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Non-conventional yeasts as hosts for heterologous protein production

Summary Yeasts are an attractive group of lower eukaryotic microorganisms, some of which are used in several industrial processes that include brewing, baking and the production of a variety of biochemical compounds. More recently, yeasts have been developed as host organisms for the production of foreign (heterologous) proteins. *Saccharomyces cerevisiae* has usually been the yeast of choice, but an increasing number of alternative non-*Saccharomyces* yeasts has now become accessible for modern molecular genetics techniques. Some of them exhibit certain favourable traits such as high-level secretion or very strong and tightly regulated promoters, offering significant advantages over traditional bakers' yeast. In the present work, the current status of *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Hansenula polymorpha* and *Pichia pastoris* (the best-known alternative yeast systems) is reviewed. The advantages and limitations of these systems are discussed in relation to *S. cerevisiae*.

Key words *Kluyveromyces lactis* · *Yarrowia lipolytica* · *Pichia pastoris* · *Hansenula polymorpha* · Heterologous protein

Introduction

Heterologous gene expression is of considerable interest for the production of pharmaceutical proteins of therapeutic (i.e. interferon, interleukins, etc.) and commercial (i.e. industrial enzymes) interest. Initially, the commercial production of heterologous proteins was achieved using *Escherichia coli* as host [42]. The dominance of *E. coli* in this field is a reflection of the depth of information about its genetic and biochemical systems accumulated over many decades of research. However, as the nature of the recombinant proteins being expressed became more complex and as transformation systems were established in more and more species, a variety of hosts—ranging from prokaryotes to transgenic animals and plants—has been developed (the baculovirus system should also be considered). In this context, yeasts are very useful hosts in that they exhibit several advantages over other microorganisms. Yeasts, mainly *Saccharomyces cerevisiae*, have been used for centuries in food production. Furthermore, there is extensive information about their safety; they are considered as Generally Recognized As Safe (GRAS) by the American Food and Drug Administration (FDA). By contrast, prokaryotic organisms may have toxic cell wall pyrogens

(endotoxins) and mammalian cells may contain oncogenic or viral DNA. The fermentation profiles of yeasts are well established and these organisms are able to grow rapidly on simple media up to high cell densities at lower cost than in any other fermentation systems. As eukaryotic microorganisms, they have the ability to perform eukaryotic processing steps on the polypeptides expressed, such as posttranslational processing and modifications (disulphide bond formation, proteolytic maturation of prohormones, N- and O-linked glycosylation, etc.), which are required for the functional synthesis of many proteins.

Most recombinant proteins produced in yeasts have been expressed using *S. cerevisiae* as the host system owing to the ease with which it can be manipulated genetically and to the extraordinary amount of information accumulated about its molecular biology and physiology. Moreover, the sequence of its entire genome has been completed [31]. Despite this, several limitations have been detected in the *S. cerevisiae* system. Examples are product yield, which is usually low, and inefficient secretion (many *S. cerevisiae* proteins are not found free in the culture medium but rather are retained in the periplasmic space or associated with the cell wall [8, 58]). The limitation to this species has now been relieved by the development of expression systems in other yeast species

known as non-*Saccharomyces* yeasts or non-conventional yeasts. This review focuses on the best known nonconventional yeasts: one dairy—*Kluyveromyces lactis*; another able to assimilate polyalcohols, organic acids or normal paraffin—*Yarrowia lipolytica*, and two methylotrophs—*Pichia pastoris* and *Hansenula polymorpha*.

of different strains of *P. pastoris* and *Y. lipolytica*. The best-known yeast strains are described in Table 1. A routine approach to test the gene constructions in several strains of a yeast species with different genetic backgrounds seems necessary to clarify the influence of the host strain in the synthesis of heterologous proteins.

Host strains

The nature of the host strain and of its genetic background is an important parameter (often neglected) governing gene expression but little attention has been paid to this aspect.

In *S. cerevisiae*, De Baetselier et al. [15], reported a 100-fold difference in the level of recombinant secreted glucose oxidase (from *Aspergillus niger*) between the lower and higher producing strains. This variation did not correlate with any known genetic marker. However, in that work the strains used were not specified. The secretion of rHSA has been analyzed in several *Kluyveromyces* strains: *K. fragilis*, *K. bulgaricus*, *K. wickerhamii*, *K. waltii*, *K. drosophilarum* and *K. lactis* (MW98-8C and CBS683). The highest levels of secretion were obtained with both *K. lactis* strains and the lowest with *K. bulgaricus* ATCC16045 [24]. In *H. polymorpha*, Veale et al. [75] improved the expression of guar α -galactosidase by genetic crossing with wild type strains and selection of segregants showing the best growth capacity in methanol. No clear data have appeared in the literature about comparisons

Life cycle and transformation systems

The *P. pastoris* and *H. polymorpha* strains usually are homothallic [27, 36, 67, 68]. Both yeasts are closely related (taking into account DNA/DNA reassociation analysis) and form hat-shaped spores. *K. lactis* is usually heterothallic, the mating types being designated as **a** and α , as in *S. cerevisiae* [78]. *Y. lipolytica* is also heterothallic and the mating type is determined by the two alleles called *MATA* and *MATB* [3]. In the two homothallic yeasts, selection of mutants, complementation and tetrad analysis poses more problems [27, 68] than in the two heterothallic yeasts, in which the standard genetic manipulations described for *S. cerevisiae* can be carried out with similar efficiency [3, 78].

Introduction of recombinant DNA into the four yeast species can be accomplished by any of the following procedures: the spheroplast method; the lithium chloride (acetate) method, and electroporation. The DNA introduced can become established in two ways: integration into chromosomal DNA by recombination, or autonomous replication as a circular plasmid.

