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MINI-REVIEW

Yeast expression platforms

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Abstract Yeasts provide attractive expression platforms. They combine ease of genetic manipulations and the option for a simple fermentation design of a microbial organism with the capabilities of an eukaryotic organism to secrete and to modify a protein according to a general eukaryotic scheme. For platform applications, a range of yeast species has been developed during the last decades. We present in the following review a selection of established and newly defined expression systems. The review is concluded by the description of a wide-range vector system that allows the assessment of the selected organisms in parallel for criteria like secretion or appropriate processing and modification in a given case.

Keywords Heterologous gene expression · Transformation · Wide-range vector system · Yeast expression platforms

Introduction

An increasing number of microbial organisms and eukaryotic cells have been employed for the production of recombinant proteins (Gellissen 2005). Production procedures based on such organisms and cells had to be developed that meet both the demand for efficient mass production in case of compounds like technical enzymes and criteria of safety and authenticity in case of pharmaceutical proteins. In this respect, yeasts offer considerable advantages over alternative eukaryotic and prokaryotic systems. Yeast-based production processes have been established with product yields in a multigram range (Gellissen et al. 2005a). The organisms meet safety aspects in that they do not harbor pathogens, viral inclusions, or pyrogens. Easy fermentation regimens have been established that allow rapid growth on simple media to high cell densities. Being eukaryotic organisms, yeasts are capable to secrete recombinant proteins and to modify them according to a general eukaryotic scheme (Gellissen et al. 2005b). Moreover, host strains have been engineered that execute humanized complex *N*-glycosylation structures in *Pichia pastoris* (Hamilton et al. 2003, 2006) and humanized high-mannose *N*-glycosylation in *Hansenula polymorpha* (Kim et al. 2006).

The initial yeast system applied to heterologous protein production was based on the baker's yeast *Saccharomyces cerevisiae*, among others to the production of insulin and HBsAg (Melmer 2005). However, certain limitations are often encountered when using this yeast. *S. cerevisiae* tends to hyperglycosylate proteins; *N*-linked carbohydrate chains are terminated via an α1,3-mannose bond which is of allergenic potential. In several cases, the preferential use of episomal vectors has led to strain instabilities resulting in batch inconsistencies of production runs (Gellissen et al. 2005a).

Alternative systems were subsequently established aimed at overcoming the described drawbacks. More recently defined systems include among others the methylotrophic species *Candida boidinii* (Sakai et al. 1996), *P. methanolica* (Raymond et al. 1998), *P. pastoris* (Ilgen et al. 2005) and *H. polymorpha* (Gellissen 2000, 2002; Kang and Gellissen 2005), *Kluyveromyces lactis* (Gellissen and Hollenberg 1997) and the dimorphic species *Arxula adeninivorans* (Böer et al. 2005) and *Yarrowia lipolytica* (Madzack et al. 2005).

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Some of the systems like *H. polymorpha* and *P. pastoris* are distinguished by an impressive track record as academic tools or as producers of valuable proteins that have already reached the market, whereas others have yet to establish themselves but demonstrate great potential for biotechnological applications. They indeed meet to a great extent the expectations for improvements: The alternatives lean mostly on vectors that are mitotically stable integrated into the genome of the respective host. Most of the species can grow on a broad range of substrates enabling the use of a variety of strong constitutive or inducible promoters for expression control and accordingly provide a versatile fermentation design. The allergenic terminal α1,3-mannose bond present in *S. cerevisiae* is known to be replaced by nonallergenic α1,2 bonds in some of the strains (*P. pastoris* and *H. polymorpha*). Some species are thermo-tolerant (*A. adeninivorans* and *H. polymorpha*), others are nitrate-assimilating organisms (*A. adeninivorans* and *H. polymorpha*), *A. adeninivorans* and *Y. lipolytica* are furthermore dimorphic yeasts (Gellissen 2005; Gellissen et al. 2005b).

The plethora of yeasts thus constitutes attractive expression platforms. Nevertheless, it is obvious that no single system is optimal for all proteins. Hence, predictions for a successful strain and process development can only be made to a certain extent. This means that the initial selection may result in costly time- and resource-consuming failures. To minimize such risks, a wide-range vector system has been established, that provides the possibility to assess several yeast platforms in parallel for criteria such as authentic processing and modification or efficient secretion in a given case (Gellissen et al. 2005b; Steinborn et al. 2006). Applied to the platforms selected for this review, a high probability of success can be envisaged.

Yeast-based expression platforms

Saccharomyces cerevisiae

To date, more than 800 yeast species have been described. *S. cerevisiae* is the species with the longest tradition of use by mankind in brewing and baking, and often the collective term “yeast” is applied to this single species. Accordingly, an overwhelming knowledge on its physiology, genetics, and biochemistry has been assembled. *S. cerevisiae* is currently a major subject in fundamental research, encompassing cell biology, genetics, biochemistry, and molecular biology, and is often used as a model eukaryotic cell. Completion of the *S. cerevisiae* genome project was accomplished in 1996 making it the first eukaryotic organism whose genome has completely been deciphered (Goffeau et al. 1996). Due to its application to food manufacturing, *S. cerevisiae* has obtained a GRAS status (Generally Recognised as Safe) in the early 1990s.

