction in *S. cerevisiae*, similar to the regulation in the homologous host *H. polymorpha* [169]. Although not a MUT pathway gene, the maltase gene promoter again demonstrates the high degree of conservation of gene regulation between different yeast species.

A very interesting report was published in 2005 about the use of the hypoxia-induced *ADH2* promoter from *Pichia stipitis* [170] in *Pichia pastoris* for the production of bacterial haemoglobin. Although some control experiments were missing in this paper, such as a comparison to non-inducing conditions and a comparison to a reference promoter from *P. pastoris* (e.g. GAP dehydrogenase promoter), this is another example of a successful transfer of

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promoters and their functionality in heterologous hosts [171,172].

In the same fashion, the transfer of *S. cerevisiae* promoters to methylotrophic yeasts has been reported. The *ScCUP1* (*S. cerevisiae* metallothionein, *CUP1*) promoter regulated expression of the *lacZ* gene in *P. pastoris* and consequently resulted in a strong increase of β-galactosidase activity after addition of Cu^{2+} to the medium. The induction level could be fine-tuned by the Cu^{2+}-concentration in the medium in a range between 1 and 200 μM just as in its native host [173]. Thus, these heterologous expression systems offer the flexibility that a researcher can combine a promoter and a host cell to attain a desired expression profile.

Conclusion

The regulation of the MUT pathway shows similarities among methylotrophic yeasts, but there are some significant differences. In general, all enzymes are induced by methanol and the methanol assimilation pathway enzymes usually are subjected to glucose repression while the dissimilation pathway enzymes are not. However, among the reviewed yeasts there are also significant differences e.g. in regulation of the alcohol oxidase genes: In *C. boidinii* and *H. polymorpha AOX* expression is strongly derepressed by glycerol, although at different levels. Addition of methanol further boosted *HpMOX* promoter activity up to levels reached by induction using methanol as sole carbon source. In presence of glycerol, *AOX* expression is still repressed in *P. pastoris* while *P. methanolica* combines both expression patterns: *MOD1* is regulated like the *H. polymorpha* and *C. boidinii* alcohol oxidase genes with derepression on glycerol as carbon source while *MOD2* is regulated similar to the *P. pastoris AOX1/2* genes where no activity is found in glycerol containing media. *P. pastoris AOX1* and *AOX2* are regulated according to the classical repression/induction mechanism which implicates the need for absence of glucose (or glycerol, ethanol) as well as the presence of methanol for full induction of the promoter-driven expression. The *C. boidinii AOD1*, *H. polymorpha MOX* and the *P. methanolica MOD1* gene regulation might be described better by a repression/derepression mechanism. Absence of the repressive carbon source is sufficient for some degree of expression which differs among the yeasts (see Table 3). Methanol enables full induction of the alcohol oxidase expression also in the presence of the non-repressing carbon source. This is also true for the *P. methanolica MOD2* gene, although no significant level of derepression was observed using glycerol as carbon source.

The differences found about the regulation of *AOX* promoters among the different yeast species are mainly attributed to the host regulatory machinery and not to the promoter regions. In *H. polymorpha* the *P. pastoris AOX1* promoter is regulated exactly like the homologous *MOX* promoter. In the *H. polymorpha* cell *PpAOX1* promoter driven expression is significantly derepressed on glycerol as carbon source. The interesting fact that both regulatory modes are combined in *P. methanolica* raises the question how this yeast is able to distinguish between the two promoters and how the exact regulation takes place. Promoter regulation and glucose repression is highly conserved among yeasts since the glucose-repressed *AOX* promoters are recognised and specifically regulated in heterologous hosts. The promoter brings in the overall regulatory feature like glucose repression and e.g. methanol induction and the yeast supplies the characteristic regulatory pattern caused by different carbon sources, as demonstrated for the *P. pastoris AOX1* and the *H. polymorpha MOX* promoters. Unfortunately, the puzzling question how *PpAOX1*, *HpMOX*, or *CbAOD1* driven expression is regulated in *P. methanolica* which combines both regulatory features, is still unanswered.

Despite their very interesting properties, the promoters of the *DAS*, *FLD* and *FDH* genes are not frequently used for recombinant protein production with the exception of the *H. polymorpha FMD* (=*FDH*) promoter, which is as frequently used as the MOX promoter. The *DAS* promoter should be a quite good alternative to the *AOX* promoters since it is as tightly regulated and seems to be even stronger than the respective *AOX* promoter, at least in *P. pastoris* and *C. boidinii* and with the reporter enzymes used so far. *FLD* and *FDH* genes are usually not subjected to glucose repression, thereby enabling induction with nitrogen sources like methylamine or choline in the presence of glucose. In the case of *C. boidinii* the *FDH1* promoter was also induced with formate in the presence of glucose. Broadening the promoter spectrum in methylotrophic yeasts with different regulatory features will strengthen the position of these yeasts for recombinant protein production and in addition open new challenges and opportunities in metabolic engineering.

In addition, identification of regulatory regions within promoter sequences and the identification of DNA binding proteins and the respective pathways and networks will help to understand the regulation of these promoters. Several transcription factor binding sites were identified in all *AOX* promoters analysed so far. Although several factors involved in glucose repression in methylotrophic yeasts have been identified so far, no complete picture has been generated about the full system of glucose repression and induction by methanol or derepression by other carbon sources works. Aided by extensive available literature, a detailed analysis of gene regulation of the MUT pathway in yeasts should be possible. However, there is just a major problem of comparability of results due to varying

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reporter enzymes and cultivation conditions. In several cases, the comparison to standard promoter systems is missing, which makes it almost impossible to directly reconcile the data of one publication with another.

Meanwhile the genome sequences of *P. pastoris* and *H. polymorpha* have been determined [174] which should accelerate research on these yeasts. In addition these genome sequences make comparative genomics possible, thereby enabling the transfer of glucose repression mechanism models from the well-characterised yeasts *S. cerevisiae* and *Kluyveromyces lactis* to methylotrophic yeasts and consequently their adaptation.

Competing interests

The author(s) declare that they have no competing interests.

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Promoter library designed for fine-tuned gene expression in *Pichia pastoris*

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ABSTRACT

Although frequently used as protein production host, there is only a limited set of promoters available to drive the expression of recombinant proteins in *Pichia pastoris*. Fine-tuning of gene expression is often needed to maximize product yield and quality. However, for efficient knowledge-based engineering, a better understanding of promoter function is indispensable. Consequently, we created a promoter library by deletion and duplication of putative transcription factor-binding sites within the *AOX1* promoter (P_{AOX1}) sequence. This first library initially spanned an activity range between ~6% and >160% of the wild-type promoter activity. After characterization of the promoter library employing a green fluorescent protein (GFP) variant, the new regulatory toolbox was successfully utilized in a 'real case', i.e. the expression of industrial enzymes. Characterization of the library under repressing, derepressing and inducing conditions displayed at least 12 *cis*-acting elements involved in P_{AOX1}-driven high-level expression. Based on this deletion analysis, novel short artificial promoter variants were constructed by combining *cis*-acting elements with basal promoter. In addition to improving yields and quality of heterologous protein production, the new P_{AOX1} synthetic promoter library constitutes a basic toolbox to fine-tune gene expression in metabolic engineering and sequential induction of protein expression in synthetic biology.

INTRODUCTION

The methylotrophic yeast *Pichia pastoris* is a well-established yeast expression host with rising popularity. Several hundred proteins from almost all kingdoms of life have been expressed in this yeast, either intracellularly or secreted into the medium (1). As a yeast, *P. pastoris* combines the advantages of eukaryotes, like efficient secretion and eukaryotic post-translational modifications (e.g. proteolytic processing, glycosylation and disulfide bond formation), with fast growth on economic, salt-based media, with little risk of contamination with endotoxins or oncogenic or viral DNAs (2). In addition, *P. pastoris* belongs to the group of Crabtree negative yeasts and has a very low maintenance energy demand, which makes it well suited for high cell density (HCD) fermentation (3–5). Along with these favourable physiologic characteristics, the availability of strong and tightly regulated promoters makes this yeast a very attractive host for recombinant protein production. In fact, in HCD processes, up to 13 g/l of secreted protein (6) or 22 g/l intracellular protein have been obtained (7).

Although several promoters, either constitutive or inducible, are available for this yeast [P_{AOX1} (8), P_{AOX2} (9), P_{FLD1} (10), P_{DAS} (8), P_{PEX8} (P_{PER3}) (11), P_{YPT1} (12), P_{GAP} (13), P_{TEF1} (14), P_{PGK1} (15), P_{ICL1} (16)], the promoter of the alcohol oxidase I gene (*AOX1*) has been employed in most studies and applications (1). *AOX1* is the predominantly expressed form of two alcohol oxidases (*AOX1* and *AOX2*) in the *P. pastoris* cell. Besides being the first well-characterized promoter of this yeast and its availability in a commercial kit (Invitrogen, Carlsbad, CA, USA), P_{AOX1}'s extraordinary strength and tight regulation by carbon sources are the main reasons for its predominant use. Being tightly repressed by glucose, its transcription is highly induced upon shift to methanol as sole carbon source (8,17). Other carbon sources which repress transcription from the *AOX1* gene are glycerol and ethanol, while very low derepression of transcription occurs upon carbon starvation (8,17). In contrast to the alcohol oxidase genes from the other prominent methylotrophic yeasts (*MOX* from *Hansenula polymorpha*, *AOD1* from

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Candida boidinii and *MOD1* and *MOD2* from *Pichia methanolica*), regulation of both alcohol oxidase genes in *P. pastoris* is strictly regulated. Although glucose repression of methanol utilization pathway genes takes place in all these yeasts, the presence of methanol is not absolutely necessary for high-level induction of alcohol oxidases in the other methylotrophic yeasts studied so far (17).

Despite its industrial importance and multiple manuscripts analyzing its expression under different fermentation strategies (18,19), little is known about the regulation of the *AOX1* promoter at the molecular level, either in terms of the *cis*- or *trans*-acting elements. Deletion analysis of P_{AOX2}, the promoter of the minor alcohol oxidase gene *AOX2*, resulted in the identification of three *cis*-acting elements in that promoter, two upstream repression sites (URS1 and URS2) and one upstream activation sequence (UAS) (9). Although some sequence similarity between the *AOX2* UAS to regions within the *AOX1* promoter was detected, no conserved function has been demonstrated so far. Lin-Cereghino *et al.* (20) identified a positive acting transcription factor (TF) (*MXR1*), which bound a P_{AOX1} region of approximately 250 base pairs. Mxr1p displayed sequence similarity to the *Saccharomyces cerevisiae* zinc-finger TF Adr1p, which is involved in regulation of the glucose-repressed *ADH2* gene and some peroxisomal proteins in baker's yeast (21,22). *Cis*-acting elements sensitive to methanol induction were also identified in the promoter sequences of the *H. polymorpha MOX* (23) and the *C. boidinii FDH* genes (24). Similar sequences exist in the *P. pastoris AOX1* promoter sequence.

Furthermore, it has been demonstrated that the endogenous Adr1p is involved in the induction of the *MOX* promoter upon shift from glucose to glycerol in *S. cerevisiae* by directly interacting with the promoter nucleotides between −245 and −112 (25). Taken together, focusing on putative transcription factor binding sites (TFBSs) within *P. pastoris* P_{AOX1} should give some insight into how this promoter is regulated and provide elements for a modular synthetic promoter design, even if the function of the respective TFs and/or their binding sites have been changed in *P. pastoris* during evolution.

In this study, we followed three major aims: (i) for the first time, we employed a comprehensive deletion analysis to identify the individual effects of small sequence elements and to surmise, which TFs might be involved in P_{AOX1} regulation; (ii) we created a simple set of promoters with regulatory properties different from P_{AOX1}, which are more desirable for heterologous protein expression and cell engineering and (iii) we demonstrated that even the extraordinarily strong *AOX1* promoter still has considerable potential for improvement by engineering.

MATERIALS AND METHODS

Sequence analysis of the *AOX1* promoter

The P_{AOX1} sequence from pPICZ B (downloaded from www.invitrogen.com) was used to search for putative TFBSs. TF analysis was done with MatInspector (26,27) release professional 6.1 January 2003 within the GenomatixSuite 1.6.1 at Genomatix Software GmbH Servers using the Matrix Family Library Version 3.1.1 April 2003 group ALL fungi.lib (www.genomatix.de). Beside this analysis, sequence modifications which were already described in literature (9,23,28) and their effects upon promoter derepression and induction were compared.

Chemicals and media

Oligonucleotides were purchased from either MWG Biotech AG (Ebersberg, Germany) or Invitrogen Corp. (Carlsbad, CA, USA). Sterile water was purchased from Fresenius Kabi Austria (Graz, Austria). Unless otherwise stated, all chemicals were purchased from Carl Roth GmbH (Karlsruhe, Germany), Becton, Dickinson and Company (Franklin Lakes, NJ, USA) and Sigma-Aldrich (St Louis, MO, USA). Media for small-scale experiments in deep-well plates were prepared essentially as described earlier (29). Complex media contained 10 g/l yeast extract, 20 g/l peptone and either 20 g/l glucose (YPD) or 10 g/l glycerol (YPG). Media for plates were solidified by addition of agar to 1.5% w/v. All five minimal media used contained per litre 200 mM potassium phosphate buffer at pH 6, yeast nitrogen base 13.4 g and D-biotin 0.0004 g, and differed with respect to the substrate concentration of 10 or 0.2 g/l of glucose, 0.5 g/l of glycerol or 0.5, 1 or 5% (v/v) of methanol for BMD (1%), BMD (0.2%), BMG (0.5%), BMM, BMM2 or BMM10, respectively.