Table 1 Available strains currently used

Strain	Description or genotype	Source or reference	
<i>Kluyveromyces lactis</i>	CBS 2359	<i>Mata</i>	(NRRL Y-1140, ATCC 8585)
	CBS 2360	<i>Matα</i>	(NRRL Y-1205, ATCC 8651)
	CBS 683		6
	2359/152	<i>Mata metA1-1</i>	32
	2360/7	<i>Matα lysA</i>	32
	2359/152F	<i>Mata metA1-1 ura 3-302</i>	21, 22
	MW98-8C	<i>Mata uraA argA lysA rag2 k⁺ pKDI</i>	23, 78
	JA6	<i>Matα trp ura ade</i>	78
<i>Yarrowia lipolytica</i>	W29	<i>MatA</i>	ATCC 20460
	E150	<i>MatB his1-1 ura3-302 leu2-270 xpr2-322</i>	3
	E129	<i>MatA lys11-23 ura3-302 leu2-270m xpr2-322</i>	3
	YB423-12	<i>MatB</i>	3
	CX161-1B	<i>MatA ade1</i>	3
	SA-1	<i>MatB</i>	73
<i>Pichia pastoris</i>	NRRL Y-11430-SC5		68
	GS115	<i>his4</i> or <i>GTS115</i>	68
	KM71	<i>his4 aox1:ARG4</i>	13
<i>Hansenula polymorpha</i>	CBS 4732		ATCC 34438
	NCYC 495		30
	LR9	<i>odc1</i>	55
	A16	<i>leu2</i>	30
	RB10		20

Targeted chromosomal integration requires homology of the DNA introduced with a chromosomal locus. Although circular DNA can become integrated, the preferred template is a linear DNA with free ends homologous to a genomic locus. Autonomous replication of circular plasmid DNA requires the presence of an autonomous replicating sequence (ARS) in the circular DNA used for transformation. Transformation can be achieved by complementary auxotrophic markers, *ura3*, *leu2*, *trp1*, *ade1*, and *his4* being the most frequently used, or by dominant selection markers against G418, phleomycin, hygromycin, etc. Usually, electroporation is the method of choice for circular plasmids and an exhaustive analysis of all the parameters involved in the process must be carried out if very high efficiencies of transformation are required [62, 69].

Autonomous replication versus chromosomal integration of the expression cassette

The usual transforming vectors for yeast species are hybrids between yeast-derived and bacterial sequences, because plasmids can be amplified and isolated with greater ease from *E. coli*, harbour an *ori*, and also have a sequence conferring resistance against a specific antibiotic.

Two types of vectors are used to express the gene of interest: (i) episomal vectors, or (ii) vectors integrated in the host chromosome. As a general rule, an ideal vector is one that is maintained in most or all cells in the population, even in the absence of selective pressure, that have a high copy-number gene for a dosage-dependent high-level expression. Episomal systems offer high copy numbers and greater flexibility, allowing, for example, the rapid curing of the host strains and/or rapid changes in the manipulation of the expression construct. Three kinds of these plasmids have been described. The first one involved linking the cloned gene to a centromer by inserting it into a YCp (centromer-based) plasmid. This approach yields a very stable persistence of the cloned gene but a per-cell copy of only one to two plasmids. A second system, using ARS-based vectors, yields high copy levels but these are extremely unstable and are rapidly lost from the yeast population in the absence of selection. The third type of episomal DNA molecule consists of circular plasmids, the 2 μ m plasmid of *S. cerevisiae*, or other similar ones. These vectors are present at high copy level and have stable propagation. However, their occurrence is restricted to only a few yeast species. Integrative expression vectors (vectors without ARS sequences integrated on the chromosome) are highly stable but present in low copy numbers [8, 58]. Several strategies have been developed to increase the copy number of integrated expression vectors. One possibility consists of the integration of heterologous genes targeted to repetitive elements such as delta sequences, Ty elements, or tRNA genes [9]. In another approach, which is probably the best in terms of copy number and expression, the recombinant DNA is targeted to a ribosomal DNA cluster [58].

Promoters

Foreign transcriptional promoters usually give aberrant initiation in yeasts [37] or are totally inactive [58]. Thus, general consensus exists about the essentiality of the use of yeast promoters for the efficient transcription of foreign genes. In general, promoters are regulated to some extent but those presenting poor regulation are called constitutive promoters (i.e. glycolytic promoters). Many experiments have been carried out with such promoters: alcohol dehydrogenase I (ADH [39]), phosphoglycerate kinase (PGK [40]) or glyceraldehyde-3-phosphate dehydrogenase (GAP [59]). However, although glycolytic promoters have been extensively used in the lab and in some cases industrially [51], the yield of heterologous protein is not very good, probably due to the accumulation of ethanol during aerobic fermentation [56]. Also, constitutive promoters are problematic when the product being expressed is toxic to the cells. In some cases hybrid promoters between ADH2 and GAL7 [63] or between invertase and glyceraldehyde-3-phosphate dehydrogenase have been described [34]. In order to circumvent this problem, currently most of the promoters chosen to drive the expression of heterologous proteins in yeast (*Saccharomyces* and non-*Saccharomyces*) systems have been derived from highly-expressed, tightly regulated host genes so that the growth stage can be separated from the expression stage. However, in non-conventional yeasts, probably due to our lack of knowledge, only a few such promoters have been used for the production of foreign proteins. The best known promoters are shown in Table 2 and our research group is currently exploring strategies for the isolation of new regulated promoters, mainly in *K. lactis* and *Y. lipolytica*.