Accordingly, the baker’s yeast was the first eukaryote applied to heterologous gene expression (for early review, see De Batselier-van Broekhoven 1994; Gellissen and Hollenberg 1997).

Vectors used for transformation of suitable *S. cerevisiae* host strains are in general hybrids between yeast-derived and bacterial sequences. The bacterial segment harbors elements required for plasmid propagation in an *Escherichia coli* host, as such an *ori* and a sequence conferring resistance against a specific antibiotic like ampicillin. The yeast portion similarly contains the elements for selection of yeast transformants such as genes for β-isopropylmalate dehydrogenase (*LEU2*) or oritidine 5’-decarboxylase (*URA3*). A comprehensive list of genetic markers can be found in early reviews (i.e., Hinnen et al. 1994; Wach et al. 1994).

Different types of *S. cerevisiae* vectors exist. Most of the production systems lean on transformants generated with plasmids of the YEp type. They are usually present in 30 copies or more. They replicate independently of the chromosomal DNA, as they contain components of the natural 2-μm yeast plasmid, namely, an *ARS* sequence and the *STB* locus. However, the preferential use of YEp vectors has led in several instances to unstable strains resulting in batch inconsistencies of production runs (Gellissen 2005).

Vectors of the YIp type do not contain *ARS* sequences and are integrated into the chromosomes. They are highly stable but are present in low copy numbers only (Hinnen et al. 1994). In a strategic approach to overcome this disadvantage, the vector can be targeted to the ribosomal DNA (rDNA) cluster, present as 150 tandem repeats. In recombinant strains engineered in this way, mitotically stable integrated vectors are present in high copy numbers (Lopes et al. 1996). Copy numbers can further be increased when using a defective promoter element for expression control of a selection marker. Such defective promoters can be created by removal of 5’-flanking sequences as executed in case of the *leu2-d* promoter (Steinborn et al. 2007b).

For expression, a heterologous open reading frame is fused to a promoter of a highly expressed *S. cerevisiae* gene, very often derived from a gene of the glycolytic pathway. Examples for such control elements are the *ADH1*, *GAP*, or the *PGK* promoter, all established within the first two decades of yeast genetic engineering.

Heterologous proteins can either be deposited intracellularly or can be targeted to the secretory apparatus by a secretion signal sequence. Most commonly, the prepro-sequence of MFα1 is employed, a signal sequence that is functional in all yeast systems assessed so far (Brake et al. 1984). Other options include alternative prepro-sequences like those derived from the *Schwanniomyces occidentalis GAM1* gene or from the gene encoding the hyperglycemic hormone of the shore crab *Carcinus maenas* (Weydemann et al. 1995), similarly pre-sequences like that derived from acid phosphatase genes of

different sources (Phongdara et al. 1998; Kaur et al. 2007). In some instances, the genuine leader of the foreign protein to be produced will work.

Often, secretion of a heterologous protein is impaired. In individual cases, this limitation can be eased by coexpression of a secretory pathway gene like *SSO1* (Ruohonen et al. 1997), *PDI* (Bao and Fukuhara 2001), *CMK2* or *CNE1* (Steinborn et al. 2006). Further improvements can be achieved by using mutant strains that influence the efficiency of various steps of protein synthesis and secretion or improve the quality of the protein. Examples include *pmr1* (Rudolph et al. 1989), *pcr1*/*kex1* (Hinnen et al. 1994), or *rgr1* strains (Sakai et al. 1988).

Various pharmaceuticals have been produced using recombinant *S. cerevisiae* strains for heterologous gene expression. The range of pharmaceuticals includes insulin, hepatitis B vaccines in various formulations, dosages and combinations, the anticoagulant hirudin, and glucagon (Brocke et al. 2005; Melmer 2005). *S. cerevisiae*-derived insulin is commercially available since 1987; yeast-derived hepatitis B vaccines were launched in 1986; an early successful example for a technical enzyme is the high-level production and secretion of an *Aspergillus*-derived glucose oxidase (de Batselier-van Broekhoven 1994).

Kluyveromyces lactis

K. lactis is an ascomyceteous budding yeast species belonging to the endoascomycetales. *K. lactis* is a heterothallic organism with a predominantly haplontic cycle. *K. lactis* was initially designated as *S. lactis* due to its close genetic relationship to *S. cerevisiae*, yet both species are physiologically very different (Barnett et al. 2000). *K. lactis* can utilize a wide range of carbon sources like galactose, raffinose, and maltose; a most discriminative feature is its ability to use lactose as a sole carbon source. The genome of *K. lactis* has been completely sequenced by the French National Centre for Sequencing (Genoscope) and annotated. Some strains contain a pair of cytoplasmic linear DNA plasmids pGKL1 (8.8 kb) and pGKL2 (13.4 kb) conferring the killer phenotype. A 2-μm-like plasmid, pKD1 (4757 bp), has been isolated in *K. lactis* strains; of particular biotechnological interest is the 1.6-μm circular pKD1 plasmid naturally occurring in a *K. lactis* strain formerly designated as *K. drosophilarum* (Chen et al. 1997).