All seed cultures (and inoculum cultures) were prepared using the buffered glycerol-complex medium (BMGY, Invitrogen), which contained per litre glycerol 10 g, yeast extract 10 g, peptone 20 g, 100 mM potassium phosphate buffer (pH 6.0), yeast nitrogen base without amino acids 13.4 g and biotin 0.0004 g.

The defined mineral medium used for the main fedbatch cultures contained per litre $CaSO_4 \cdot 2H_2O$ 0.17 g, K_2SO_4 2.86 g, KOH 0.64 g, $MgSO_4 \cdot 7H_2O$ 2.3 g, EDTA 0.2 g, H_3PO_4 7.225 g, $FeSO_4 \cdot 7H_2O$ 0.2826 g, H_3BO_3 0.00087 g, $CuSO_4$ 0.0167 g, $MnSO_4 \cdot H_2O$ 0.013 g, $Na_2MoO_4 \cdot 2H_2O$ 0.00087 g, $ZnCl_2$ 0.087 g, NaI 0.00035 g, $CoCl_2 \cdot 6H_2O$ 0.004 g, H_2SO_4 (Synopharm, Basel, Switzerland) 0.04 g and biotin 0.00087 g. In total, per litre either 784.2 g glucose monohydrate or 773.24 g methanol was completed with 0.0024 g biotin and 12 ml PTM1 mineral salts solution [i.e. containing per litre 5.0 ml of 69% H_2SO_4 (Synopharm), $CuSO_4$ 3.84 g, NaI 0.08 g, $MnSO_4 \cdot H_2O$ 3.0 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, H_3BO_3 0.02 g, $CoCl_2 \cdot 6H_2O$ 0.92 g, $ZnCl_2$ 20.0 g and $FeSO_4 \cdot 7H_2O$ 65.0 g].

Strains and plasmids

Standard molecular biology procedures were performed according to ref. (30). *Escherichia coli* DH5α-T1^R or TOP10F' (Invitrogen Corp.) were used for all *E. coli* cloning experiments.

The cycle-3-GFP (31) coding sequence was amplified from pTRACER-CMV2 (Invitrogen Corp.) using Phusion^{TM} polymerase (Finnzymes Oy, Espoo, Finland) with primers GFPZeo forw and wtGFP-XhoI-r according to the supplier's manual. The PCR product was TOPO

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cloned into vector pCR4blunt-TOPO (Invitrogen Corp.) according to the supplier's manual. Unfavourable BamHI and XhoI restriction sites within the GFP coding sequence were removed using primers BamHI-del-f and XhoI-del-f using the QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene Corp.) according to the supplied protocol. The GFP gene was subsequently cloned into vector pGAPZ A (Invitrogen Corp.) employing EcoRI and XhoI as restriction enzymes. The *AOX1* promoter was synthesized according to the sequence of the plasmid pPICZ B and cloned into vector pUC18. The plasmid was cut with BglII and EcoRI, the promoter fragment was purified via agarose gel electrophoresis and the Wizard® SV Gel and PCR Cleanup System (Promega Corp., Madison, WI, USA) and ligated into the BglII- and EcoRI-cut vector pGAPZA-GFP. The resulting vector was named pPICZ-GFP.

Generation of promoter mutants

Short deletions or insertions within the *AOX1* promoter of pPICZ-GFP were generated by site-directed mutagenesis (SDM) using PfuUltra™ polymerase (Stratagene Inc.) according to the two-step SDM protocol (32). All other modifications which were not introduced by SDM were generated by overlap extension PCR (oePCR). 5′ arms of P_{AOX1} were synthesized by PCR using P(AOX1)forw as forward primer and appropriate reverse primers, 3′ arms using the appropriate forward primers and P(AOX1)rev as reverse primer. Truncated *AOX1* promoter variants were generated using an appropriate forward primer and P(AOX1)rev as reverse primer.

All arms were synthesized using 12 ng of pPICZ B as template and 10 pmol of each primer. The reaction also contained 200 µM of each dNTP and 0.6 U *Pwo* DNA polymerase (MWG Biotech GmbH) in appropriate buffer conditions in a final volume of 50 µl.

PCR was performed in an GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA) for 30 cycles (95°C, 1 min; 55°C, 1 min; 68°C, 1 min 30 s) with an initial denaturation step of 5 min at 95°C and a final extension step of 10 min at 68°C. All arms were purified by agarose gel electrophoresis and gel extraction prior use for oePCR. The reaction contained 10 ng of each purified arm, 200 µM of each dNTP and 0.6 U *Pwo* DNA polymerase in appropriate buffer conditions in a final volume of 50 µl. PCR was performed according to the manufacturer's suggestion. After 10 cycles, 20 µl of a mixture containing 10 pmol of the outer primers P(AOX1)forw and P(AOX1)rev, again 200 µM of each dNTP and 0.6 U *Pwo* DNA polymerase in appropriate buffer conditions were added. The PCR was continued as programmed after addition of this amplification mixture.

The obtained PCR products representing P_{AOX1} variants with the desired size of about 898–947 bp were purified on an agarose gel and cloned into the pGAPZ-GFP backbone replacing P_{GAP}.

A basal Leu2 promoter was generated by mixing 25 pmol of oligos Leu2basal1fw, Leu2basal 1rv, Leu2basal 2fw and Leu2basal 2rv in a total volume of 20 µl. The mixture was incubated at 95°C for 2 min followed by a slow cooling to room temperature. Three microlitres of the mixture was ligated into BglII/EcoRI cut pGAPZ-GFP backbone vector thereby replacing P_{GAP}. The resulting vector was named pLeu2basal-GFP. Basal promoter variants AOX194 and AOX176 were generated by amplifying the basal fragment from vector pPICZ-GFP using reverse primer AOX1basalrv and forward primers AOX1basalfwn and AOX176fw, respectively. After purification, the PCR products were cut with BglII/EcoRI and ligated into vector pGAPZ-GFP, thereby replacing P_{GAP}. The resulting vectors were named pAOX194-GFP and pAOX176-GFP. Truncated promoter fragments added to basal promoter variants were amplified from pPICZ-GFP using reverse primer AOX1oe176rv and forward primers P(AOX1)forw, AOX1fw-SacBgl and 415BglIIn, thereby generating fragments with a 5′ end at −940, −731 and −411, respectively. After purification, the PCR fragments were cut with BglII/BspTI and ligated into vectors containing basal promoters Leu2, AOX176 and AOX194. The resulting vectors were named pLeu2-176-GFP-full, pLeu2-176-GFP-SacI, pLeu2-176-GFP-d1.0, pAOX176-GFP-full, pAOX176-GFP-SacI, pAOX176-GFP-d1.0, pAOX194-GFP-full, pAOX176-GFP-SacI and pAOX176-GFP-d1.0 (Supplementary Figure 1A). The promoter variants truncated at −203 were amplified from plasmids pLeu2-176-GFP-full and pAOX176-GFP-full using primer pairs 214BglII/LEU2basalrv and 214BglII/AOX1basalrv, respectively. After purification, the PCR products were cut with BglII/EcoRI and ligated into vector pGAPZ-GFP, thereby replacing P_{GAP}. The resulting vectors were named pLeu2-176-GFP-214 and pAOX176-GFP-214.

Synthetic promoter variants were generated by amplifying the basal AOX176 promoter with forward primers 201-214AOX176, 737-38AOX176, ADR-AOX176, Stre-AOX176, Mat-AOX176 and Gcr-AOX176 and reverse primer AOX1basalrv. The PCR fragments were purified, cut with BglII/EcoRI and ligated into vector pGAPZ-GFP, thereby replacing P_{GAP}. The resulting plasmids were named pAOX176-GFP-201-214, pAOX176-GFP-737, pAOX176-GFP-Adr1, pAOX176-GFP-Stre, pAOX176-GFP-Mat and pAOX176-GFP-Gcr1. All primers are listed in Supplementary Table 1.

Generation of plasmids for the expression of alternative reporter genes

An NdeI restriction recognition site was introduced by SDM 5′ of the BglII site at the 5′ end of the *AOX1* promoter in the plasmid pPICZαB-HRP (a kind gift of Frances Arnold, Caltech) containing the gene coding for the mature protein of horseradish peroxidase, isoenzyme C1A (HRP) fused to the *S. cerevisiae* α-factor signal sequence and the wild-type P_{AOX1} (33). This modification enabled the exchange of promoter variants in this plasmid employing NdeI and HindIII. A SbfI and an AscI restriction site were introduced employing the QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene Corp.) according to the supplied protocol. The final plasmid was called pHRP-AOX1. The newly introduced NdeI

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restriction site can also be used for plasmid linearization for *Pichia* transformation.

The *Pa*HNL5α gene (34), where the native hydroxynitrile lyase, isoenzyme 5 (HNL5) leader signal was replaced by the *S. cerevisiae* mating factor α leader sequence was cloned cloned into pGAPZ-A (Invitrogen Corp.) employing the EcoRI restriction sites flanking this gene construct on both ends. The resulting plasmid with the open reading frame in correct orientation was named pGAPPaHNL5α. All promoter variants were introduced into pGAPPaHNL5α employing the BglII and the EcoRI restriction sites. The resulting vectors were named pHNL5-AOX1 for the wild-type promoter and for promoter mutants the respective mutant name was added at the end.

The gene for the intracellular *Hevea brasiliensis* hydroxynitrile lyase gene (*Hb*HNL) was amplified from plasmid pHNL-400 (7) by PCR using primers HNL-Ecofw and HNL-Ecorv and ligated into pPICZ B (Invitrogen Corp.) employing EcoRI sites. The resulting plasmid was named pSHNL-AOX1. The *AOX1* promoter was replaced with promoter mutants via BglII and HindIII.

The series using the β-galactosidase reporter was constructed as follows. The 3.3-kb fragment of pLG178F [(35), kindly provided by Leonard Guarente, MIT, Cambridge, MA, USA], containing the *S. cerevisiae CYC1* TATA box fused to a partial *lacZ* coding sequence, was inserted into the same restriction sites in pBLHIS (36) to create pGC140. The 350 bp EcoRI–HindIII fragment, containing the *AOX1* transcription termination sequence of pHILD2 (Invitrogen), was then inserted into the same site of pGC140 to produce pGC141. A 1.3-kb PCR fragment, containing the *S. cerevisiae LEU2* TATA box fused to the 5′ end of the *lacZ* coding sequence, was amplified from pMC2019 [(37), kindly provided by Malcolm Casadaban, University of Chicago, Chicago, IL, USA] using the primers LEU1X and LEUR5. The PCR product was digested with XhoI and EcoRV and used to replace the XhoI–EcoRV fragment, containing the *CYC1* TATA element, of pGC141 to construct pGC146. The full-length *lacZ* coding sequence was cloned into pGC146 by amplifying a 2.1-kb fragment from pMC2019 with primers LACZX5 and LACZX3 and inserting it into the EcoRV and EcoRI sites of pGC146 to produce pGC187. The pGC187 contains the basal promoter of *S. cerevisiae LEU2*, with no upstream promoter region, fused to the complete coding sequence of *lacZ* followed by the *AOX1* transcription termination region. For amplification of *AOX1* promoter regions and cloning of sufficiency constructs, pHILD-2 was used as the template in PCR. The pGC179 was produced by inserting the 245-bp *AOX1* promoter region created by PCR with the primers SUFFS1 and SUFFX into the SpeI and XhoI sites of pGC187. The pGC180 was created by digesting pGC187 with SpeI and XhoI, blunt ending both ends with Klenow fragment and ligating the ends together. The pGC181 was produced by inserting the 742-bp *AOX1* promoter region created by PCR with the primers SUFFS0 and SUFFX into the SpeI and XhoI sites of pGC187. The pGC183 was constructed by inserting the 70-bp *AOX1* promoter region created by PCR with the primers SUFFS3 and SUFFX into the SpeI and XhoI sites of pGC187. pGC191 was produced by inserting the 45-bp *AOX1* promoter region created by PCR with the primers SUFF170S and SUFFAUS214X into the SpeI and XhoI sites of pGC179, replacing the 245-bp *AOX1* fragment.

Pichia transformation

Pichia pastoris was transformed according to a condensed protocol (38) after linearization of the plasmid with BglII and subsequent purification and concentration using the Wizard® SV Gel and PCR Cleanup System (Promega Corp.). The mixture was transferred to ice-cold electrotransformation cuvettes (0.2 cm, Bio-Rad, Munich, Germany) and pulsed at 200 Ω, 25 μF and 1.5 kV. 0.5 ml of ice-cold 1 M sorbitol was added immediately. The suspension was transferred to a sterile 12 ml PP-tube (Greiner, Frickenhausen, Germany). After regeneration for 1 h at 30°C without shaking, 0.5 ml of YPD was added followed by incubation for an additional hour. After regeneration, aliquots were plated on selection plates.