Table 2 Homologous promoters used in *Saccharomyces cerevisiae* and in non-conventional yeasts for the expression of heterologous proteins

Strain	Promoter	Reference
<i>S. cerevisiae</i>	<i>ADH2</i>	58
	<i>CUP1</i>	58
	<i>GAL1</i>	58
	<i>GAL7</i>	78
	<i>GAL10</i>	78
	<i>GAP</i>	58
	<i>PGK</i>	58
	<i>PHO5</i>	58
	<i>Mfα</i>	78
	<i>K. lactis</i>	<i>LAC4</i>
<i>PHO5</i>		22
<i>Y. lipolytica</i>	<i>XPR2</i>	3, 71
	<i>MTPI/II</i>	*
<i>P. pastoris</i>	<i>AOX1</i>	68
	<i>GAP</i>	77
<i>H. polymorpha</i>	<i>MOX</i>	27
	<i>FMD</i>	28

*Promoter corresponding to metallothioneins 1 and 2 (Prado M, García S, Domínguez A, unpublished results)

mRNA 5'- and 3'-untranslated region(s) [UTR]

The nucleotide sequence, the structure, and the length of the 5' and 3' UTR may influence optimal protein production. By adjusting the 5'-UTR region of the promoter to that of *AOX1*-mRNA, Sreekrishna et al. [66] obtained a 50-fold increase in the level of expression of human serum albumin (HSA) in *P. pastoris*. Also, Demolder et al. [16] reported that in *S. cerevisiae* the expression of a prepro-MF α /murine IL-2 fusion under the control of different yeast promoters produced ten-fold higher yields when the major part of the mammalian 3' UTR was deleted.

Codon bias, RNA elongation and RNA stability

Codon usage does not appear to be an essential parameter for gene expression [46]. However, some observations suggest that the preferred sequence context observed in yeast may be optimal for the expression of foreign proteins in *S. cerevisiae*: a 50- and a 3-fold increase in the expression of an immunoglobulin κ chain and in guar α -galactosidase using codon-optimized genes has been described [45].

With regard to codon bias, it should be noted that *K. lactis* does not differ much from *S. cerevisiae* [78]. The codon bias of *Y. lipolytica* is more similar to that of *Aspergillus* but almost no data exist for *P. pastoris* and *H. polymorpha*.

The yield of full-length transcripts may also be affected by fortuitous transcriptional termination, mainly in genes with high A + T contents. Several examples of genes that give rise to truncated mRNA species when expressed in *S. cerevisiae* have been described [58]. Probably the clearest example of this is the expression of the *Clostridium tetani* gene encoding tetanus toxin fragment C in *S. cerevisiae*. It was found that the native AT-rich gene contained at least six fortuitous polyadenylation sites that produce truncated mRNA species [57]. Recently, this problem has also been described in *P. pastoris*. The ATTATTTTATAAAA sequence found in HIV-gp20 led to premature termination [64]. Thus, changing the primary sequence of a gene in order to optimise codon bias is recommended [66, 68], although its influence on other parameters, such as the primary and secondary structure of the resulting mRNA, its translation stability and 3' end formation, must also be considered.

Glycosylation

An important modification taking place in the secretory apparatus is the glycosylation of translated polypeptides. Many proteins of therapeutic interest are glycosylated—IFN's,

hormones, growth factors, etc. Yeasts only synthesize oligosaccharide chains of the high mannose type and are unable to synthesize oligosaccharide chains of the mammalian complex type [58]. In some cases, foreign proteins are glycosylated and while *S. cerevisiae* shows a tendency to hyperglycosylate proteins (heterologous glycosylation by addition of Man >40), in many cases *P. pastoris* and *H. polymorpha* produce glycoproteins with average Man chain lengths of 8 to 14 [8, 27]. Also, tissue plasminogen activator (tPA) secreted from *Y. lipolytica* has been found to contain only short oligosaccharide chains (Man 8–14) [8]. No specific data have been described for *K. lactis*. It seems that the glycosylation patterns of at least some non-conventional yeasts are more similar to the mammalian high mannose type glycosylation and this must be taken into account when attempting to improve heterologous protein expression in yeast.

Secretion signals

Secretion is the preferred mode of protein production due to ease of product recovery. As in higher eukaryotes, protein secretion in yeasts is directed by an amino terminal signal sequence, which mediates co-translational translocation into the endoplasmic reticulum. For a protein without these sequences that is not normally secreted, it may be difficult or even impossible to reach the culture medium. It is also very difficult to obtain normally secreted proteins in soluble intracellular form.

Topogenic signals, such as secretion signals, are functionally conserved throughout nature (i.e., bacteria, plants or mammals). Some authors have used the protein's own signal sequence to direct the secretion of foreign proteins in yeasts in a somewhat arbitrary way—*E. coli* β -lactamase [56], wheat α -amylase [61], human α and γ interferon [40], human salivary α -amylase [52], human serum albumin [2], etc. Other authors have argued that the homologous leader sequences of the yeast itself are preferable for this purpose [58]. It has also been suggested that choice of the signal sequence to be used should be based on the protein to be secreted or on the size of the product [38]. In general, in order to obtain efficient heterologous protein secretion, DNA sequences encoding signal peptides are usually included in the expression cassette [38]. Until now, few systematic approaches to elucidate the effect of the signal peptide on the total amount of heterologous protein have been explored. Using five different leader sequences for the secretion of human serum albumin (HSA) in *S. cerevisiae*, in 1990 Sleep et al. [65] showed that the choice of the signal peptide and its relationships to the structural protein are essential elements in the process.

The most extensively used signal sequence for heterologous protein production is based on that found to be present in the *S. cerevisiae* alpha factor (MF α 1) [7, 38, 58]. The structural gene encodes a 165-residue protein—prepro-alpha factor—,

which contains the signal peptide, 19 amino acids, a pre-region and a pro-region. In the expression of fusions of the alpha factor with heterologous proteins, some authors have recommended retaining the Glu-Ala spacers adjacent to the KEX2 protease cleavage site [7]. This introduces a new element to be considered in the use of leader sequences for heterologous protein production: the utilization of "pro-regions".