During growth on lactose-containing substrates like whey, genes of the lactose-utilizing pathway are strongly induced. Lactose is taken up by lactose permease (encoded by *LAC12*) and metabolized to glucose and galactose by an intracellular β-galactosidase (encoded by *LAC4*). Having the strong inducible *LAC4* promoter in hand, a most promising element for heterologous gene expression became available (Hollenberg and Gellissen 1997).

The first transformation system for *K. lactis* was described in the early 1980s using vectors containing a Km^R gene for selection. When containing the *S. cerevisiae*-derived ARS sequence, the fate of such constructs can be altered into a chromosomal integration instead of maintaining an episomal state in case of a genuine KARS sequence. A genomic integration can be achieved targeting the foreign DNA to the *LAC4* gene (van den Berg et al. 1990) or to the rDNA (Bergkamp et al. 1992). In the first case, a prochymosin sequence was fused to the MFα1-prepro-leader and inserted between promoter and terminator of the *LAC4* gene. A transformant containing several integrated copies was selected for large-scale production at a 40,000-l scale. The purified enzyme is commercially available (trade name MAXIREN) and applied to cheese production (van den Berg et al. 1990). In the second case, successful production of β-galactosidase has been achieved (Bergkamp et al. 1992).

Alternative expression systems are based on the pKD1 plasmid from *K. drosophilarum* mentioned before (Fleer et al. 1990). Expression vectors have been developed containing the *S. cerevisiae*-derived *URA3* gene or a Tn903-derived Km^R gene for selection in suitable hosts. Such episomal vectors are present in about 70 copies per cell and remarkably stable in the absence of selection pressures. Production processes based on such transformants have been established for human interleukin-1α and human serum albumin (HSA; Fleer et al. 1990). Recombinant HSA has been produced in *K. lactis* using a pKD1-based expression system or an integrated pGB-HSb20 vector. Production levels of about 3 g l^{-1} in fermenters at cell densities of 100 g l^{-1} (dry cell weight) were obtained.

Other proteins produced in *K. lactis* are guar α-galactosidase, mouse α-amylase, and D-amino acid oxidase, etc. Surprisingly, most of the promoters used are *S. cerevisiae* promoters (*PHO5*, *GAL1*, *GAL7*, *GAL10*, *GPD*, *PGK*, *MFα1*).

Arxula adeninivorans (*Blastobotrys adeninivorans*) and *Yarrowia lipolytica*

The characteristics of the two dimorphic species as platforms for heterologous gene expression have recently been reviewed (Gellissen et al. 2005a).

The first description of *A. adeninivorans* was provided by Middelhoven et al. (1984) who selected a yeast species from soil by enrichment culturing and designated it as *Trichosporon adeninovorans*. Recently, *A. adeninivorans* was renamed as *Blastobotrys adeninivorans* after detailed phylogenetic comparison of a range of gene sequences (Kurtzmann and Robnett 2007).

A particular strain CBS 8244T was found to exhibit unusual biochemical activities being able to assimilate a range of amines, adenine, and several other purine compounds as sole energy and carbon source. A second strain, LS3 (PAR-4), was isolated in Siberia (Kapultsevich, Institute of Genetics

and Selection of Industrial Microorganisms, Moscow, Russia) from wood hydrolysates with characteristics similar to the CBS strain, yet another seven strains were isolated during the 1990s in The Netherlands and in South Africa (Van der Walt et al. 1990). They all share properties like nitrate assimilation and xerotolerance; all representatives of the new proposed genus are ascomycetous, anamorphic, and arthroconidial (Van der Walt et al. 1990). Special features of biotechnological impact are the thermo-tolerance and temperature-dependent dimorphism which is especially pronounced in the Siberian wild strain *A. adeninivorans* LS3. This strain can grow at temperatures of up to 48°C without previous adaptation to elevated temperatures and is able to survive some hours at a temperature of 55°C (Wartmann et al. 1995). Strain LS3 exhibits a temperature-dependent morphological dimorphism. At temperatures above 42°C, a reversible transition from budding cells to mycelial forms is induced. Budding is reestablished when cultivation temperature is decreased below 42°C (Fig. 1).

Wartmann et al. (2000) selected mutants that grew already as mycelia at 30°C, thus enabling a distinction of temperature-mediated and morphology-related effects on gene expression. Like in other dimorphic yeasts, different contents of RNA and soluble protein and different dry weights are observed. During the middle and end phases of the exponential growth, mycelia are found to be of lower RNA and protein content. In contrast, synthesis of secreted proteins including the enzymes glucoamylase and invertase is more pronounced in mycelia resulting in a twofold higher extracellular protein accumulation.