Promoter library characterization

A master plate containing representative strains bearing the promoter variant-GFP expression cassette was generated by inoculating 300 μl YPG with single colonies of the strains. After a 24 h incubation period at 28°C, 320 r.p.m., 80% humidity, 150 μl 60% glycerol was added and further incubated for 30 min. Then the library was transferred in 100 μl aliquots to 96-well microtitre plates and stored at −80°C. These glycerol stock master plates were used for all subsequent experiments.

A pre-culture in BMD (0.2%) was grown for 48 h to obtain uniform cell densities in all individual wells. The glycerol stock master plates were replica stamped to a preculture plate with 300 μl BMD (0.2%). The plate was incubated after inoculation at 28°C, 80% humidity and 320 r.p.m. for 48 h.

For determination of the promoter activity under repressing and derepressing conditions, three parallel deep-well plates containing 500 μl of appropriate growth medium [BMD (1%), BMG (0.5%)] was inoculated with 10 μl of the pre-cultures. Growth and GFP expression was followed by measuring optical density at 595 nm and fluorescence intensity for up to 70 h.

For determination of the promoter activity under methanol inducing conditions, three parallel deep-well plates containing 300 μl of appropriate growth medium, [BMD (1%) or BMG (0.5%)] was inoculated with 10 μl of the pre-cultures and incubated for 60 h. After this time, 20 μl were taken for determination of the GFP fluorescence and OD measurements. Induction was performed by adding 250 μl BMM2/well followed by a further incubation for 72 h. Additional methanol was fed by adding 50 μl BMM10 after 10, 24, 36, 48 and 60 h. Twenty microlitre samples were taken every 12 h before methanol addition for determination of fluorescence intensity and optical density.

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Quantification of GFP fluorescence and cell density measurements

A total of 20 or 50 µl of culture were added to 180 or 150 µl of water in fluorescence microtitre plates with clear bottom (Greiner Bio-One GmbH, Kremsmünster, Austria). Expression of GFP in *P. pastoris* was analysed by fluorescence measurements in a Spectramax Gemini XS plate reader (Molecular Devices Corp., Sunnyvale, CA, USA) with excitation at 395 nm and emission at 507 nm. Optical density was measured at 600 nm using a Spectramax Plus 384 plate reader (Molecular Devices Corp.). The GFP fluorescence was calculated by subtracting the blank value (*P. pastoris* X-33 grown and measured under the same conditions) from the sample value. The measured values were corrected for the dilution during measurement and for the dilution due to addition of the methanol induction medium.

Copy number determination by quantitative PCR

Copy number of the expression cassette was determined by quantitative real-time PCR using the *P. pastoris ARG4* gene as reference gene. Quantitative real-time PCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) in an ABI PRISM 7300 Real Time PCR System (Applied Biosystems). GFP-RTfw/GFP-RTrv and ARG4-RTfw/ARG4-RTrv were used as primers at concentrations of 200 nM with 1 ng of genomic DNA as template. Temperature conditions were 10 min at 95°C, 40 cycles at 15 s at 95°C and 60 s at 60°C followed by a dissociation step (15 s 95°C, 30 s 60°C, 15 s 95°C) at the end of the last cycle.

Activity assays for additional reporter systems

The HNL activity and HRP activity was determined essentially as described earlier (29), with just a few modifications: instead of an endpoint assay for the HRP activity, the activity was determined with a kinetic assay following the absorption at 405 nm for 5 min. The 15 µl of supernatant was transferred to a microtitre plate and the reaction was started with 140 µl of the substrate solution as described earlier (33). For the *Hb*HNL activity 100 µl culture were centrifuged and the pellet was lysed with 50 µl "Y-PER reagent" (Pierce, Rockford, IL, USA) for 30 min at 25°C. After centrifugation, 20 µl of supernatant was diluted 1:5 with 5 mM potassium phosphate buffer, pH 6.5. A 50 µl of the diluted sample was taken for the activity assay as described previously (29) for the secreted *Pa*HNL5 (100 µl 0.1 M phosphate buffer was used instead of 130 µl).

Fedbatch cultures in bioreactor and process monitoring

All cultivation processes were performed in a 14 l stirred tank bioreactor (MAVAG, Neunkirch, Switzerland). The initial batch working volume was 6 l and increased to total 12 l at the time of harvesting. The set-points of all control variables were maintained the same during the entire process duration and, thus, the cultivations were accomplished under the conditions of constant temperature 30 C, 18 l/min airflow (i.e. without any oxygen enrichment), 1400 r.p.m. agitation, 0.5 bar overpressure and pH 5. Ammonia solution 25% and phosphoric acid 8.5% were used to automatically control the pH.

The process consisted of a phase of biomass growth (i.e. in both batch and exponentially fed fedbatch cultures) and production phase with constant feed addition. The cultivation began at time 0 h with a batch (containing 33 g/l glucose monohydrate), during which the pO_2 value continuously decreased (i.e. no control of the pO_2 set-point applied). After some 12–14 h, the pO_2 rose again rapidly, and at this point the exponential addition of glucose solution was started and continued over 13.5 h in accordance with the function $f(t) = 4.655\ e^{0.2 \cdot t}$ (in gram of carbon per hour). During the subsequent production phase, either glucose or methanol as sole substrate was added at a constant rate of 29.54 g of carbon h^{-1} for at least 32.5 h.

Biomass concentration was determined gravimetrically as cell dry weight (CDW). Samples were centrifuged for 5 min at 14 000 r.p.m. (5417C, Nethler, Hamburg, Germany) in pre-weighted 2 ml Eppendorf tubes, washed and re-centrifuged, and subsequently dried for 24 h at 105°C (Heraeus Instruments, Zurich, Switzerland) to constant weight. Concentrations of glucose, methanol and other metabolites were analysed by HPLC using an LC-20AB device equipped with autosampler SIL-20A, thermostated column cell CTO-20A and RID-10A refractometer detector (all Shimadzu). Aminex HPX-87H column of $300 \times 7.8\ mm^2$ I.D. (Biorad) was operated at 40°C, flow rate of 0.6 ml/min under isocratic conditions with 2.5 mmol/l H_2SO_4. The injection volume was 25 µl.

RESULTS

Sequence analysis of the *AOX1* promoter

To carry out a comprehensive analysis of the *AOX1* promoter, we first summarized all sequences previously demonstrated to be involved in promoter regulation by methanol and for which we could find a homologous region in the P_{AOX1} sequence. In addition, we performed an *in silico* analysis of the P_{AOX1} sequence for putative TFBSs for eukaryotic TFs (Figure 1A and Supplementary Table 2).

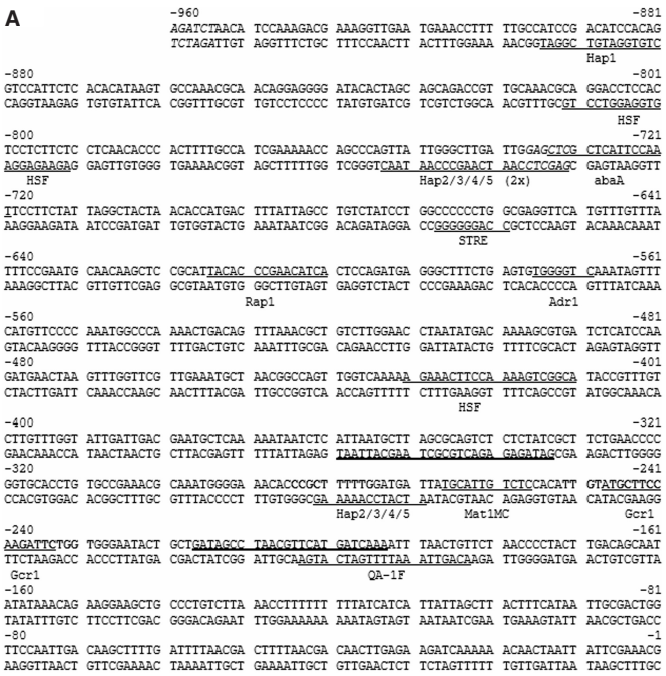
The putative TFBSs found within the *AOX1* promoter sequence can be divided into three general groups by the effects of the TFs:

- glucose repression or regulation of glucose-repressed genes, especially those related to oxygen consumption (e.g. Adr1p and the Hap2/3/4/5p complex);
- stress response (e.g. Msn2p/Msn4p and HSF);
- developmental programs and general TFs (e.g. abaA, Rap1p, QA-1F, Mat1-Mc).

Based on the sequences identified, we first generated a deletion library where small internal sequence stretches, including putative TFBSs, were deleted. The library contained both small deletions of 4–11 nt and larger stretches of up to 66 bp, which were surmised to encompass several putative TFBSs.

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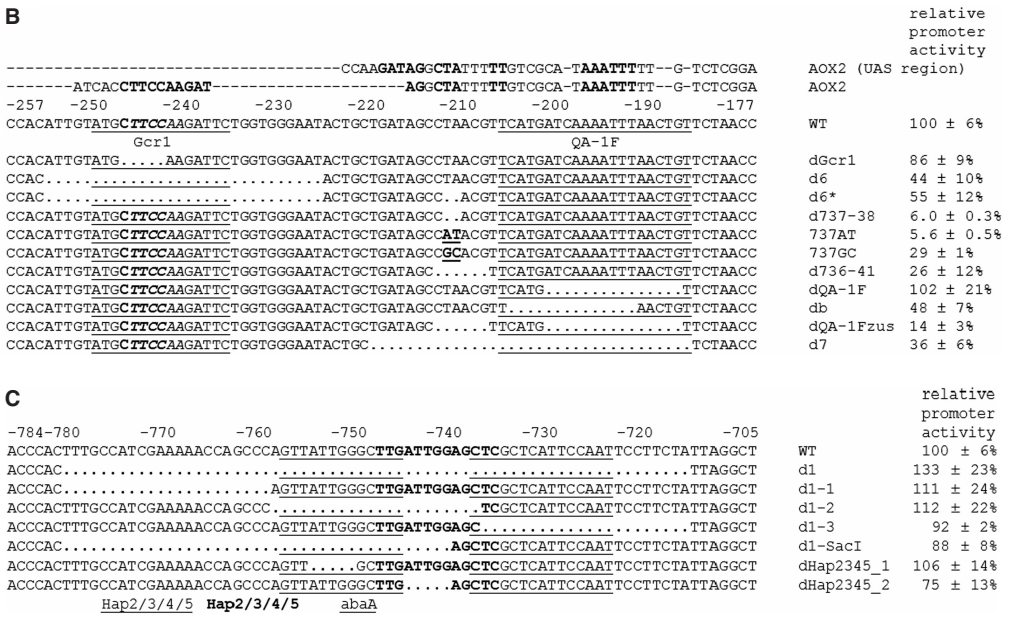


Figure 1. (**A**) Sequence of the *P. pastoris AOX1* promoter sequence (www.invitrogen.com). Putative fungal TFBSs as predicted by the MatInspector software are underlined. The frequently used BglII restriction site and the SacI restriction site are written italic. Putative regulatory sequences as predicted by Ohi *et al*. (9) based on P_{AOX2} sequence analysis are double underlined. Putative regulatory sequences as predicted by Kumagai *et al*. (28) based on *P. pastoris ZZA1* sequence analysis are written bold. Putative regulatory sequences as predicted by Gödecke *et al*. (23) based on *H. polymorpha MOX* sequence analysis are marked in grey. The TATA box is marked in dark grey. (**B**) P_{AOX1} deletions and their effects on *AOX1*-driven expression using methanol as sole carbon source during the induction phase following growth on glucose and glucose depletion. The *AOX2* UAS as proposed by Ohi *et al*. (9) is also aligned. Identical nucleotides between the *AOX2* and the *AOX1* sequence are written bold. Deletions performed are shown as dots instead of the nucleotides. Putative TFBSs predicted using MatInspector are underlined. The TTCCAA sequence proposed by Kumagai *et al*. (28) is italicized. Mutations introduced into the sequence are written bold and underlined. Relative promoter activity is given as mean % of the wild-type ± SD of three independent measurements. (**C**) P_{AOX1} deletions in the d1 region and their effects on *AOX1*-driven expression using methanol as sole carbon source during the induction phase following growth on glucose and glucose depletion. Deletions performed are shown as dots instead of the nucleotides. The first putative Hap2/3/4/5p-binding site and the putative abaA-binding site are underlined. The second putative Hap2/3/4/5p-binding site is written bold. Relative promoter activity is given as mean percentage of the wild-type ± SD of three independent measurements.