In conclusion, it is currently difficult to propose a general strategy for the choice of a signal peptide for the secretion of a heterologous protein and more systematic studies are required to elucidate this point.

Kluyveromyces lactis: general characteristics, vectors and examples of foreign gene expression

The FDA [1, 54] considers *K. lactis* as a GRAS organism. *K. lactis* was used first to produce biomass in whey to supplement food and feed [76] and to produce β -galactosidase, the main enzyme used in commercial preparations for the production of low-lactose milk for intolerant populations [41]. Recent interest in the utilization of *K. lactis* for heterologous protein production seems to derive especially from the ability of this yeast to secrete high molecular weight protein [78].

In *K. lactis* the *LAC4* locus, the *RAG2* locus [24] and the ribosomal RNA repeats [4, 60] have been used for the integration of expression cassettes (Fig. 1). Transformation of *K. lactis* using the *SrtII* linearized pGB-HSA20 results in targeting of the genomic *LAC4* promoter region in about 70% of the transformants. Most transformants contain only 1 or 2 copies of the expression cassette, although in some of them up to 10 copies are found in the *LAC4* promoter region. An alternative system for increasing the copy number of the integrated DNA, analogous to the pMIR42 type vector system described for the integrants in the rDNA of *S. cerevisiae*, also exists for *K. lactis* [50]. The plasmid pMIRK1 (Fig. 1) was present in about 60 copies per cell and persisted stably for over 70 generations under non-selective conditions. However, the expression or the presence of a heterologous gene in this vector reduced the copy number (15 versus 60 integrated copies) [4]. In *K. lactis*, killer linear DNA plasmids (pGKL1 and pGKL2) have been described [35]. Although killer plasmids are a possible source of gene vectors, this involves two major problems. The first is that these plasmids contain covalently attached proteins at their 5' termini, which complicates manipulations in vitro and hinders their amplification in *E. coli*. The second drawback lies in the cytosolic location of the killer plasmid; gene expression on the linear plasmid requires the specific transcription signals of the plasmid. Although some attempts have been made to solve this problem [44], the expression levels of the heterologous proteins (interleukin 1 β) were low in comparison with the results obtained with circular vector [12].

Plasmids containing *K. lactis* autonomously replicating sequences of chromosomal origin (*KARS*) have also been described [18]. Some *KARS* can also replicate in *S. cerevisiae* [18] and have been used in gene expression studies [49]. The main problem in using these plasmids for heterologous protein production is their mitotic instability; after 20 generations less than 40% of the cells contain the plasmid. A stable plasmid, pKD1, has been isolated from a strain (4CD-31-130; CBS2105) formerly called *Kluyveromyces drosophilorum* (hence the name pKD1 [19]) now classified as *K. lactis*. The pKD1 plasmid belongs to the 2 μ m plasmid family, is stably maintained at high copy number (70–100 per cell [19]), and recombinant vectors carrying the plasmid replication origin can be constructed (Fig. 1). The mitotic stability of the pKD1 expression vectors was found to be strain—and species—dependent (among 50 strains tested HSA production yielded from negligible to 300 mg/l in shake flask cultures [24]) and was influenced by promoter strength and culture conditions [24].

Chymosin, the active constituent of the cheese rennet, is the only heterologous protein produced by this yeast that has reached the market place under the name of MAXIREN. High levels of biologically active chymosin can be recovered from the culture supernatant because *K. lactis* does not secrete appreciable amounts of endogenous proteins. The transformants selected for large-scale production contain several integrated copies under the *LAC4* promoter and only require the α -factor leader from *S. cerevisiae* as the signal sequence [74].

Recombinant HSA (rHSA) has been produced in *K. lactis* using a pKD1-based expression system [24] or an integrated pGB-HSb20 vector [70]. Production levels of about 3 g/l in fermentors at cell densities of 100 g/l (dry cell weight) were obtained [24]. The production of rHSA by *K. lactis* in chemostat cultures at 75 mg/l per h has also been reported [6]. Other proteins expressed in *K. lactis* are guar α -galactosidase [4], human interleukin-1 β [23], mouse α -amylase [72] and D-amino acid oxidase [33], etc. Surprisingly, most of the promoters used are *S. cerevisiae* promoters (*PHO5*, *GALI*, *GAL7*, *GAL10*, *GPD*, *PGK*, *MF α*), the exception being the *LAC4* promoter [24, 74].

In most of the cases, the secretion signal fused to the heterologous protein is the MF α leader sequence of *S. cerevisiae*. Only in the case of the human interleukin 1 β has the pre-region of the *K. lactis* killer toxin been used. To improve heterologous protein secretion in *K. lactis*, a promising approach is to clone homologous tightly-regulated promoters and secretion signals. Our research group has isolated several *K. lactis* promoters—*KIPHO5*, regulated by phosphate [21]; *KIPHO3*, regulated by thiamine (Domínguez et al., in preparation), and *KIMAL22* (Domínguez et al., in preparation) which is regulated by maltose. Using the *KIPHO5* promoter and the signal peptide of the repressible acid phosphatase, we were able to secrete up to ten times more trout growth hormone into the culture medium than when the *S. cerevisiae* *PGK* promoter was used [22].