The first transformation system based on *A. adeninivorans* has been developed using the *LYS2* genes from *A. adeninivorans* and *S. cerevisiae* as selection markers. In these instances, transformation vectors inconsistently either integrated into the chromosomal DNA or were of episomal fate displaying an altered restriction pattern. Therefore, this system was replaced by alternatives based on a stable integration of heterologous DNA into the rDNA (Rösel and Kunze 1998). For rDNA targeting, it is equipped with an *A. adeninivorans*-derived 25S rDNA fragment. Further elements are selection markers like the *E. coli*-derived *hph* gene inserted between the constitutive *A. adeninivorans*-derived *TEF1* promoter and the *PHO5*-terminator conferring resistance to hygromycin B or the *A. adeninivorans*-derived *ALEU2* and *AILV1* genes for complementation of a respective auxotrophic strain. The resulting transformants were observed to harbor two to ten plasmid copies stably integrated into the ribosomal DNA (Wartmann et al. 1998, 2003a). Transformants could be obtained from both the wild-type strain and mutant strains. A new attractive host/vector system is based on *atrp1* complementation (Steinborn et al. 2007a). Under control of a defective *ALEU2* promoter, transformants with up to 20 copies can be obtained (Steinborn et al. 2007b). Further improvements in vector design resulted in plasmids that leave behind upon integration all initially contained bacterial sequences. The range of alternative promoters includes elements derived from the *GAA* (Wartmann and Kunze 2000) and the *AHSB* gene (Wartmann et al. 2003b). For secretion, a set of secretion leader sequences is available similar to those described before for *S. cerevisiae*.

Based on recombinant *A. adeninivorans* strains, production processes for *E. coli*-derived β-galactosidase (Wartmann and Kunze 2000), green fluorescent protein, and HSA (Wartmann et al. 2003b) and enzymes like invertase (Böer et al. 2004) have been established. Furthermore, strains have been engineered that coexpress two or more heterologous genes. As such, a system for the production of polyhydroxyalkanoates (PHA) has been established cointegrating and coexpressing the three *Ralstonia eutropha*-derived *phbA*,

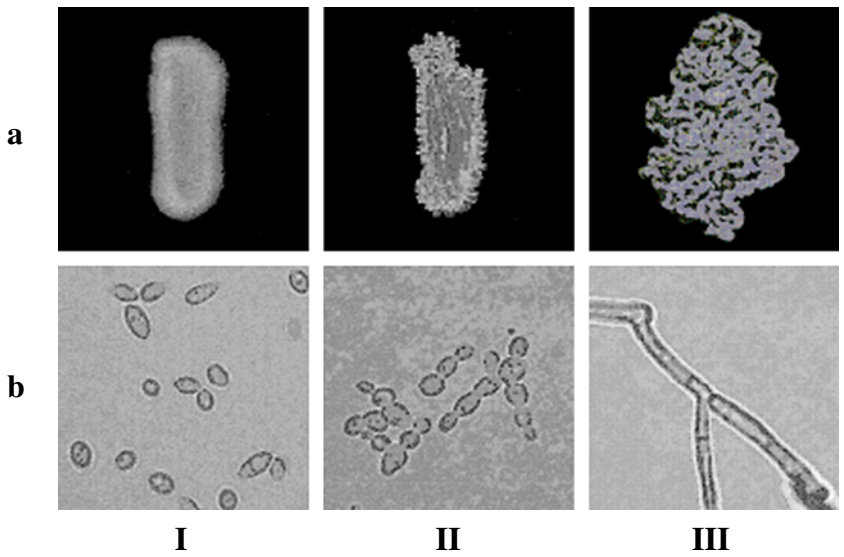

Fig. 1 **a** Colony form and **b** cell morphology of *A. adeninivorans* LS3 grown at 30°C (*I*), 42°C (*II*) and 45°C (*III*). The cells were cultured in YEPD medium for 18°h

phbB, and *phbC* genes (Terentiev et al. 2004; Böer et al. 2005; Steinborn et al. 2006). In yet another application, a biosensor for the detection of estrogenic activities in wastewater has been developed coexpressing the gene for the human estrogen receptor α (*hERα*) and a *Klebsiella*-derived phytase (*phyK*) reporter gene fused to a *GAA* promoter which had been modified by the insertion of estrogen-responsive elements (Hahn et al. 2006; Steinborn et al. 2006).

The yeast *Y. lipolytica* is the only known species in its genus, but the asexual taxon *C. neoformans* appears phylogenetically closely related (Bigey et al. 2003). Nearly all natural isolates from *Y. lipolytica* are hemiascomycetous, haploid, heterothallic, and belong either to the A or B mating type. Crossing of A and B strains results in the formation of a stable diploid, which can be induced to sporulate, forming one to four spored asci. *Y. lipolytica* metabolizes only few sugars (mainly glucose, but not sucrose), alcohols, acetate, and hydrophobic substrates such as alkanes, fatty acids, and oils and is strictly aerobic (Barth and Gaillardin 1997). *Y. lipolytica* is a dimorphic organism which, depending on the growth conditions, is able to form predominantly either yeast cells, hyphae, or pseudohyphae.