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Internal deletions within the *AOX1* promoter

In *P. pastoris*, *AOX1* expression is tightly repressed by glucose and glycerol, while methanol is absolutely necessary for high-level induction of this gene (39). In the absence of any carbon source, the promoter is derepressed, showing a small increase in transcriptional activity which is still much lower than the activity found in the presence of methanol. To characterize the *cis*-acting elements in P_{AOX1} that are necessary for this behaviour, a promoter library, fused to cycle-3-GFP as a reporter, was constructed. The library, consisting of *AOX1* promoter variants with internal deletions (as indicated in Supplementary Figure 2 and Supplementary Table 3), was used to inoculate 96-well deep-well plates (29). The library was cultivated in dextrose or glycerol containing media, i.e. BMD (1%) and BMG (0.5%), respectively. A 60 h growth phase was followed by a 72 h methanol induction phase as described in Materials and methods section. In neither the presence of glucose nor glycerol was any significant GFP expression detected for any strain with *AOX1* promoter deletion variants (as exemplified for some variants shown in Figure 2). Once the glucose or glycerol was used up, the wild-type *AOX1* promoter became slightly derepressed, which resulted in GFP expression and an increase in fluorescence. In some variants, a significant upregulation of GFP expression compared to the wild-type was detected during this time of derepression. After 60 h of growth and glucose depletion, the strongest derepression variants (d6 and d6*) showed more than a 4-fold higher GFP fluorescence intensity than the clone with P_{AOX1} wild-type driven expression. A similar but even more enhanced effect was observed with multicopy transformants of these constructs, where high expression of GFP could be easily monitored (data not shown). This increase was observed only under derepressing conditions and not in the presence of glucose or glycerol. The same mutations (d6 and d6*) resulted in a decreased fluorescence intensity (compared to the wild-type) after 70 h of methanol induction in deep-well plates.

After methanol addition, most promoter variants were induced to some degree, causing an increase in GFP fluorescence. A range of promoter activity from ~6 to 166% compared to the wild-type activity was observed with this library consisting of 46 promoter variants (including wild-type) having either one deletion or insertion or a combination of 2–3 such mutations (Figure 3 and Supplementary Table 3).

Most remarkably, a deletion of nucleotides −209 and −210 (TA), referred to as d737-38 in Figure 1B, within the P_{AOX1} sequence resulted in a drop of GFP fluorescence to ~6% of wild-type. The same drop in activity occurred when the TA base pair was interchanged to AT (737AT). This decrease was even more dramatic than the change caused by the deletion of the putative TATA box (−159 to −154) within the d8 region (18% residual activity). When the TA base pair was changed to GC (i.e. 737GC), the fluorescence intensity was five times higher, resulting in ~30% of the wild-type promoter driven expression activity. In addition, a deletion of nucleotides −184 to −218, called d7, which spans this region, resulted in a promoter with 36% of the wild-type activity. As a fourth observation, when the d737-38 deletion was introduced in addition to the d6 mutation, thereby generating mutant d6*, no change in activity under derepressing conditions and a comparatively less dramatic change in activity under induction conditions occurred (55% activity) (Figure 1B).

Within these two regions, sequences similar to the putative binding sites for *S. cerevisiae* TF Gcr1p and the *Neurospora crassa* QA-1F were found. Gcr1p is a major player in the regulation and coordination of glycolytic genes in baker's yeast and absolutely necessary for their high-level expression (40,41). In addition, the sequence to which this factor binds contains the central motif CTTCC, proposed by Kumagai *et al.* (28) to be involved in the

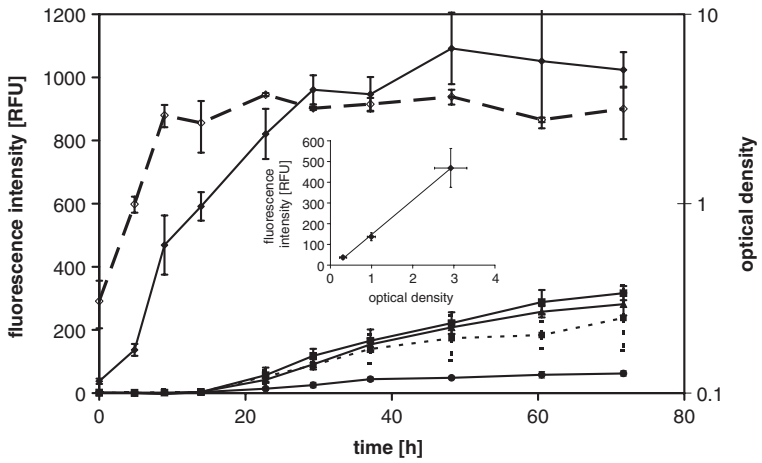


Figure 2. Time course of GFP expression driven by WT, d6 and d6* *AOX1* promoter variants and the *GAP* promoter in glucose containing media and during the glucose depletion phase in deep-well plates. The insert in the figure shows the correlation of the P_{GAP} driven GFP expression and biomass as represented by the optical density of the culture. Closed rhomboids, P_{GAP}; closed circles, P_{AOX1}; closed triangles, P_{AOX1}d6*; closed squares, P_{AOX1}d6; closed squares and dashed line, P_{AOX1}2xMat; open rhomboids, optical density of P_{GAP} strain. Fluorescence data and optical density represents mean ± SD of single measurements of four independent cultivations. Optical density curve of the P_{GAP} clone is shown as a representative.

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regulation of the *AOX1* promoter. However, deletion of this core CTTCC motif (dGcr1) resulted in only minor decrease in promoter activity upon methanol induction, while deletion of a larger region (d6) resulted in a 55% decrease in activity of the *AOX1* promoter. However, deletion of the CTTCC motif resulted in an increase in promoter activity comparable to the d6 deletion under derepression conditions. In addition, the QA-1F protein is the activator protein of the glucose-repressed quinic acid cluster in *N. crassa* (42). Deletion of the core sequence of this putative regulatory region (dQA-1F) had no effect, while a deletion positioned 4 nt to the 5′ of this sequence (db) reduced fluorescence intensity ~50%. Other deletions 5′ to the QA-1F binding site (dQA-1Fzus, d7) resulted in further decreases in promoter activity as shown in Figure 1B. This region, in which no putative TFBS were predicted by the MatInspector analysis showed a high similarity to the UAS region proposed by Ohi *et al.* (9).

Taken together, the above results demonstrate that we closely located a major *cis*-acting element within the region between −253 and −184 of the *AOX1* promoter sequence positioned around −210 and also minor *cis*-acting elements surrounding the major −210 region. Remarkably, the expression effects of the individual deletions are largely dependent on their extension and exact location (Figure 1B).

Furthermore, we identified several other regions where either short deletions of the core region of putative TFBSs or larger deletions spanning this region resulted in a strong decrease in promoter activity upon methanol addition. The responsible short putative binding sites and the corresponding larger deletion (d)-regions include Rap1p like sequence (34% residual activity) with the corresponding d2 region (33%), the Adr1p like sequence (30%) and d3 (70%), Hsf like sequences (60%) and d4 (46%) and Hap2/3/4/5p (67%), Mat1-Mc like sequences (42%) and d5 (40%). While the first three combinations are directly overlapping, i.e. the core sequence deletion of the putative TFBS is integrated into the larger deletion, this is not the case for the combination d5 and Mat1-Mc, which are only partly overlapping (Supplementary Figure 2). Taken together, we identified five regions based on the prediction of putative TFBSs where a single deletion of such a region of 2–59 bp resulted in a significant reduction in promoter activity, in most cases >50%.

While most variants of the *AOX1* promoter resulted in decreased expression, a few mutants actually enhanced its already strong expression in methanol containing medium. Notably, deletions in the region called d1, where three putative TFBSs were found, caused higher expression (Figure 1C). Two of these sequence motifs are overlapping and show sequence similarity to the *S. cerevisiae* Hap2/3/4/5p complex binding site (22). The second one of these binding sites is overlapping with a putative *Aspergillus nidulans abaA* binding site (43). The core consensus sequence of the Hap2/3/4/5p complex, CCAAT, is also present twice within this *abaA* motif. Deletion of the whole d1 region resulted in an increase of promoter activity of about 30%. To further delineate this *cis*-acting region, we narrowed the d1 deletion by dividing it into three parts, called d1-1 to d1-3 (Figure 1C). A positive effect of the deletions d1-1 and d1-2 of ~110% could be observed, while deletion of region d1-3 resulted in a reduction of promoter activity of ~10%. Deletion of the core sequence of the first putative Hap2/3/4/5p-binding site also resulted in a small increase, while deletion of the second resulted in a decrease in activity of ~75%. The effect of the d1 deletion seems not to be the sum of the effects of deletions of subfragments of d1. In case of the d1-3 deletion a new putative Hap2/3/4/5 site was generated due to the deletion (including the abaA site), which might explain that we did not see the expected increase of the promoter activity in this case.

Addition of putative *cis*-acting elements

After we made deletions of putative TFBSs, we next examined if we can utilize the results of our deletion analysis to engineer new promoter variants. Therefore, we duplicated putative positive *cis*-acting elements at their natural location. We chose three such elements: nucleotides 201–214 (−203 to −190), Mat1-Mc (−271 to −254) and Rap1p (−615 to −601). While duplicating the putative Rap1p-binding site resulted in no significant change in promoter activity under the conditions tested, doubling the putative Mat1-Mc-binding site resulted in a ~3-fold improvement of promoter activity under derepressing conditions (data not shown). Duplicating the 201–214 regions resulted in an increase in promoter activity of 139% of the wild-type in presence of methanol (Supplementary Table 3). This rise in transcriptional activity, together with the increase produced by the deletion of region d1, is the best promoter activity observed by single modifications within the *AOX1* promoter sequence. Both mutations, deleting d1 and doubling 201–214, resulted also in an increase in activity under derepression conditions, but less significant compared to the d6 and d6* modifications.

Next, we asked if we could further improve the *AOX1* promoter by combining both mutations, deletion of d1 and doubling 201–214. In doing so, we achieved an increase in promoter activity of >60% under methanol induction conditions (Figure 3 and Supplementary Table 3), which is remarkable for two reasons: (i) we started with an already very strong promoter and (ii) we did not find additivity in other cases as e.g. in the case of d6 and d6* variants or combinations of d1, d2, d6, d6*, dHap2/3/4/5p and the second dHsf deletion (Supplementary Table 3) by simple combination of the mutations.

Alternative reporters

To test the transferability of the results obtained with the laboratory reporter GFP to a real case scenario, we selected five promoter variants (wt, d1, d2, d6* and d2d6) with different activities to express three additional reporters: the first is the hydroxynitrile lyase isoenzyme 5 of *Prunus amygdalus* (almond) (*Pa*HNL5α) (34). This FAD-containing enzyme can be produced and secreted by *P. pastoris* in the range of ~1 g/l in bioreactor cultures. The second enzyme, HRP (33) has so far been produced by *P. pastoris* only in the lower microgram per litre range. For both of these secreted enzymes, the native signal sequence was replaced by the *S. cerevisiae* mating factor α signal sequence. The third enzyme, the hydroxynitrile

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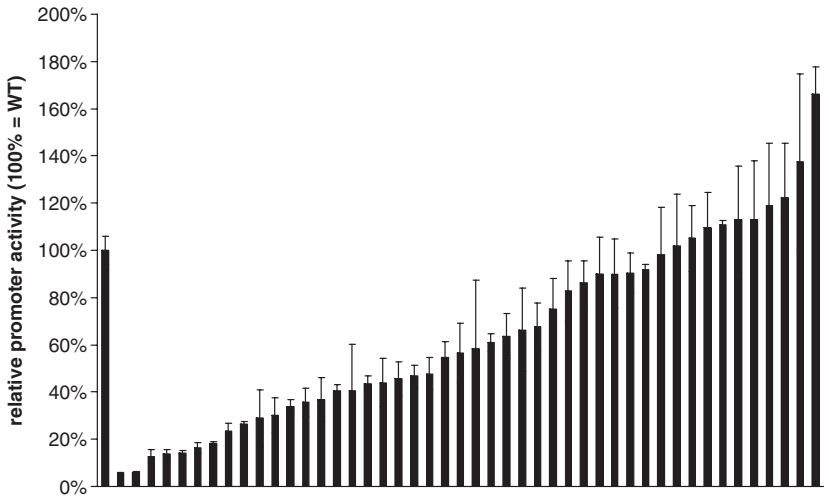


Figure 3. Promoter activity as represented by the fluorescence intensity of reporter clones after cultivation in 96-well deep-well plates and methanol induction for 72 h. The data represent mean ± SD of single measurements of three independent cultivations in separate deep-well plates.