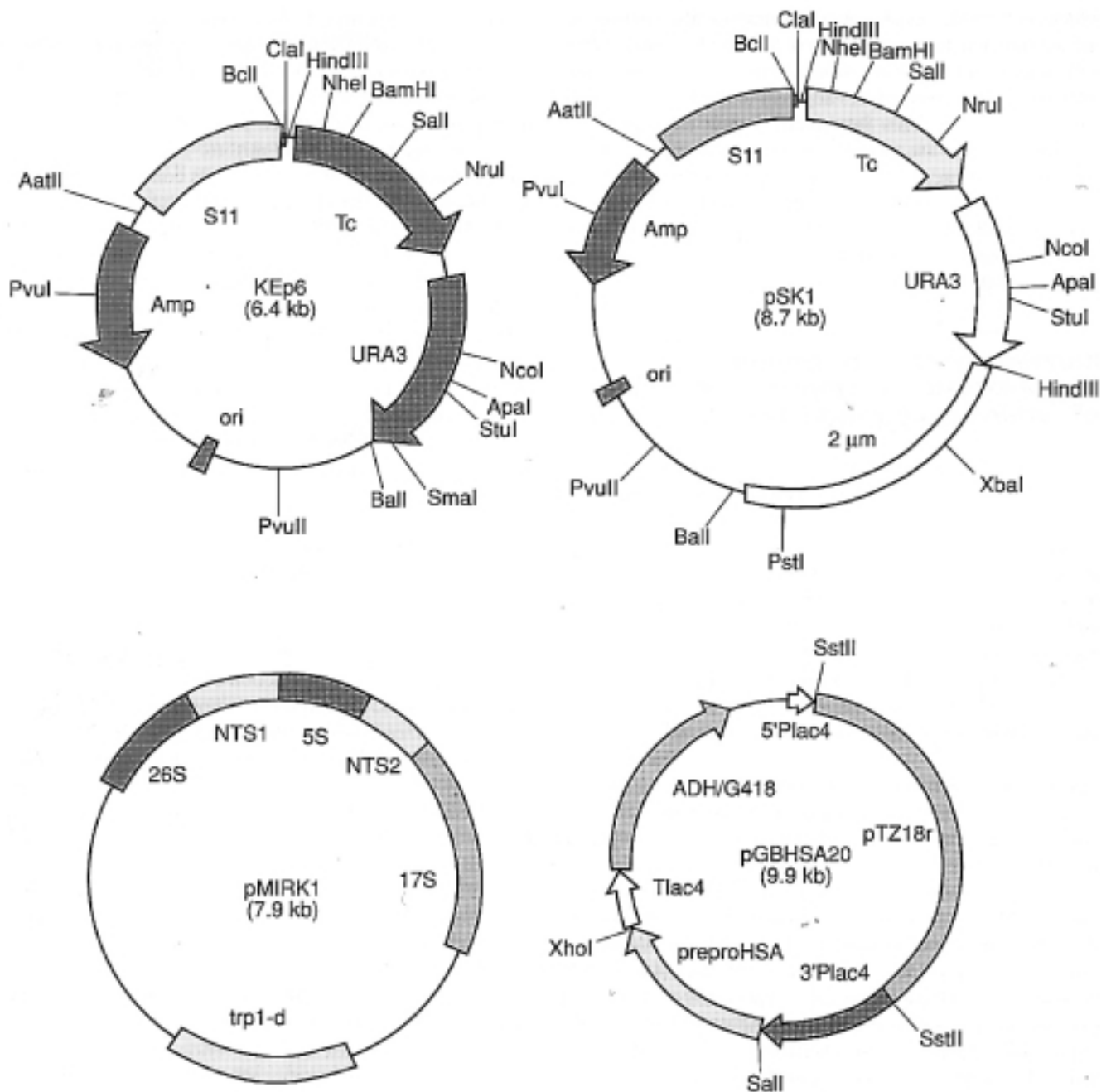


Fig. 1 Typical *Kluyveromyces lactis* vectors. KEp6 is a standard multicopy vector obtained by introducing the replication origin of pKD1 into the *EcoRI* site of pBR322-derived plasmid YIp5. pSK1 is a shuttle vector (a 2- μ origin of replication was added to the KEp6 plasmid). pMIRK1 is a multicopy integration vector, 5S, 17S and 26S designate the rDNA-encoding genes. NTS- non transcribed space. Selection with a *trp1-d* gene with a defective promoter. pGBHSA20 is a plasmid containing a human serum albumin (HSA) expression-cassette for integration into the *LAC4* locus of the *K. lactis* genome

Yarrowia lipolytica: general characteristics, vectors and examples of foreign gene expression

In *Y. lipolytica* integration occurs almost exclusively by homologous recombination [3, Domínguez, unpublished work]

and integrative vectors using the *YILEU2* or the *YIURA3* genes as selective markers have been described [3]. In *Y. lipolytica*, transplacement (double homologous recombination to replace yeast chromosomal DNA, resulting in stable structures without duplication) can be carried out. Experiments to test this approach by replacement—at the same time and with the same construction—of six auxotrophic markers are under way in our