An efficient integrative transformation system became available in the mid-1980s. It results from (generally) homologous recombination events between vector and the chromosomal target sequences. Single-copy integration is the rule, permitting gene complementation and gene replacement or invalidation. No natural episomal DNA was ever found in *Y. lipolytica*. Replicative vectors have been engineered based on ARS. They behave like mini-chromosomes, segregate 2:2 at meiosis and can be stably maintained as one to three copies per cell (Madzak et al. 2000). The initial tools, promoter, signal sequence, and terminator for efficient production and secretion were isolated from the highly expressed *XPR2* gene encoding the secreted alkaline extracellular protease AEP. First attempts to increase the copy number in *Y. lipolytica* leant on homologous multiple integrations into the rDNA cluster, together with a defective selection marker, i.e., the *ura3d4* allele (Mougin et al. 2003). Using this combination, transformants carrying up to 60 integrated copies could be obtained, present as tandem repeats located on one or two genomic sites. However, only around ten copies were found to be stable in cases of detrimental gene products. Other potential genomic regions for targeting are the Ylt1 retrotransposon (up to 35 copies) with very large (714 bp) long terminal repeats (LTRs) and the "zeta" sequences, which exist also as solo copies (up to 60; Schmid-Berger et al. 1994).

The native form of the *XPR2* promoter (p*XPR2*) was initially applied to the expression of heterologous protein genes (Schmid-Berger et al. 1994; Juretzek et al. 2001). However, a complex regulation imposed restriction on general industrial use: It is active at pH above 6, and its full induction requires high levels of peptones in the culture medium (Nicaud et al. 1991). The functional dissection of the p*XPR2* showed that one of its upstream activating sequences, UAS1, was poorly affected by environmental conditions (Müller et al. 1998). Consequently, this element was used to construct a hybrid promoter composed of four tandem copies of the UAS1 and inserted upstream of a minimal *LEU2* promoter (reduced to its TATA box). This recombinant promoter, *hp4d*, is almost independent from environmental conditions such as pH, carbon and nitrogen sources, and presence of peptones (Blanchin-Roland et al. 1994; Madzak et al. 1999). *Hp4d* is able to drive a strong expression in virtually any medium. It retains yet unidentified elements that drive a growth phase-dependent gene expression because *hp4d*-driven heterologous gene expression was found to occur at the beginning of stationary phase. This newly acquired characteristic enables a dissociation of growth and expression phases. *Hp4d* has been used successfully for the production of various heterologous proteins in *Y. lipolytica* (Madzak et al. 1999, 2000). The secretion pathway appeared to be closer to that of mammalian cell (cotranslational secretion of secretory polypeptides) than to that of the baker's yeast *S. cerevisiae* (Beckerich et al. 1998). Two strong constitutive promoters, derived from the *Y. lipolytica TEF* and *RPS7* genes, have been identified (Müller et al. 1998), and a number of inducible promoters with interesting properties have been described. Dominguez et al. (1998) reported the use of the bidirectional metallothionein promoter, but again, the requirement of metal salts for induction imposes restriction on general use. *Y. lipolytica* capacity to grow on hydrophobic substrates promoted the search of promoters of genes for key enzymes from this pathway. As such, promoters from isocitrate lyase (*ICL1*), 3-oxo-acyl-CoA thiolase (*POT1*), and acyl-CoA oxidases (*POX1*, *POX2*, and *POX5*) were isolated and tested. p*ICL1*, p*POT1*, and p*POX2* were found to be the strongest inducible *Y. lipolytica* promoters. They are highly inducible by fatty acids and alkanes and repressed by glucose and glycerol. p*ICL1* is also inducible by ethanol and acetate, but it is not completely repressed in the presence of glucose and glycerol. p*ICL1* and p*POX2* have been used successfully for heterologous production (Dominguez et al. 1998; Madzack et al. 2005; Gellissen et al. 2005b).

Candida boidinii, *Hansenula polymorpha* (*Pichia angusta*), *Pichia methanolica*, and *Pichia pastoris*

A limited number of yeast species is able to utilize methanol as sole energy and carbon source. These include *H. polymorpha*, *P. pastoris*, *C. boidinii*, and *P. methanolica* (Gellissen 2000). The first methylotrophic yeast described was *Kloeckera* sp. No 2201, later reidentified as *C. boidinii*. Subsequently, other species, including *H. polymorpha* and *P. pastoris*,

were identified as having methanol-assimilating capabilities (Middelhoven 2002).