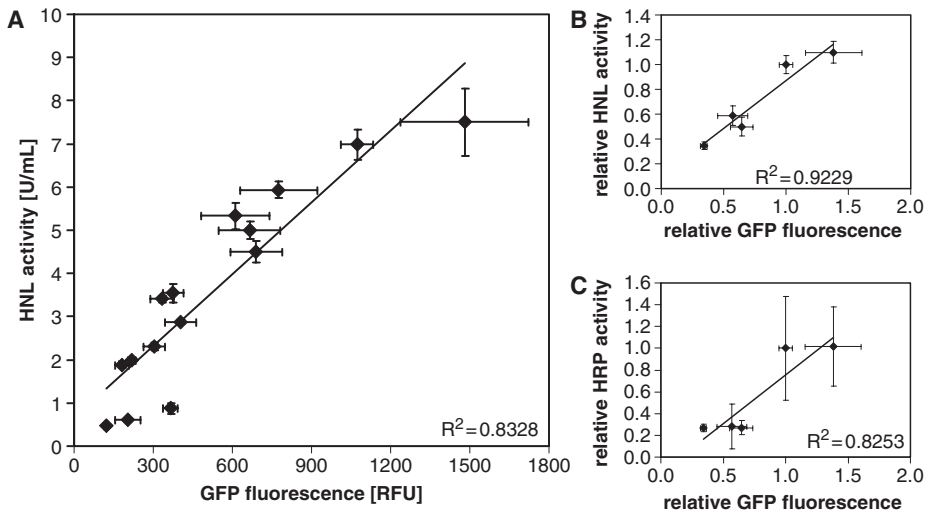


Figure 4. Correlation of P_{AOX1}-driven reporter enzyme expression and GFP expression driven by the same promoter variants. (**A**) *Pa*HNL5α activity of five different promoter variants at three different time points during methanol induction (after 24, 28 and 72 h of methanol induction) compared as a function of the GFP expression using the same promoter variants at the same time points. HNL activity is given in U/ml in the culture supernatants. Data represent mean ± SD of four independent cultivations. (**B**) Relative *Hb*HNL activity of cell lysates compared to the wild-type promoter transformant after 72 h of methanol induction. The relative *Hb*HNL activities were normalized to the activity obtained with the wild-type P_{AOX1} and are represented by the mean ± SD of four independent cultivations. (**C**) Relative HRP activity of culture supernatants compared to the wild-type promoter transformant after 72 h of methanol induction. The relative HRP activities were normalized to the activity obtained with the wild-type P_{AOX1} and are represented by the mean ± SD of four independent cultivations. The GFP fluorescence intensities or relative GFP fluorescence are represented by the mean ± SD of three independent cultivations.

lyase from *Hevea brasiliensis* (tropical rubber tree) (*Hb*HNL) had been produced by *P. pastoris* to a level of more than 20 g/l previously (7). All three are industrially important enzymes. For these three enzymes and the five promoter variants the same trend was observed as for the GFP expression strains (Figure 4).

For HCD cultivations, Mut^S strains, where the *AOX1* gene is disrupted, are often preferred since they are easier to handle in terms of oxygen consumption, heat production and control of methanol concentration in the bioreactor. Thus, we tested the three promoter variants (wt, d1 and d6*), including the derepressed d6* variant, for the GFP and the *Pa*HNL5α expression in a Mut^S phenotype background (KM71H). The promoter activities found in the mutant library were fairly independent of the reporter used and the methanol utilization phenotype of the strain that harboured them (Supplementary Figure 3).

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Basal promoter fragments

As a first step towards the creation of useful fully artificial *P. pastoris* promoters by using modular building blocks, several *cis*-acting elements described above were added to different basal promoters. Since the core promoter containing the transcription initiation site is also a key *cis*-acting element crucial for efficient transcription (44), we first analysed three different core promoter fragments. The first one was the basal *S. cerevisiae LEU2* promoter fragment (20), and the other two were *AOX1* promoter fragments which where truncated at positions −10 from the 5′ end of the TATA box, called AOX176, and at −25, called AOX194. First we tested these three core promoters by fusing them to full-length *AOX1* promoter fragments (−170 to −940) and truncated *AOX1* promoter variants with a 5′ end at position −731 (truncSacI), −411(trunc411) and −203 (trunc203). Therefore, P_{AOX1} was cut at position −10 from the TATA box for the *LEU2* and the AOX176 fragment, and cut at −25 from the TATA box for the AOX194 variant. In doing so, we introduced three additional nucleotides at −10 of the TATA position to generate a BspTI restriction site (for AOX176) and we changed two outer nucleotides in the TTTAAC hexanucleotide to CTTAAG, again to generate the BspTI site for AOX194 (Supplementary Figure 1B). In addition, we constructed reporter genes with the *LEU2*-variants and the *E. coli lacZ* as a reporter gene. Table 1 clearly demonstrates the superior performance of the basal AOX fragments in comparison to the *S. cerevisiae LEU2* fragment. AOX176 and AOX194, when fused to the full *AOX1* promoter regions, showed a greater induction than the *LEU2* in methanol medium. While no promoter activity of any mutated P_{AOX1} variant could be found as long as glucose (or glycerol) was present in the medium, all three basal promoter variants showed some basal activity in presence of glucose (Figure 5). Since there was no difference between the AOX194 and the AOX176 fragment in terms of methanol-sensitive induction, we chose the AOX176 as a core fragment for further studies. In addition, the AOX176 core promoter showed the lowest basal GFP expression on methanol of the three variants tested (data not shown), making it the best suited to study induction effects by glucose depletion or addition of methanol.

Synthetic promoters using small *cis*-acting elements

We were interested in utilizing short synthetic promoter variants with several *cis*-acting elements to design useful tailor-made promoter variants, which would optimize

Table 1. Fluorescence intensities of basal promoter fragments with added *AOX1* promoter parts

Construct	5′ promoter region added	Fluorescence intensities [RFU]			lacZ activity [10^3 U μg^{-1}]
		LEU2	AOX194	AOX176	LEU2
Full-length	−940 to −170[a]	310 ± 30	849 ± 120	845 ± 81	ND
truncSacI	−731 to −170[a]	345 ± 31	1095 ± 123	593 ± 158	5.82
trunc411	−411 to −170[a]	115 ± 18	175 ± 8	121 ± 13	5.11
trunc203	−203 to −170[a]	47 ± 3	ND	48 ± 4	0.54
Basal	No addition	17 ± 7	11 ± 2	7 ± 4	0.27

The fluorescence intensity of the wild-type AOX1 promoter is 889 ± 70.
[a]Constructs used for fusion with AOX194 were truncated at the 5′ end at –193.

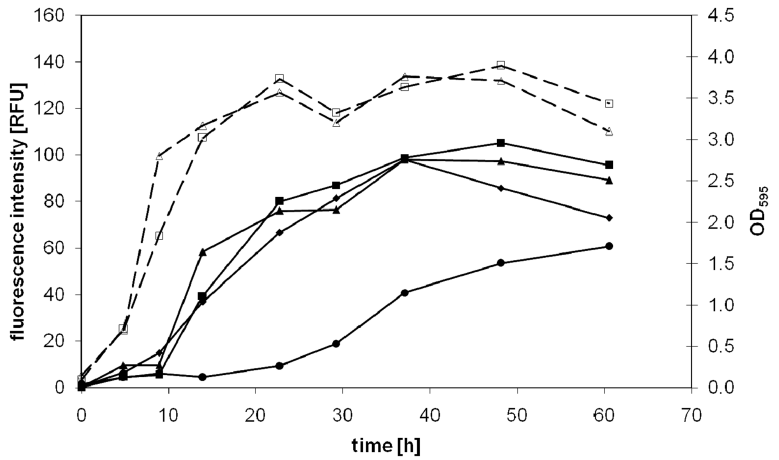


Figure 5. Time course of GFP expression driven by basal *S. cerevisiae LEU2*, two *P. pastoris AOX1* basal promoter variants (AOX176 and AOX194) and the wild-type *AOX1* promoter in glucose-containing media and during the glucose depletion phase in deep-well plates. Closed rhomboids, $P_{LEU2basal}$; closed circles, P_{AOX1}; closed triangles, P_{AOX194}; closed squares, P_{AOX176}; open squares, optical density of P_{AOX176}; open triangles, optical density of P_{AOX194}. Fluorescence data and optical density represents mean of single measurements of four independent cultivations.

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gene expression for applications such as protein manufacturing (45,46), metabolic engineering (47–50) and synthetic biology (51–53). In addition, for some specific applications such as directed evolution of enzymes using *P. pastoris* (54), short promoters are preferable since they usually enable a more efficient PCR-based generation of expression cassettes or mutant libraries. Therefore, our next goal towards the design of fully synthetic promoter variants was to determine the effect of selected putative *cis*-acting sequences on the expression of the basal AOX176 region. Based on the results of the deletion libraries as well as the similarity of predicted sites to the matrix of the TFBS, we chose: (i) the 201–214 bp fragment, (ii) the 737 region, (iii) the STRE element, (iv) the putative binding site for Adr1p, (v) the putative binding site for Gcr1p and (vi) the putative binding site for Mat1-Mc to be inserted upstream of the basal promoters.

While the fragments 201–214, STRE and Mat1-Mc enhanced expression constitutively under both the conditions [the absence of glucose (derepression) and the presence of methanol (induction)], the other fragments displayed only an effect when methanol was used for induction (Figure 6). Adding the 737 element to a basal promoter resulted in a decrease of GFP expression when glucose is present; therefore, the induction ratio was further increased with this variant compared to the sole basal promoter element. The same effect was found when the 737 element was added to the basal *LEU2* promoter (data not shown). Therefore, the verified five *cis*-acting elements within the *AOX1* promoter cause different effects. While the elements 201–214, STRE and Mat1-Mc seemed to enhance transcription from the basal promoter equally in absence of glucose and presence of methanol, the 737 element had a repression effect in presence of glucose and an induction effect when methanol was present in the medium. Adr1p and Gcr1p, exerted effects only when methanol was present, but activity under derepressing conditions remained unchanged.

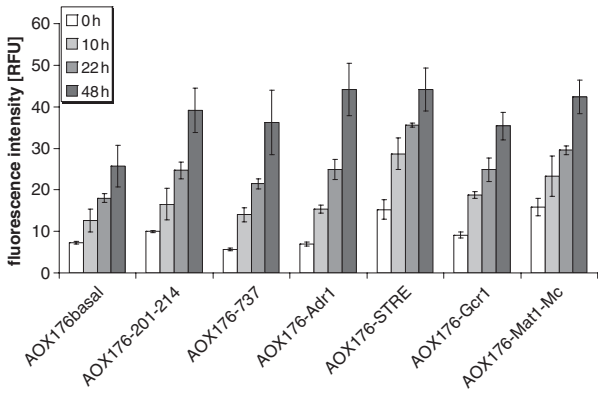


Figure 6. Promoter activity as represented by the fluorescence intensity of reporter clones after cultivation in 96-well deep-well plates and methanol induction for 72 h. The data represent mean ± SD of single measurements of three independent cultivations in separate deep-well plates. Samples were taken at 0, 10, 24 and 48 h after methanol addition. Values at 0 h represent promoter activities under derepression conditions. Fluorescence intensities of the wild-type *AOX1* promoter are: 0 h, 7 ± 2 RFU; 10 h, 110 ± 3 RFU; 24 h, 262 ± 32 RFU; 48 h, 457 ± 55 RFU.

Product formation in HCD fedbatch cultures

To evaluate the scalability and the potential for industrial application of our findings with new promoter variants showing interesting technological features, we analysed the d6* promoter in a laboratory-scale bioreactor experiment. In 96-well format, this promoter variant caused enhanced protein expression capability without the need of methanol for induction. In addition, we faced the challenge of keeping the derepression effect observed upon glucose depletion over a long lasting production phase in the bioreactor, where some cell growth and energy metabolism has to be maintained by feeding limiting amounts of glucose. In this part of our study, two multicopy strains in which HRP was expressed either under the control of the wild-type (wt) or the d6* *AOX1* promoter variant were cultured in a HCD fedbatch, and compared with respect to both biomass formation and extra-cellular secretion of the recombinant product. The following two fedbatch procedures were carried out on each strain: (i) glucose as the sole carbon and energy source was added during the entire process duration to determine product levels under derepressing conditions, where the carbon source was limiting, and (ii) after an initial biomass growth with glucose, the composition of the original feed solution was changed to pure methanol, which caused further induction of the heterologous protein expression under the control of the wild-type P_{AOX1} and most of its variants.

After 32.5 h, the fedbatch cultures grown with glucose addition during the entire process were stopped as the working volume limit was reached. As both processes (i.e. either with glucose or methanol) were designed in such a way that the same total amount of carbon was added to the bioreactor, only data obtained at this particular time (32.5 h) were appropriate for direct comparisons of the process performance. During both, the glucose and the methanol processes, product titres approx. twice as high were reached in cultures with the d6* *AOX1*-promoter variant (i.e. given as volumetric activity in U/l) than in those where wt was used (Figure 7). Furthermore, compared to glucose in methanol processes a titre enhancement was observed for each of the particular strains/promoters, i.e. at a factor of 1.7 and 2.1 for the d6* and wt strains respectively. Thus, the highest amount of product produced per biomass [i.e. ~107 U (g CDW)$^{-1}$ at the end of 51.5 h production period and 63.0 U (g CDW)$^{-1}$ during the first 32.5 h], which corresponds to an average specific product formation rate of 2.08 U (g/CDW)$^{-1}$ h^{-1} (51.5 h), 1.94 U (g CDW)$^{-1}$ h^{-1} (32.5 h) was demonstrated in the experiment with the d6* promoter operating under full induction by methanol at pH 5 and 30°C. However, even without induction by methanol 4133 U/l were obtained with the d6* promoter-based expression construct corresponding to 129% of the maximal titre of the wild-type P_{AOX1}-based strain in the presence of methanol and 2.5 times more than in case of derepression of the wild-type P_{AOX1} system. Due to

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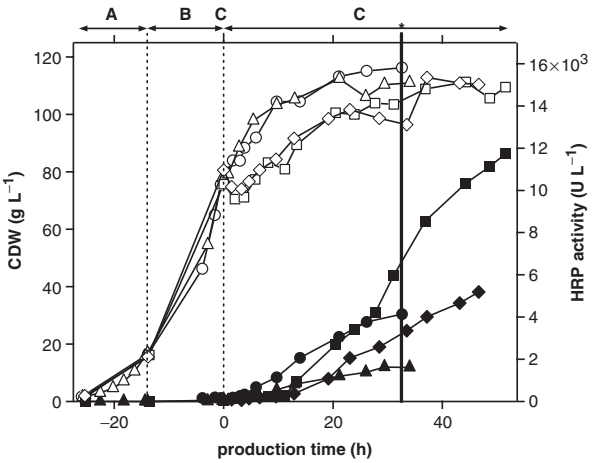


Figure 7. Time courses of biomass concentration and volumetric product (HRP) activity as determined in HCD-fedbatch cultures. Marked with an asterisk: vertical line at 32.5 h, the time when the final titres were compared. Open symbols, biomass concentrations determined as cell dry weight; closed symbols, product measured as volumetric activity in a photometric assay; squares and circles, for methanol and glucose processes with d6* AOX1-promoter variant, respectively; rhomboids and triangles, for methanol and glucose processes with wild-type promoter, respectively. The capital letters show the following process phases: A—batch growth with glucose; B—fedbatch growth over 13.5 h with exponential addition of glucose at a feed rate $F(t) = 4.655 \cdot e^{0.2 \cdot t}$ g C h^{-1}; C—production phase of about 44 h with a constant addition of substrate, i.e. at $F(t) = 29.54$ g C h^{-1}.

specific differences in the mode of operation between microscale and bioreactor experiments, batch-wise induction in microscale and continuous induction with limiting amounts in bioreactors, together with the fact that the d6* promoter variant offers faster induction kinetics at the beginning of the induction phase resulted in a higher overall productivity of the d6* promoter, even under methanol induction conditions (Figure 7).