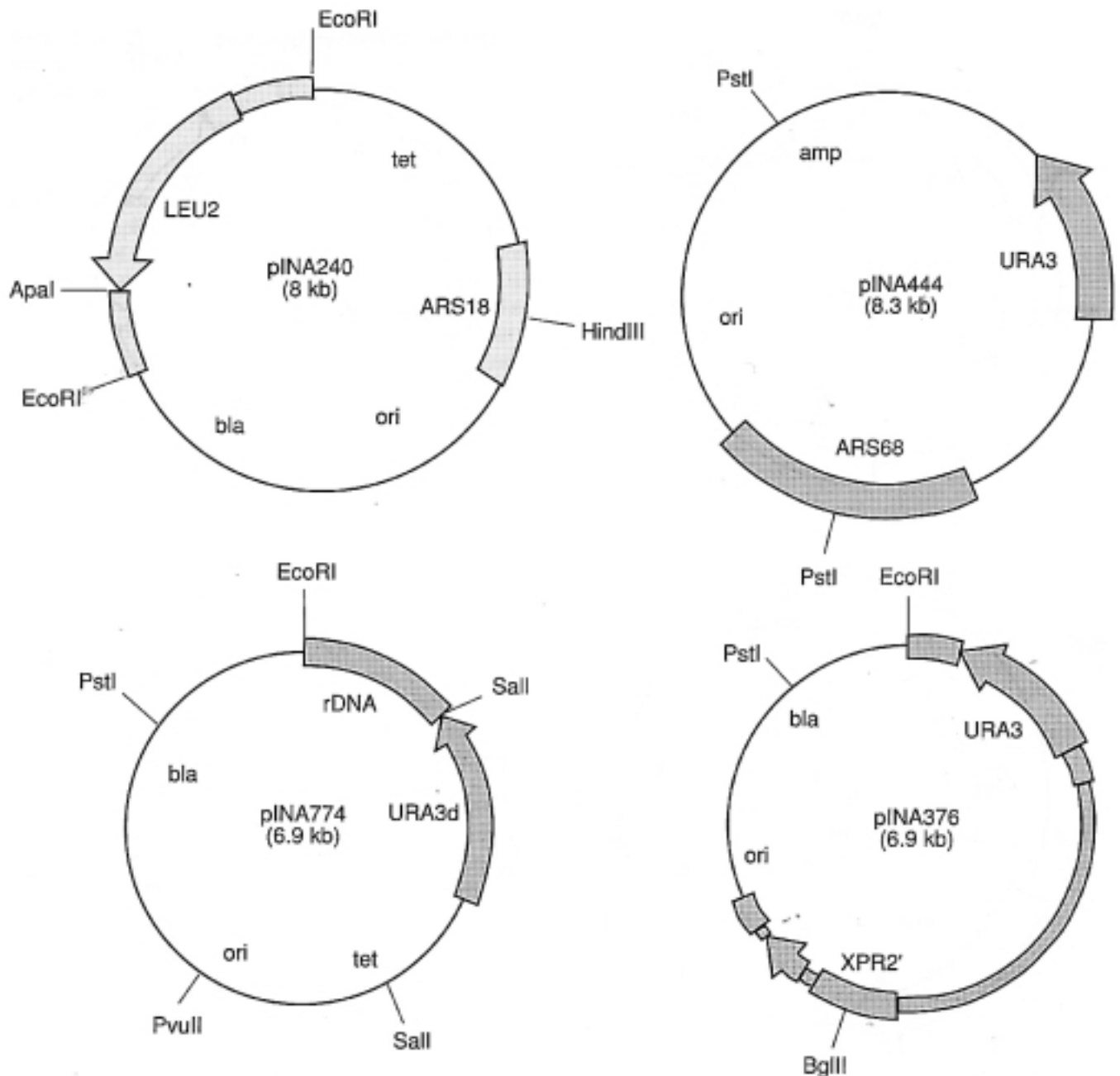


Fig. 2 pINA240 and pINA444, replicative *Yarrowia lipolytica* vectors based on pBR322. ARS18 and ARS68 (Autonomous Replicating Sequences). URA3 and LEU2 markers. pINA774, vector for multicopy integration. It bears a defective URA marker and a fragment of rDNA. pINA376, vector for the expression of secreted proteins containing the XPR2 promoter and terminator sequences, the AEP prosequences and a polylinker sequence

laboratory. The integration of foreign genes in rDNA-based vectors has also been described in *Y. lipolytica* [47]. However, the expression cassette (under the control of the XPR2 promoter) rapidly destabilized high copy number transformants under induction conditions [47]. Plasmids containing *Y. lipolytica* ARS sequences [25] have been isolated (Fig. 2). Vectors containing these sequences were present in only 1–3 copies per cell and were very stable, being lost at a rate of 0.5–5% per

generation. Expression vectors based on these ARS elements are currently being developed [3, Domínguez et al., unpublished results].

Under optimal physiological conditions, wild type strains of *Y. lipolytica* can secrete 1–2 g/l of an alkaline extracellular protease (XPR2) into the medium [3]. The XPR2 promoter and signal sequence have been used to express and secrete human anaphylatoxin C5a [14] and bovine prochymosin [26].