All *P. pastoris* expression strains are derived from strain NRRL-Y 11430. In case of *H. polymorpha*, three basic strains with unclear relationships, different features, and independent origins are used in basic research and biotechnological application: Strain CBS4732 (CCY38-22-2; ATCC34438, NRRL-Y-5445) was initially isolated from soil irrigated with waste water from a distillery in Pernambuco, Brazil. Strain DL-1 (NRRL-Y-7560; ATCC26012) was isolated from soil; a strain designated NCYC495 (CBS1976; ATAA14754, NRLL-Y-1798) is identical to a strain first isolated from spoiled concentrated orange juice in Florida and initially designated *H. angusta* (Middelhoven 2002). In basic research, it is additionally used as model organism for research in peroxisomal function and biogenesis (van der Klei and Veenhuis 2002), as well as nitrate assimilation, a feature not shared by *P. pastoris* and the other methylotrophs (Siverio 2002).

Methylotrophic yeast species share a compartmentalized methanol metabolic pathway which has been detailed elsewhere (Guengerich et al. 2004). During growth on methanol, key enzymes of this metabolism are present in high amounts, and peroxisomes proliferate. An especially high abundance can be observed for alcohol oxidase (AOX), formate dehydrogenase (FMD), and dihydroxyacetone synthase. Their synthesis is regulated at the transcriptional level of the respective genes. In the two *Pichia* species, two *AOX* genes are present, designated *AOX1* and *AOX2* in *P. pastoris* (Cregg et al. 1989) and *AUG1* and *AUG2* (alcohol-utilizing gene) in *P. methanolica* (Raymond et al. 1998). In *C. boidinii* and *H. polymorpha*, a single gene is present (*AOD1*—Sakai and Tani 1992, and *MOX*—Kang and Gellissen 2005). The respective genes are induced in the presence of methanol except for the *MOX* gene in *H. polymorpha*, which is derepressed in absence of glucose (Kang and Gellissen 2005). Promoter elements derived from these genes constitute attractive components for the control of heterologous gene expression that can be regulated by carbon source addition to a medium. The possibility of eliciting high promoter activity with glycerol as sole carbon source and even with limited addition of glucose (glucose starvation) in *H. polymorpha* is unique among the methylotrophic yeasts. In *P. pastoris*, the active status of the promoter is strictly dependent on the presence of methanol or methanol derivatives (Ilgen et al. 2005). In addition, *FLD1* (formaldehyde dehydrogenase) promoter is available for *P. pastoris* (Shen et al. 1998), and both *DAS1* (dihydroxyacetone synthase) and *FMD* (formate dehydrogenase) promoters in *C. boidinii* (Sakai et al. 1995, 1996) and *H. polymorpha* (Hollenberg and Gellissen 1997).

Promoters aside from those derived from methanol metabolism genes can also be utilized for efficient heterologous gene expression in the various methylotrophic yeast species. These include the *GAP1* promoter in *P. pastoris* (Waterham et al. 1997; Doring et al. 1998) and the *GAP1* (Sohn et al. 1999) and *PMA1* promoter (Heo et al. 2003) in *H. polymorpha* as constitutive elements. In *H. polymorpha*, promoters derived from nitrate assimilation pathway genes are available (Brito et al. 1999) as well as the trehalose-6-phosphate synthase (*TPS1*)-derived promoter. The latter elicits excellent levels of heterologous gene expression at normal and elevated temperatures (Amuel et al. 2000).

Established production systems lean on suitable host strains and effective components mostly derived from genes of the methanol metabolism pathway. Furthermore, vectors used for transformation are stably integrated into the genome providing for a consistent production process.

Plasmids are targeted to specific genomic loci. In *P. pastoris*, foreign DNA is targeted to the *AOX1*, the *HIS4* genes (Cregg et al. 2000; Ilgen et al. 2005), or rDNA (Steinborn et al. 2006), in *P. methanolica* to the *AUG1* gene (Raymond et al. 1998). In most recombinant *H. polymorpha* strains, foreign DNA is integrated using HARS-sequence-harboring circular plasmids for transformation (Hollenberg and Gellissen 1997). In contrast to earlier assumptions of a random integration of such plasmids, it was found that an expression vector containing a gene for HBsAg under control of a *FMD* promoter was integrated into the *FMD* gene via homologous recombination (unpublished results). Targeted integration can be achieved using the *MOX/TRP3* locus for disruption/integration (Agaphonov et al. 1995). Other target sequences include HARS36 (Agaphonov et al. 1999), the *LEU2* gene (Agaphonov et al. 1999), or the rDNA cluster (Cox et al. 2000; Steinborn et al. 2005, 2006). Transformations in *H. polymorpha* typically result in a variety of individual mitotically stable strains containing single to multiple copies of the expression cassette in a head-to-tail arrangement.

Strains with up to 100 copies have been identified (Hollenberg and Gellissen 1997). Furthermore, several genes can be coexpressed in *H. polymorpha* cotargeting several vectors to the rDNA in as single transformation step (Klabunde et al. 2002). Alternatively, expression cassettes can be transferred in subsequent transformation steps using different marker genes for selection. Using this approach, strains can be isolated that harbor different genes in a fixed copy number ratio. Resulting from these fixed copy numbers, a gene dosage-dependent production of different recombinant proteins can be envisaged. This makes feasible the production of complex composite structures like mixed hepatitis B particles (Janowicz et al. 1991) as well as the creation of new metabolic pathways coproducing suitable recombinant enzymes in an optimal stoichiometric ratio (Gellissen et al. 1996). Despite of homologous recombination with linearized recombinant DNA, multiple copies can also be present in *P. pastoris* and *P. methanolica*.