DISCUSSION

A promoter library consisting of several *AOX1* promoter variants fused to GFP displayed a broad range of activities (from ~6% to >160%) with small increments between the individual promoter variants. Although we started with one of the most powerful promoters described to date, the P_{AOX1} (55), we obtained some variants which were even stronger. Several important *cis*-acting elements responsible for high P_{AOX1} activity were identified and characterized. Using our approach starting with computational TFBS prediction, we: (i) achieved a high success rate in changing the promoter strength by introducing mutations within the TFBSs and (ii) localized the *cis*-acting elements and their individual effects precisely by the first deletion series.

We identified seven regions within the *AOX1* promoter where a deletion of either small core sequences of the putative TFBS or the deletion of a larger stretch covering the whole putative TFBS resulted in a decrease in promoter activity of >50%. Additionally, the prediction of putative TFBSs suggests possible target TFs, where a search for homologues in the *P. pastoris* genome might facilitate future analyses about P_{AOX1} regulation (and regulation of other genes of the methanol utilization pathway). While we expected to strongly downregulate the promoter activity upon deletion of the putative TATA box, we did not expect such a large number of regions where a mutation can decrease promoter activity by >50%, some of the effects being even more dramatic than in the case of the TATA box deletion. For instance, when we deleted (or mutated) nucleotides surrounding position −209 to −210, the promoter activity dropped to ~6–30% of the wild-type activity, depending on the mutation (Figure 1B). In addition, these results are consistent with previous studies where promoter activity dropped with stepwise truncations (20) and which effects observed in the case of larger deletions of the whole *AOX1* promoter sequence (56,57).

Our approach of predicting putative TFBSs followed by deletion or duplication of the respective region within the promoter sequence was highly successful in generating a library spanning a broad range of activities. This not only delivered mutants with strongly altered activity but also suggested which TFs might be involved in the regulation of the *AOX1* promoter. Our results also demonstrated the complex architecture of the *AOX1* promoter with several activator/repressor sites, which might explain its strong and tight regulation.

To identify TFs involved in methanol-sensitive gene regulation, past studies have focused on the behaviour of these types of promoters in heterologous hosts that are better characterized than most methylotrophic fungi. *Pichia pastoris AOX1* and *DAS1* and *H. polymorpha MOX* promoter regions were shown to promote expression of the reporter enzyme β-galactosidase (*lacZ* of *E. coli*) in *S. cerevisiae* (8,25). The regulation patterns of these genes in *S. cerevisiae* are similar, though not the same, as in their natural hosts: glucose represses gene expression, under carbon starvation conditions expression is slightly derepressed, and glycerol as carbon source induces expression [however, the latter is contradictory to what happens in *P. pastoris* (8)]. *MOX* promoter driven expression was induced by ethanol, methanol and oleic acid in *S. cerevisiae* (21). Furthermore, Adr1p, a positive effector of *ADH2* (alcohol dehydrogenase 2) and some peroxisomal proteins in *S. cerevisiae* (21), was shown to be an activator of the *MOX* promoter in *S. cerevisiae* when glucose is lacking in the medium (25). Interestingly, the only identified TF involved in *AOX1* regulation, Mxr1p, shows similarity at the structural level to the *S. cerevisiae* Adr1p (20). However, Mxr1p was identified to bind to a region between −170 and −411, but the putative Adr1p-binding site we found with MatInspector is located at −576 (5′ end of the binding site). Thus, the exact localization of the Mxr1p-binding site in the *AOX1* promoter remains to be elucidated.

We introduced deletions into the promoter sequence, which possibly modified positioning of *trans*-acting factors that work in a concerted fashion to achieve full induction (e.g. facilitating the binding of partner factors or bending of DNA). Thus, the observed effects are not necessarily

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caused by the deletion of a *cis*-acting element, but may result simply from changes in spacing between authentic elements. Whether this characteristic P_{AOX1} regulation pattern (glucose repression, derepression in absence of carbon source and methanol induction) is caused by the presence of one or several repressor proteins, by inaccessibility of the promoter packed in nucleosomes or by the absence of activator proteins, or a combination of those possibilities remains to be elucidated in further studies.

However, a rough model can be presented based on our preliminary results in addition to other previous studies. Cells lacking *MXR1* function cannot induce the *AOX1* promoter at all. Mxr1p has many similarities to *S. cerevisiae* Adr1p and can be considered a homologue. Having several domains, Adr1p not only binds to and activates target promoters but also modifies the activity or expression level of other TFs which modulate the same target promoters. Adr1p can be considered both a global and local regulator of transcription. In parallel fashion, we hypothesize that Mxr1p not only binds the *AOX1* promoter but also can regulate at least one additional *P. pastoris* TF protein additionally involved in regulation of the *AOX1* promoter. Such a model helps to explain the presence of so many different binding sites in the same promoter.

Furthermore, we demonstrated the transferability of promoter strength of the new promoter variants to the expression of industrial enzymes that are produced by *P. pastoris* in a wide variety of concentrations either intracellularly (*Hb*HNL) or extracellularly (*Pa*HNL5α, HRP).

Since *P. pastoris* is one of the major protein production host, a detailed understanding of the regulation of its most commonly used promoter at a molecular level will have a dramatic impact on the optimization of protein production processes in the future. This should be supported further by the availability of the *P. pastoris* genome sequence, which could enable a straight-forward isolation and study of *trans*-acting factors involved in regulation of P_{AOX1}-driven gene expression. There is no general optimal promoter: the regulatory properties of the promoter variant, the target protein and the production process have to be seen as a whole. The new promoter variants are especially useful for tailoring a production strategy that is specific to the needs of the individual protein. For example, stronger variants would be useful for the expression of non-toxic proteins, as in the case of reporters where fewer copies of the expression cassette would be sufficient to obtain a maximum of expression. Weaker variants could be favourable for the expression of proteins, which cause deleterious effects to the host cell. Within this group, we include proteins which are toxic to the cell per se due to their biological activity (e.g. proteases, toxins) or which induce an unfolded protein response (UPR) or ER-associated degradation (ERAD) due to incomplete folding in the ER during secretion (58–61) or which face other bottlenecks where an excess of transcript would ultimately end up in less active protein. Another group of 'difficult' proteins that needs optimization of expression are integral membrane proteins, which are intrinsically hard to produce (62,63).

For the first time, we succeeded in the generation of promoter mutants which displayed increased promoter activity without the need for any methanol for strong expression while, in contrast to constitutive promoters like P_{GAP}, still are strongly repressed in presence of glucose. Such an expression profile was also found for short synthetic promoter variants, which were constructed using P_{AOX1}-derived *cis*-acting elements. Such promoters can be used to express proteins in carbon starvation phases like the stationary phase in batch cultures, under carbon-limited conditions like fedbatch or by continuous cultivation below the maximum specific growth rate, as demonstrated by HRP production employing a glucose-limited fedbatch process.

Briefly, we demonstrated that specific mutations introduced at putative TFBSs can be employed to generate a promoter library with a broad range of activities and thereby, identified regulatory regions that provide a basis for designing short, synthetic promoter variants for metabolic engineering and synthetic biology. Further work is needed to understand the biological role of the different *cis*-acting elements so that a complete characterization of the molecular basis of P_{AOX1} regulation can be achieved. Nevertheless, considering the results obtained during this study and the advantages of *P. pastoris* in recombinant protein production processes, especially proteins needed in the pharmaceutical, chemical and biofuel industry, we are convinced that our library will be broadly applicable to optimize production yields.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. A patent application covering the work described in this article has been filed by the Graz University of Technology. Graz University of Technology, who was the employer of the authors and inventors Franz Hartner and Anton Glieder could potentially gain financially from the publication of the studies described in this work. The authors do not see any other conflict of interest.

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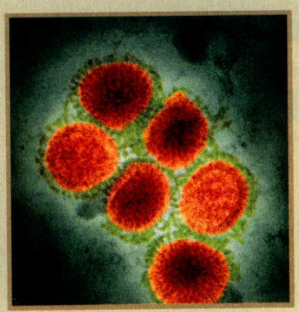

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Scanning electron micrograph of *Pichia pastoris*. De Schutter *et al.* present the complete genomic sequence of this important protein expression system (p 561). Credit: Dennis Kunkel Microscopy, Inc.



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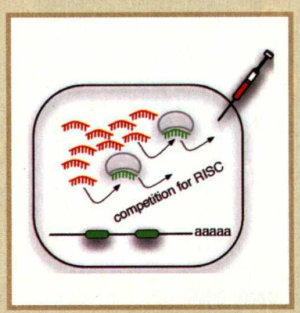
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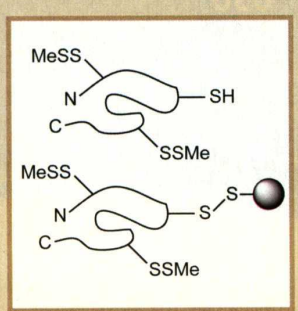
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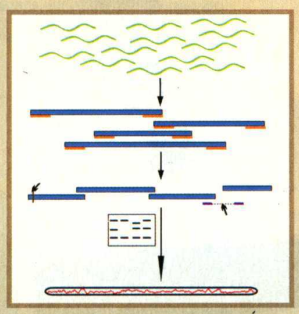
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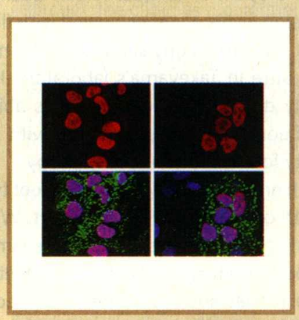
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ARTICLES

nature biotechnology

Genome sequence of the recombinant protein production host *Pichia pastoris*

Kristof De Schutter[1,2,7], Yao-Cheng Lin[3,4,7], Petra Tiels[1,5,7], Annelies Van Hecke[1,5], Sascha Glinka[6], Jacqueline Weber-Lehmann[6], Pierre Rouzé[3,4], Yves Van de Peer[3,4] & Nico Callewaert[1,5]

The methylotrophic yeast *Pichia pastoris* is widely used for the production of proteins and as a model organism for studying peroxisomal biogenesis and methanol assimilation. *P. pastoris* strains capable of human-type N-glycosylation are now available, which increases the utility of this organism for biopharmaceutical production. Despite its biotechnological importance, relatively few genetic tools or engineered strains have been generated for *P. pastoris*. To facilitate progress in these areas, we present the 9.43 Mbp genomic sequence of the GS115 strain of *P. pastoris*. We also provide manually curated annotation for its 5,313 protein-coding genes.

The methylotrophic yeast *Pichia pastoris* is by far the most commonly used yeast species in the production of recombinant proteins[1] and is employed in laboratories around the world to produce proteins for basic research and medical applications. It is also an important model organism for the investigation of peroxisomal proliferation and methanol assimilation. The *P. pastoris* expression technology has been commercially available for many years. *P. pastoris* grows to high cell density, provides tightly controlled methanol-inducible transgene expression and efficiently secretes heterologous proteins in defined media. Several *P. pastoris*–produced biopharmaceuticals that are either not glycosylated (such as human serum albumin[2]) or for which glycosylation is needed only for proper folding (such as several vaccines[3]) are already on the market. An important recent breakthrough has been the development of *P. pastoris* strains with human-type N-glycosylation[4–6]. Humanized glycosylation will further increase the importance of *P. pastoris* for biopharmaceutical production; indeed, proteins produced with this system are moving into clinical development[7]. Moreover, monoclonal antibodies can be made at gram-per-liter scale in the humanized glycosylation-homogenous strains[8].