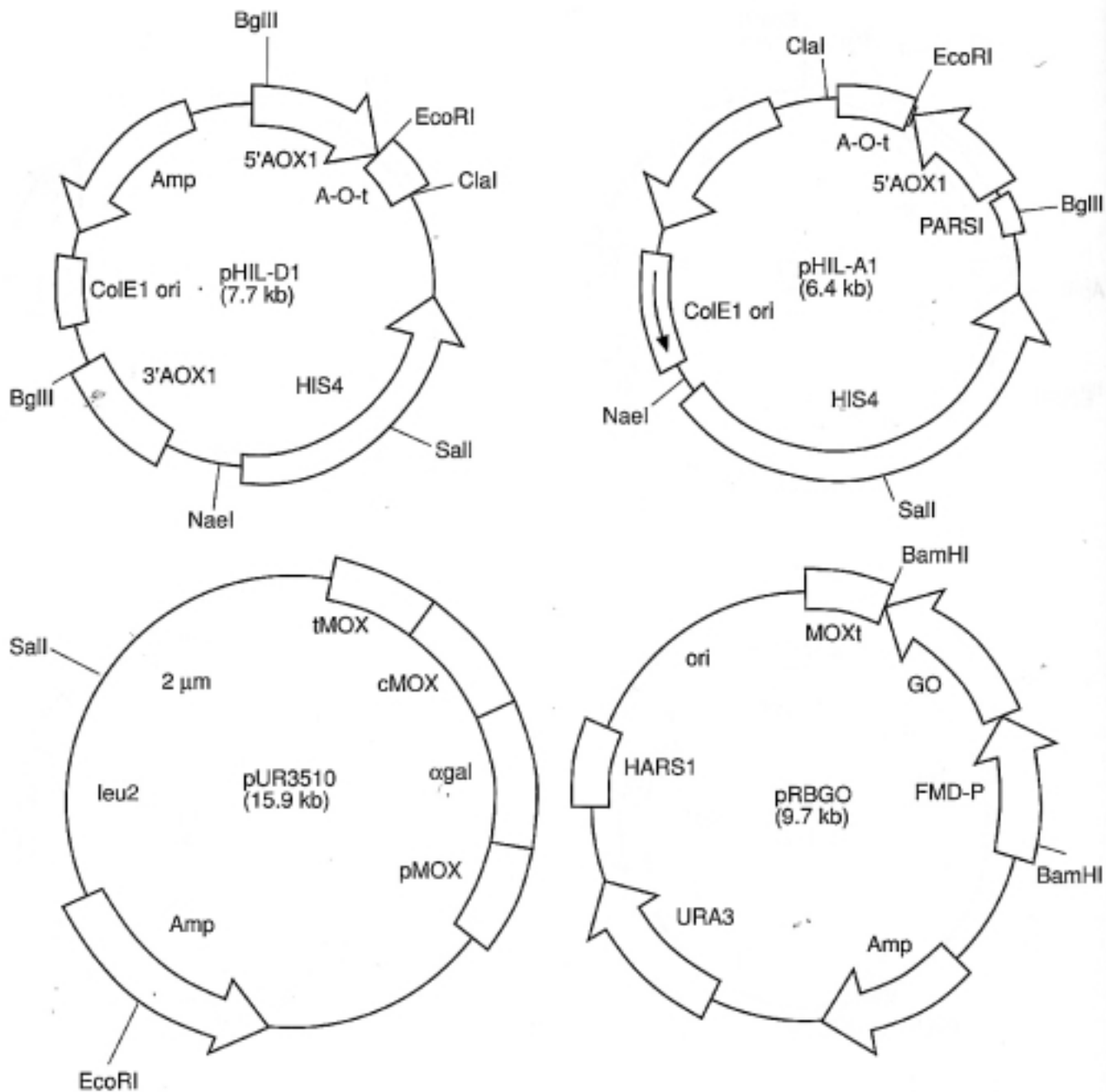


Fig. 3 pHIL-D1 is an *Escherichia coli* (pBR322)-*Pichia pastoris* (*AOX1* promoter and terminator containing *HIS4* as selective marker) shuttle vector. pHIL-A1 is a similar plasmid containing an autonomous replicating sequence from *P. pastoris* (*PARS1*). pUR3510, *Hansenula polymorpha* plasmid for the expression of guar α -galactosidase. It contains a fragment from pBR322, the *LEU2*, the invertase signal sequence and the 2- μ fragment of *S. cerevisiae* and the promoter and terminator of the *H. polymorpha* methanol oxidase gene (*pMOX* and *tMOX*). pRBGO, *H. polymorpha* vector designed for the production of glycolate oxidase. The vector contains the following components: *ori* (*ori*) and a chloramphenicol resistance (*cam*) gene for selection in *E. coli*, the *URA3* of *S. cerevisiae* for selection, a *HARS*-sequence (*HARS1*) for autonomous replication in *H. polymorpha*, and the *FMD*-promoter and a *MOX*-terminator sequences

Gaillardin's group has carried out a detailed analysis of the *XPR2* promoter [5] and has developed two types of expression vectors [3], permitting either expression of cytosolic proteins

or secretion to the culture medium (Fig. 2). These constructions have been used to drive the secretion of porcine α -interferon [53] and human coagulation factor XIIIa [71].

Pichia pastoris: general characteristics, vectors and examples of foreign gene expression

P. pastoris was initially developed by Phillips Petroleum Company for the production of single cell protein and is now probably the non-conventional yeast in which the most proteins have been expressed. Since *P. pastoris* has no native plasmids, expression of the foreign gene was achieved by chromosomal integration, using integrative plasmids such as pHIL-D1 or autonomous vectors such as pHIL-A1 (Fig. 3). A set of derivative vectors has been described [68]. All authors agree that the preferred mode of expression of *P. pastoris* is by integration. Such constructions are stable and, additionally, stable multicopy integrands can be obtained using the spheroplast method of transformation, which renders transformants with a wide range of copy numbers. In practical terms, an analysis of about 100 individual clones for protein production is necessary to obtain a good producer strain. Autonomous vectors such as pHIL-A1 are of low copy number, unstable and a high frequency of plasmid integration is seen, usually at one or more of the chromosomal loci (*AOX1*, *HIS4* or *ARS1*, targeted chromosomal integration). Also, multicopy constructs of the expression cassette have been obtained by transformation with DNA concatemers or using multicopy vectors [66].

As a methylotrophic yeast, *P. pastoris* can grow in methanol as the sole carbon and energy source. Key enzymes in this pathway may comprise up to 20–35% of the total intracellular protein content in methanol-grown cells.

In this system, almost all the foreign genes have been expressed under control of the *P. pastoris* alcohol oxidase 1 (the first enzyme of the pathway) promoter. This promoter is tightly regulated by a carbon source-dependent repression/induction mechanism. Its expression is undetectable in cells grown on glucose or glycerol, but is maximally induced during growth on methanol. Foreign genes whose products are toxic to the cell can be introduced and maintained in *P. pastoris* by culturing the yeast under repressed growth conditions. As mentioned in the previous paragraph, in *P. pastoris* the foreign gene expression cassette is normally integrated in the host genome using *HIS4* as a selectable marker. For optimal genetic stability, the expression cassette is cleaved from the vector and transplanted into the chromosomal *AOX1* locus [13]. This results in the site-specific eviction of the *AOX1* structural gene (Fig. 4) affording a strain that grows slowly in methanol (*Mut*⁻). Alternatively, the vector can be linearized and targeted to integrate by non-disruptive insertion into *HIS4*, generating a strain that grows normally on methanol (*Mut*⁺). For intracellular expression, it is preferable to use *Mut*⁻ cells because they have a lower level of alcohol oxidase protein. As an alternative constitutive promoter, the gene encoding the glyceraldehyde 3-phosphate dehydrogenase enzyme—GAP—in *P. pastoris* has recently been isolated [77].