As the *P. pastoris* has become an academic tool, hundreds of expression examples exist for this platform

(Ilgen et al. 2005). A range of *H. polymorpha*-derived pharmaceuticals like Hepatitis B vaccines (Brocke et al. 2005), IFNα-2a (Müller II et al. 2002), or insulin (Kang and Gellissen 2005) have already reached the market, a recombinant hexose oxidase is applied to baking (Cook and Thygesen 2003).

Comparative aspects

Vector design

In the previous section, a selection of yeast expression systems has been presented. Although design of the plasmids presented so far meets the demands and requirements of the specific hosts, they follow similar construction principles and may even share to large extent similar or identical elements. Most of them are shuttle vectors with a bacterial part (*ori*) and selection marker for plasmid propagation in *E. coli*. The yeast part may lean on two basic options: A first possibility leads to constructs which result in an episomal fate of the transformation plasmid. Such vectors contain elements of naturally occurring plasmids, such as the 2-μm plasmid-derived vectors for *S. cerevisiae* or the pKD1-derived vectors for *K. lactis*. Alternatively, vectors are mitotically stable integrated into the host's genome targeting the foreign DNA to specific genes of the host by homologous recombination as it is the case for most of the host/vector systems described before. Examples for such genes are AOX or the *FMD* genes in the methylotrophs, the *LAC4* gene in *K. lactis*, or the rDNA cluster in all described species. For selection of a recombinant yeast strain, a dominant selection marker may be present or a sequence for complementation of a particular auxotrophy. The expression cassette for incorporation of a heterologous gene usually contains a strong inducible or constitutive promoter. A selection of such elements has been presented in the previous sections for the different hosts.

Secretion, processing, and glycosylation

Secretion vectors can be equipped with a variety of prepro- or pre-leader sequences. The most commonly applied element is that derived from MFα1 as described before. However, differences exist in the basic mechanism of secretion. In *S. cerevisiae*, a posttranslational translocation mechanism is preferred; in other yeasts like *Y. lipolytica*, cotranslation is predominant (Boasmaré et al. 1998). Several genes involved in quality control and secretion efficiency of proteins have been assessed for process improvements, like *KAR2*, *PDI1*, *SSO2*, and others (Steinborn et al. 2006). A particular interesting gene is *SLS1* that controls the activity of Kar2p in *Y. lipolytica* (Kabani et al. 2003).

Protein glycosylation represents an important protein modification having a potential impact on stability, activity, or immunogenicity. Mammals generate different types of *N*-linked glycosylation: high-mannose, complex, and hybrid types. Lower eukaryotes, including yeasts, are only able to perform *N*-glycosylation of the high-mannose type adding only mannose residues to the outer chains. Detailed analysis of glycans has only been performed for *S. cerevisiae*, *P. pastoris*, and *H. polymorpha*. Moreover, the yeast mannose chains can be much longer than in mammals and of heterogeneous length: In *S. cerevisiae*, they are typically of 50 to 150 mannose residues, a situation referred to as hyperglycosylation. In some alternative yeasts, the extent of hyperglycosylation is less pronounced: *P. pastoris* and *H. polymorpha* preferentially produce chains of 8 to 14 mannose residues (Jigami and Odani 1999; Guengerich et al. 2004). Detailed data addressing this aspect of characterization in *Y. lipolytica* are restricted to a single recombinant protein of human origin where short oligosaccharide chains of about eight to ten mannose residues have been observed (Madzack et al. 2005). Terminal mannose residues in *N*-linked glycans are added in *S. cerevisiae* by an α-1,3 bond which is suspected to be allergenic; in *H. polymorpha* and *P. pastoris*, a nonallergenic α-1,2 bound is present (Jigami and Odani 1999; Guengerich et al. 2004). In *A. adeninivorans*, patterns of *O*-glycosylation vary, depending on the morphological status (Wartmann et al. 2002). In case of *P. pastoris*, strains with humanized complex *N*-glycosylation have been engineered and applied in a recent example to the production of terminally sialylated EPO (Hamilton et al. 2006); strains with humanized *N*-glycans of the high-mannose type exist for *H. polymorpha* (Kim et al. 2006).