For further strain engineering, a better understanding of all aspects of the yeast's protein production machinery is needed, and a number of studies relating to *P. pastoris*'s secretory system and engineered promoters have been forthcoming[9,10]. To facilitate the investigation of *P. pastoris* and other methylotrophic yeasts, we present the 9.43 Mbp genomic sequence of the GS115 strain of *P. pastoris*.

RESULTS

Genome sequencing and assembly

Very little is known about the genomic features of *P. pastoris*. The *P. pastoris* genome has been shown to be organized in four chromosomes with a total estimated size of 9.7 Mbp by pulsed-field gel electrophoresis[11]. In addition they assigned 13 *P. pastoris* genes to the different chromosomes. The absence of a genetic map makes chromosome assembly a challenging task, which we completed according to the strategy outlined in **Figure 1a**. We made use of 454/Roche sequencing[12] (GS-FLX version) to highly oversample the genome (20× coverage) and generated 70,500 paired-end sequence tags, to enable the assembly of all but seven contigs into nine 'supercontigs' (plus the mitochondrial genome) using automated shotgun assembly and BLASTN-based contig end-joining (Online Methods and **Supplementary Fig. 1** online). Upon assigning these (super)contigs to the four chromosomes (Online Methods and **Supplementary Fig. 2** online), the order of the supercontigs was determined through PCR and Sanger sequencing of the amplification products. These finishing experiments allowed the reconstruction of the four chromosomal sequences (**Fig. 1b** and **Table 1**), with only two gaps remaining (one each on chromosomes 1 and 4). A ribosomal DNA (rDNA) repeat sequence was present in the assembly as a separate contig of 7,450 bp, with exceptionally high coverage (328.8-fold). Given that sequence coverage all over our assembly very closely approximates 20×, we interpret that there are ~16 copies of the rDNA repeat region, thus accounting for about 119 kbp in sequence. We detected these rDNA loci on all chromosomes (Online Methods, **Fig. 1b** and **Supplementary Fig. 2**). The rDNA locus contains the 18S, 5.8S and 26S rRNA coding sequences. Unlike the *Saccharomyces cerevisiae* 5S rRNA gene, which is localized to the repeated rDNA locus, the 21 copies of the *P. pastoris* 5S rRNA are spread across the entire length of all chromosomes. Based on pulsed-field gel electrophoresis (PFGE), the chromosomes of *P. pastoris* GS115 were estimated to be 2.9, 2.6, 2.3 and 1.9 Mbp[11], whereas we obtained 2.88 (2.8 + 0.08), 2.39, 2.24

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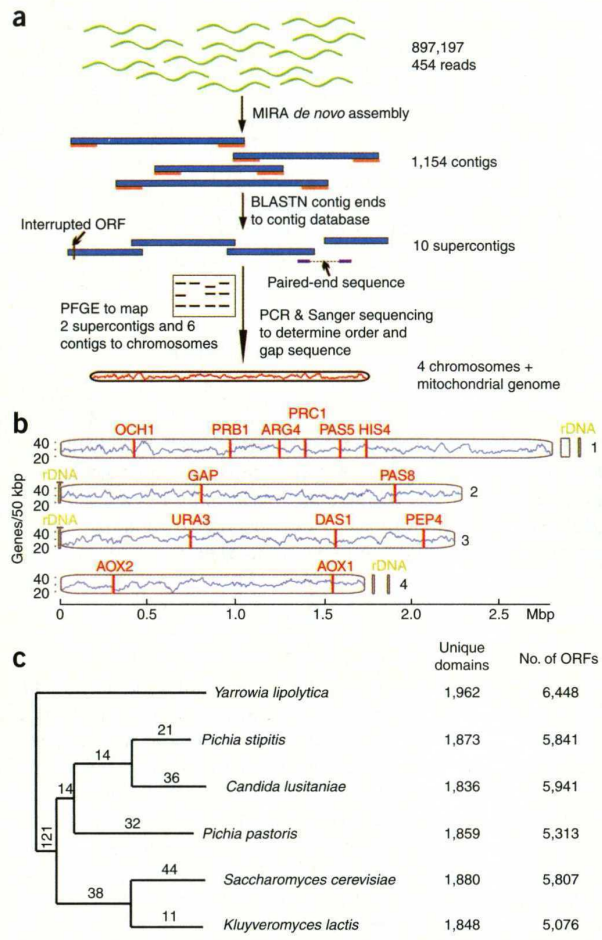


Figure 1 *Pichia pastoris* genome sequencing and overview. (**a**) Genome sequencing and assembly strategy. (**b**) *P. pastoris* gene density and known markers position. Gene density is plotted as a histogram, showing a uniform distribution of genes across each chromosome. The gene density is calculated in a window size of 50 kbp with 5 kbp sliding window. Genes that had been previously mapped to the chromosomes through PFGE are indicated in red, and rDNA repeats in green. (**c**) Phylogenetic tree. The phylogenetic tree was built on the concatenated sequence of 200 single-copy orthologous genes in all of the six species. Numbers next to each branch correspond to the number of Pfam domains uniquely present in the corresponding lineage.

and 1.8 (1.78 + 0.017) Mbp after assembly (assembled chromosome + assigned contig). Including the estimated 0.12 Mbp of rRNA repeats, we calculate a genome size of 9.43 Mbp.

Genome sequence accuracy estimation

A concern with genome sequences largely generated through 454 sequencing is the potential for 'indel errors' at homopolymeric sequences[13]. An analysis of the occurrence of such sequences in the *P. pastoris* genome is provided in **Supplementary Figure 3** online. Two approaches were followed to estimate the accuracy of our genome sequence. First, we retrieved 39 peer-reviewed Genbank coding sequences of *P. pastoris* strain GS115 (**Supplementary Table 1** online; total sequence length 70,295 bp). These sequences were compared to our genome sequence, and 84 differences were encountered. To establish which sequence was correct, we amplified these genes by PCR and Sanger-sequenced the PCR products. In all but two cases, the Sanger sequences confirmed our genome sequence, and we thus estimate the error rate to be 1 in 35,147 bp. In an alternative approach, we analyzed all open reading frames (ORFs) encoding proteins with at least one clear homolog in the databases. Where we found an interrupted ORF with clear homology to the 5′ part of the homologs, immediately followed by a coding sequence with clear homology to the 3′ part, the most logical interpretation was that there was a frameshift error mutation in our genome sequence (that is, both coding sequences are extremely likely to be linked into one open reading frame (ORF)). We found such frameshift errors in 2.7% (108) of the 3,997 genes for which such analysis could be made, totaling 6.11 Mbp of coding sequence. Conservatively estimating that we would only have detected such error if it occurred in the first two-thirds of the ORF, we then calculated a frameshift error rate in the coding sequences of 1 in 37,716 bp. Both estimates show that high-coverage 454 sequencing can indeed yield highly accurate genome sequences.

Pichia pastoris phylogenetic position

Phylogenetic analysis (**Fig. 1c**; Online Methods) shows that *P. pastoris* diverged before the formation of the CTG clade (yeasts which translate the CUG codon into serine instead of leucine[14]).

Genome sequence annotation: protein-coding genes

Protein-coding genes were automatically predicted using EuGène[15] (Online Methods and **Supplementary Fig. 4** online). The gene models were manually curated for functional annotation, accurate translational start-and-stop assignment, and intron location. This resulted in a 5,313 protein-coding gene set of which 3,997 (75.2%) have at least one homolog in the National Center for Biotechnology Information protein database (BLASTP e-value 1e-5, sequence length $\leq$20% difference and sequence similarity $\geq$50%). The protein-coding genes occupy 80% of the genome sequence. According to recently proposed measures for genome completeness, we searched the genome for highly conserved single (or low) copy gene sets: core eukaryotic genes (CEGs) with 248 genes across six model organisms[16] and FUNYBASE[17] with 246 genes with orthologs in 21 fungi. All genes from both gene sets were present in our proteome with full domain coverage.

We assigned 1,285 genes to the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways, and 4,262 of the genes were annotated with Gene Ontology (GO) terms[18]. The GO slim categories of *P. pastoris* are presented in **Supplementary Figure 5** online. A secretion signal peptide was predicted in 9% of the genes[19], and 4,274 of proteins contain InterPro domains. These include 2,320 distinct Pfam domains. In comparing the presence and absence of protein domains with five other yeast proteomes, 32 domains in 32 genes are identified as specific to *P. pastoris* (**Supplementary Table 2** online). The two fungi in the CTG clade whose genomes have been sequenced (*P. stipitis* and *C. lusitaniae*) share 71 gene families that are absent in *P. pastoris* (**Supplementary Table 2**).

Codon (pair) optimization of transgenes to the expression host organism often yields substantial improvements in recombinant protein yield[20]. *P. pastoris*'s codon usage is shown in **Figure 2a**, which will guide synthetic gene design for protein production in this organism. Overall, the codon usage is similar to the one for *S. cerevisiae*. Some synonymous codon pairs are also more or less frequently used than expected (the codon pair bias)[21]. As reported for

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Table 1 Genome sequencing and assembly statistics and contents overview

a. Genome sequencing and assembly statistics

454 Sequencing						
Sequenced reads		Sequenced length (bp)		Paired-end reads		
897,197		218,602,026		11,538		
MIRA assembly						
Assembled reads	Assembled contigs	Contigs (>500 bp)	Length (bp)	N50	L50	Average coverage
885,659	1,154	230	9,658,092	40	77	20
Contig joining		**Chromosomes**				
Joined contigs	Supercontigs	Length (Mbp)				
203	10	9.3	4			

b. Genome contents overview

General information	Coding genes	RNA genes	Mitochondrial genome
Size (Mbp): 9.3 (not including rDNA loci, estimated at 0.12 Mbp)	Coding genes: 5,313	tRNA genes: 123	Size (bp): 36, 119
Genome GC content (%): 41.1	Coding (%): 79.6	5S rRNA genes: 21	Genome GC content (%): 22
Assembled chromosomes: 4	Coding GC (%): 41.6		Coding genes: 16
	Mean gene length (bp): 1,442		tRNA genes: 31
	Single exon genes: 4,680		

N50, number of contigs that collectively cover at least 50% of the assembly. L50, length of the shortest contig among those that collectively cover 50% of the assembly.

S. cerevisiae[22], under-represented and over-represented codon pair clusters were observed (**Fig. 2b**). It remains untested in *P. pastoris* whether optimizing genes to this codon pair bias results in higher protein expression levels.

Genome sequence annotation: tRNA genes

tRNA coding genes were automatically predicted and manually confirmed by BLASTN with *S. cerevisiae* homologs, which identified 123 nuclear tRNA genes (**Supplementary Table 3** online), compared

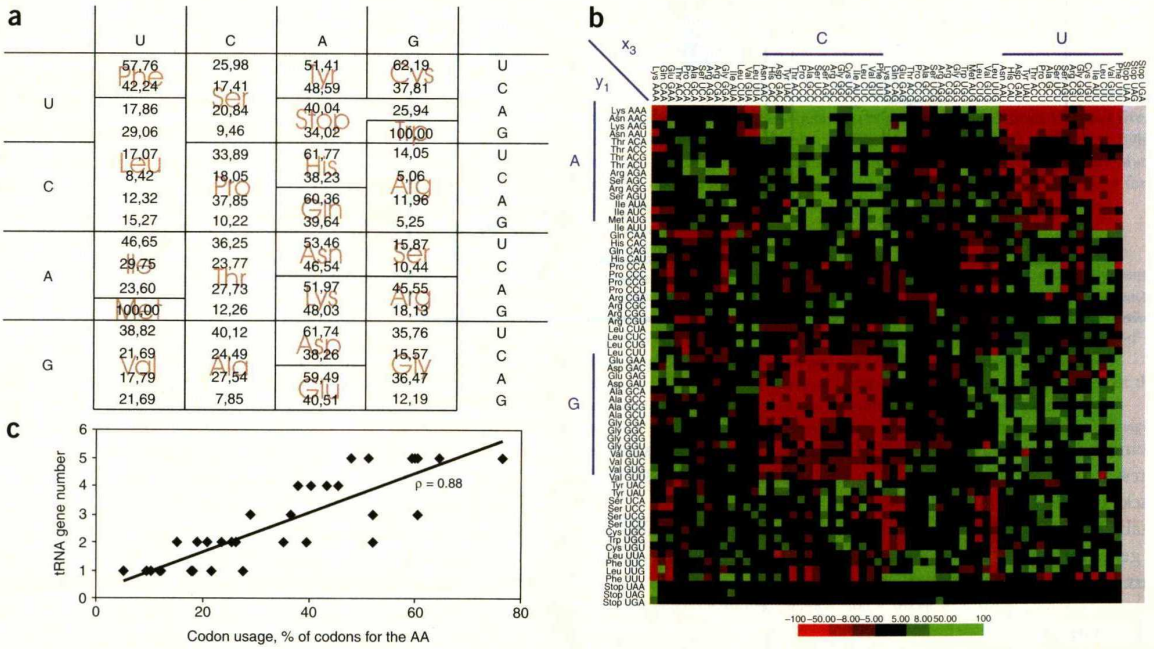


Figure 2 *Pichia pastoris* codon usage. (**a**) Codon usage. Codon usage in the *P. pastoris* ORFeome. The relative abundance of a codon is represented as a percentage of the total codon usage for the amino acid. (**b**) Codon pair usage. Codon pair residual values for *P. pastoris*. The horizontal and vertical axis show, respectively, the 5′ P-site and 3′ A-site codon. Each pixel represents a codon pair residual value. Favored codon pairs are represented in green, under-represented pairs in red. Grouping codon pairs by the x_3 and y_1 nucleotides in the $x_1x_2x_3$ and $y_1y_2y_3$ codon pair reveals over- and under-represented clusters. (**c**) Correlation of tRNA genes and codon usage. Graph shows correlation between the codon usage in relation to the number of genes coding for tRNAs recognizing this codon (Spearman $\rho = 0.88$, $P < 0.0001$).

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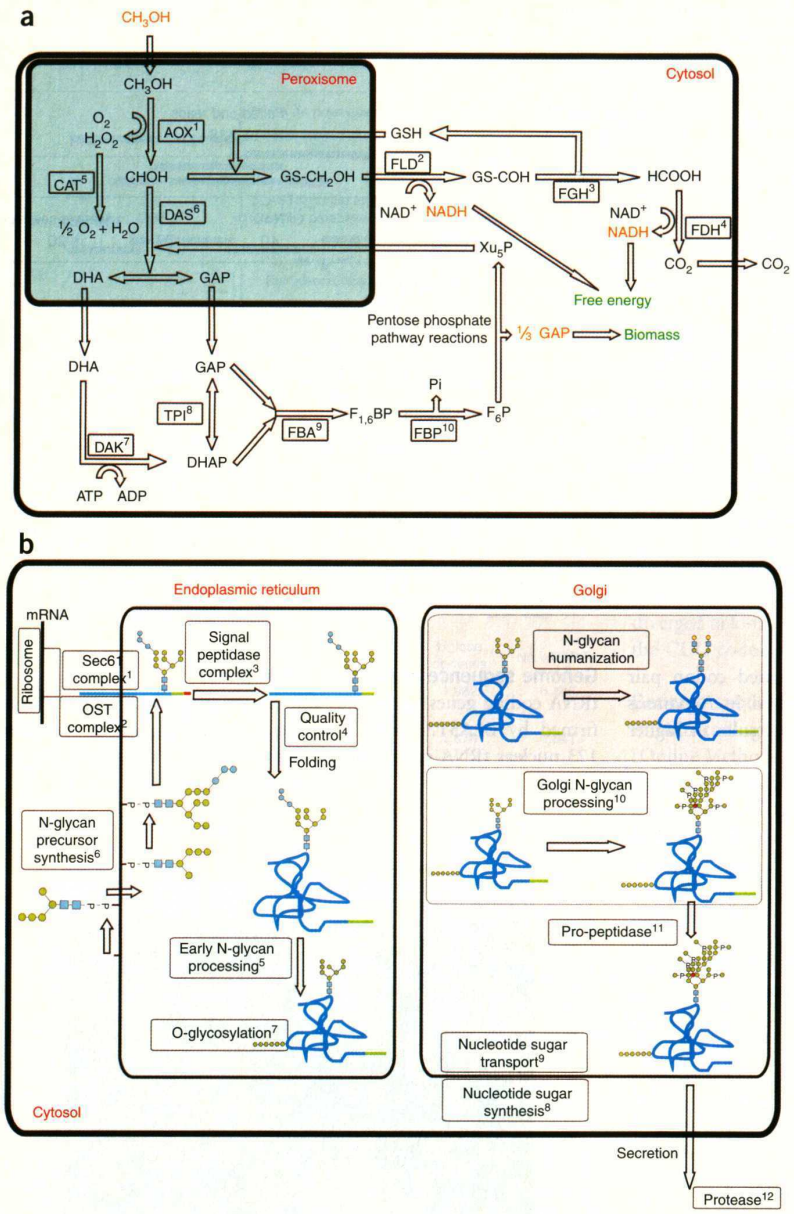


Figure 3 *Pichia pastoris* pathways. (**a**) Methanol utilization pathway in *Pichia pastoris*. A detailed table with the genes coding for the respective enzymes is shown in **Supplementary Table 4a**. [1]AOX, alcohol oxidase; [2]FLD, formaldehyde dehydrogenase; [3]FGH, S-formylglutathione hydrolase; [4]FDH, formate dehydrogenase; [5]CAT, catalase; [6]DAS, dihydroxyacetone synthase; [7]DAK, dihydroxyacetone kinase; [8]TPI, triosephosphate isomerase; [9]FBA, fructose-1,6-bisphosphate aldolase; [10]FBP, fructose-1,6-bisphosphatase; DHA, dihydroxyacetone; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; $F_{1,6}BP$, fructose-1,6-bisphosphate; F_6P, fructose-6-phosphate; P_i, phosphate; Xu_5P, xylulose-5-phosphate; GSH, glutathione. (**b**) Protein secretion pathway. Schematic representation of the secretion pathway in *P. pastoris*. A detailed table with the genes coding for the components involved in the represented complexes or processes is shown in **Supplementary Table 4b**. The nascent protein is translocated to the ER by the Sec61 complex, and N-glycosylation sites are glycosylated with the dolichol-linked $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor by the OST complex. After processing of the signal peptide, the protein is folded with the aid of chaperones. ER N-glycan processing results in $Man_8GlcNAc_2$ type glycan. O-glycosylation is also initiated in the ER by the protein-*O*-mannosyltransferases. After transport to the Golgi apparatus, the N-glycans are further processed to the yeast-typical hypermannosyl-type glycans. In strains with humanized glycosylation pathways,[4–6] the hypermannosylation is abolished and the glycans are processed to $Gal_2GlcNAc_2Man_3GlcNAc_2$. After processing of the pro-domain, the protein is secreted in the growth medium, where it may be a substrate for yeast proteases.

to 274 in the *S. cerevisiae* genome[23]. *P. pastoris* has three tRNA families not present in *S. cerevisiae* (tR(UCG), tL(CAG) and tP(CGG)), but also lacks one tRNA family (tL(GAG)).

Notably, a positive correlation was found between the number of tRNA genes for a given codon and the frequency of use of this codon (Spearman $\rho = 0.88$; $P < 0.0001$, **Fig. 2c**).

DISCUSSION

The genomic sequence of *P. pastoris* presented here will facilitate the development of improved strains with customized properties for high-yield protein production with defined post-translational modifications. Promising targets for genetic engineering include inducible promoters for transgene expression, chaperones that assist protein folding, proteins involved in the secretory pathway and enzymes catalyzing protein glycosylation, proteolytic processing and other post-translational modifications.

The commonly used methanol-inducible promoters in *P. pastoris*—the alcohol oxidase I promoter[10,24] and the formaldehyde dehydrogenase promoter[25]—drive the production of enzymes needed for methanol assimilation and therefore produce extremely high levels of these transcripts upon switching the carbon source to methanol. The genome sequence has allowed identification of all genes coding for enzymes involved in methanol assimilation and their promoters (**Fig. 3a** and **Supplementary Table 4a** online), which can now be studied for their suitability for transgene expression in *P. pastoris*. A first comparative analysis of these promoters did not reveal obvious commonalities in sequence motifs or promoter organization (data not shown).

Secretion of heterologous proteins rather than cytoplasmic accumulation is most often the preferred option in *Pichia*-based production processes. The yeast secretory system (overview in **Fig. 3b**; **Supplementary Table 4b** summarizes the genes discussed in the remainder of the text) is thus an important engineering target to obtain optimized strains that are capable of folding and processing a large flux of recombinant protein. However, many aspects of the secretory pathway are insufficiently characterized. For example, the

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knowledge on the *Pichia* chaperones is incomplete, and we here provide the complete catalog of orthologs to the *S. cerevisiae* endoplasmic reticulum (ER) folding machinery, which should enable more efficacious folding-system engineering in the future[26].

The heterologous preprot signal sequence of the *S. cerevisiae* alpha-mating factor is most often used to induce Sec61p-mediated translocation of the protein into the endoplasmic reticulum of *P. pastoris* (http://faculty.kgi.edu/cregg/). This signal sequence works in most cases, although there have been almost no studies to compare it to other signal sequences. Moreover, the Kex2p/Ste13p-mediated processing of the propeptide in this *S. cerevisiae* sequence is often problematic in *Pichia*[27], resulting in nonnative amino acids at the N-terminus of the heterologous protein. The genome sequence now reveals a multitude of endogenous signal sequences (**Supplementary Fig. 6** online shows a subset of such signal sequences, derived from homologs of functionally annotated secreted *S. cerevisiae* proteins). This database of secretion signals will allow screening for the optimal signal-ORF combination, which may result in augmented protein expression levels. Multiple sequence alignment also allowed derivation of a consensus signal sequence (**Supplementary Fig. 6**), which may be suited for mediating heterologous protein secretion.

The secretory system is also the site of post-translational modification (especially glycosylation), and yeasts differ substantially from higher eukaryotes in this respect. In terms of N-glycosylation, yeasts such as *P. pastoris* modify proteins with a range of heterogenous high-mannose glycans[28], which introduce a large amount of heterogeneity in the protein (reducing downstream processing efficiency and complicating product characterization) and induce fast clearance from the bloodstream. The highly immunogenic terminal alpha-1, 3-mannosyl glycotopes that are abundantly produced by *S. cerevisiae* are not detected on *Pichia*-produced glycoproteins[29]. Indeed, we did not find an ortholog of the *S. cerevisiae* gene *MNN1* (encoding the alpha-1,3-mannosyltransferase) in the *Pichia* genome. However, *Pichia* glycoproteins can in some cases be modified with β-1,2-mannose residues[30], reminiscent of antigenic epitopes on the *Candida albicans* cell wall[31]. We find the patented *P. pastoris* AMR2 β-mannosyltransferase in the genome, and three homologs, thus providing the basis for reducing the levels of this undesired glycan modification.

To overcome the difficulties with *Pichia*'s glycosylation, strains have been developed with an entirely re-engineered glycosylation pathway to produce human IgG–type N-glycans (N-glycosylation humanization technology; **Fig. 3b**)[4–6]. The heterologous glycosyltransferases needed for this use the sugar-nucleotides UDP-GlcNAc and UDP-Gal as monosaccharide donors. Although UDP-GlcNAc is synthesized in yeasts for the synthesis of cell wall chitin (we have identified a UDP-GlcNAc transporter in the genome), no galactosylated glycoconjugates in *P. pastoris* have been described. We have shown previously that the mere overexpression of a *Pichia* Golgi-targeted version of human β-1,4-galactosyltransferase I is sufficient to achieve galactosylation of secreted glycoproteins, indicating that *Pichia* produces UDP-Gal and transports it into the Golgi apparatus[32]. Indeed, we now find an endogenous cytoplasmic UDP-Glc-4-epimerase and clear homologs of Golgi UDP-Galactose transporters in the *P. pastoris* genome (**Supplementary Table 4b**). These findings are relevant to glycan engineering in this yeast as researchers have previously overexpressed a heterologous UDP-Glc-4-epimerase in fusion to the galactosyltransferase to achieve higher levels of UDP-Gal in the yeast Golgi apparatus[6,33].

Yeasts also O-glycosylate secreted proteins with oligomannosyl-glycans that differ from the mucin-type O-glycosylation in humans[34]. No robust engineering approach has yet been developed to overcome this issue. The identification of the *Pichia* protein-O-mannosyltransferases that initiate this modification in the ER in the genome will help toward this goal.

Finally, an often-observed problem is degradation of the product by endogenous proteases. If the heterologous protein is toxic to the cell, much of this proteolytic activity can be of vacuolar origin (released in the growth medium upon cell lysis), but *Pichia* also expresses secreted proteases. It would be of great interest to have a panel of *P. pastoris* strains in which the most active proteases had been disrupted. Only few such strains are currently available because knowledge on the protease gene sequences was unavailable. We here provide a catalog of the *Pichia* vacuolar and secreted proteases (**Supplementary Table 4b**), which will speed up the development of protease-deficient strains.

The wealth of information provided by a full genome sequence will enable a more rapid development of *P. pastoris* as a protein expression host, building on its exceptional natural capacity for heterologous protein production. With a large academic and industrial user base, human-type N-glycosylation already in place, gram-per liter monoclonal antibody production recently reported[8] and the genome now publicly available, the stage is set for *Pichia pastoris* to become an even more important expression system for biopharmaceutical proteins.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Accession numbers. The *P. pastoris* genomic sequence has been deposited in the EMBL Nucleotide Sequence Database (Accession numbers FN392319–FN392325).

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

K.D.S. and P.T. assembled and finished the genome sequence, manually curated the computer-generated annotation, analyzed the annotation and wrote parts of the manuscript. Y.-C.L. performed all post-shotgun assembly bio-informatics aspects of the study under guidance of Y.V.d.P. and P.R. and wrote parts of the manuscript. A.V.H. assisted in gap closure and in determining sequence accuracy. S.G. performed the 454/Roche sequencing and J.W.-L. processed the raw data and performed shotgun assembly and contig scaffolding. Both provided the corresponding methods sections of the manuscript. N.C. designed and coordinated the study, initiated the BLAST-based contig joining approach and wrote parts of the manuscript.

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