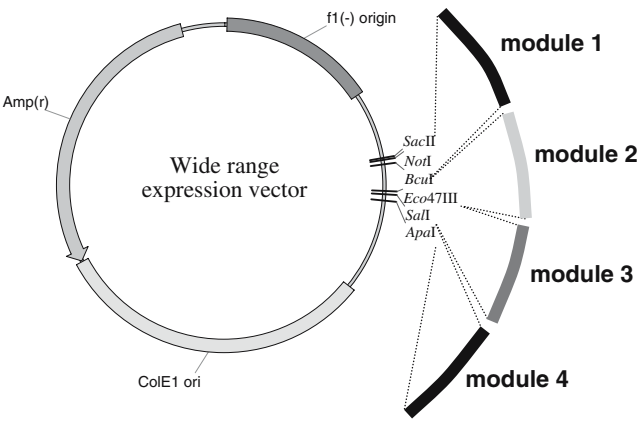

Fig. 2 Design and functionality of the wide-range yeast vector (CoMed™) system. The vector contains all *E. coli* elements for propagation in the *E. coli* system and an MCS for integration of yeast fragments like ARS, rDNA, selection marker, and expression cassette modules. For this purpose, all fragments are flanked by specific restriction sites (rDNA regions—*Bcu*I/*Eco*47III, selection markers—*Eco*47III/*Sal*I, expression cassettes—*Sal*I/*Apa*I ARS—*Sac*II/*Bcu*I

Differences exist among the different yeast platforms in processing capabilities of particular precursor proteins. In a recent example, it was shown that the IL-6 processed from a MFα1/IL-6 precursor was *N*-terminally truncated in *S. cerevisiae* and *H. polymorpha*, but not in *A. adeninivorans* (Steinborn et al. 2006; Böer et al. 2007).

A wide-range yeast vector system

Despite the superior characteristics of yeast hosts in various developments, there is clearly no single system that is optimal for production of all possible proteins. Predictions of a successful development for a given protein can only be made to a certain extent when restricting the initial strain engineering to a single species. They described platforms exhibit differences in productivity, processing, or glycosylation. The availability of a wide-range yeast vector system enables the assessment of several yeasts in parallel for their capability to produce a particular protein in desired amounts and quality. The design of a vector suited for a wide range of fungal organisms has to meet several prerequisites. Such a plasmid has to contain a targeting element suitable for all test species. The promoter that drives heterologous gene expression has to be functional in all these organisms. The vector/host system has to employ a dominant selection marker or a sequence that can complement the auxotrophy in all selected organisms. Some yeast-derived sequences fulfill all these criteria. For selection, the *A. adeninivorans*-derived *LEU2* gene or an *E. coli*-derived resistance marker (*hph*; Wartmann et al. 2003a; Rösel and Kunze 1998) that confers resistance against hygromycin B were chosen. rDNA is an obvious universal target for integration, and rDNA targeting has been described for a range of yeast species including *S. cerevisiae* (Lopes et al. 1996), *K. lactis* (Bergkamp et al. 1992), and *Y. lipolytica* (Madzack et al. 2005), but only recently conserved rDNA sequences of both organisms have been defined as targeting elements with appropriate characteristics (Steinborn et al. 2005). For expression control, an *A. adeninivorans*-derived *TEF1* promoter was chosen (Rösel and Kunze 1995). The basic design is shown in Fig. 2. The system is being supplemented with additional auxotrophic strains as such of a *trp1* background and modular vector elements for complementation (Steinborn et al. 2007a, b). Additional modular promoter element will be incorporated into the system that can drive heterologous gene expression in all or in a subset of the yeast platforms included in the system.

The wide-range yeast vector (CoMed™) system has recently been applied to the comparative assessment of yeasts for several product developments (Steinborn et al. 2006). For proof of concept, an expression cassette was engineered harboring the *Aequoria victoria*-derived *GFP* gene under control of the *A. adeninivorans*-derived *TEF1* promoter and incorporated in a particular vector construct equipped with the *E. coli*-derived *hph* gene for dominant selection and a conserved rDNA sequence for targeting. The final construct was addressed in parallel to *S. cerevisiae*, *H. polymorpha*, *P. pastoris*, *Debaryomyces polymorphus*, *D. hansenii*, and *A. adeninivorans* and successful expression could be demonstrated for all selected platforms. In a second example, an expression cassette for an MFα1/IL-6 fusion was integrated into a similar vector now containing the *A. adeninivorans*-derived *LEU2* gene for complementation of respective auxotrophic hosts. When comparing the products secreted by recombinant *S. cerevisiae*, *H. polymorpha*, and *A. adeninivorans* strains, the mature cytokine was found to be *N*-terminally truncated in the baker's yeast and the methylotroph (Steinborn et al. 2006; Böer et al. 2007).

rDNA integration provides the option to cointegrate several independent vectors in parallel into different yeast hosts. This was successfully executed among others for the assessment of different yeasts for the production of PHA.

Outlook

We have presented a selection of established and novel yeast expression platforms. The biodiversity of the respective species and the derived elements adds to the versatility of yeast expression systems. The growing knowledge about the genetics, physiology, and biochemistry of these organisms and the emerging genomics is expected to provide further improvements. The attractiveness of these microbes, the future advances, and the inclusion of yet unknown species will secure to the yeasts a prominent place within the range of expression platforms.

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