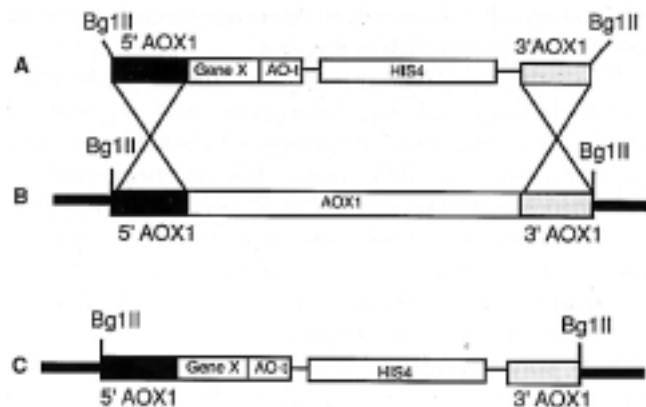


Fig. 4 *Pichia pastoris* *AOX1* gene-containing region and deletion substitution in *AOX*. (A) *Bgl*III-digested DNA derived from plasmid pHIL-D1 (Fig. 3). (B) *P. pastoris* *AOX1* locus. (C) Replacement of the complete *AOX1* locus by the DNA transformation and homologous recombination

An exhaustive list of the heterologous proteins expressed in *P. pastoris* has been published [68]. Three examples are of especial interest: the production of epidermal growth factor, yielding 450 mg/l [10]; the intracellular production of tetanus toxin fragment (yield 12 g/l [11]), and *B. pertussis* pectatin (yield >3 g/l [57]). The *P. pastoris* expression system is available from Invitrogen (San Diego, CA) and data about the fermentation processes involved are available [68].

Hansenula polymorpha: general characteristics, vectors and examples of foreign gene expression

H. polymorpha is a methylotrophic yeast able to grow on methanol as the sole energy and carbon source. In *H. polymorpha* integration of plasmids within the genome and autonomous replicative vectors have been described. Stable integration into *H. polymorpha* chromosomal locations has been reported for the expression cassette pUR3510 based on the *S. cerevisiae* plasmid Yep13 [75]. Targeted vector integration (1 to 22% of the cases) by single or double crossover recombinants in the alcohol oxidase gene (*AO*) has also been described [17]. Stable integration was observed with the integrative vector YIp5 [55]. The same authors demonstrated that the *ARS1* replication origin of *S. cerevisiae* allows unstable plasmid replication and isolated two *ARS* (*HARS1* and *HARS2*) elements from *H. polymorpha* which also allow autonomous replication with high copy number (30 to 40 per cell). Both vectors are shown in Fig. 3.

The key enzyme for methanol utilization is methanol oxidase (*MOX*), which is regulated by a powerful regulon [48]. *H. polymorpha* is subject to carbon catabolite repression; it is repressed by glucose and induced by methanol, formaldehyde

and formate [29]. It is also susceptible to derepression by certain other carbon sources such as glycerol.

Currently, the *MOX* promoter is almost exclusively employed to express heterologous genes in *H. polymorpha* [36]. Another gene, encoding formate dehydrogenase (*FMD*), has been cloned and its promoter has also been used for heterologous protein secretion [28]. *H. polymorpha* is able to secrete large amounts of heterologous products. In comparison with *P. pastoris*, *H. polymorpha* requires short fermentation times and high levels of protein expression can be obtained using glycerol as the carbon source.

In *H. polymorpha*, a high frequency of integration even in the presence of an *ARS* sequence in the vector has been observed, and strains with up to 100 copies of the integrated expression cassette have been identified [27]. Target homologous integration in the *MOX* site is more difficult than in *P. pastoris* and optimal growth and induction of heterologous gene expression are better on mixed substrates (glucose/choline; glucose/methanol/ammonium sulphate) [27].

Most of the heterologous proteins expressed in *H. polymorpha* are described by Gellisen and Hollenberg [27]. Some interesting examples are the production of S and L surface antigens of the hepatitis B virus (intracellularly to up 2–8% of the total soluble protein [43]) and recombinant hirudin, now under clinical trial [79].

Concluding remarks

Upon choosing appropriate experimental organisms for heterologous gene expression, one must carefully consider the advantages that each of the available bacterial and eukaryotic expression systems offers. Considering only yeasts, *S. cerevisiae* is the best known organism, although it does have some drawbacks, such as limited secretory capacities, hyperglycosylation, etc. Non-conventional yeasts are alternative systems for the expression of heterologous proteins. For the four non-conventional yeasts described in this review, the most important tools required for heterologous gene expression have already been developed. In two of them—*K. lactis* and *Y. lipolytica*—knowledge of their general physiology is reasonably advanced. In the other two—*P. pastoris* and *H. polymorpha*—most work has been directed specifically to the production of recombinant proteins. Regarding secretion efficiency, generally all four yeasts seem to have better secretory capacities than *S. cerevisiae*. *K. lactis* offers the advantage of being a GRAS organism. Finally, it should be noted that *K. lactis* and *Y. lipolytica* do not require explosion-proof fermentation equipment and that episomal vectors exist for both.

Acknowledgements This work was partially supported by grants from the CICYT (BIO92-0304 and BIO 95-0518) and EU (BIO4-CT96-0003). We wish to thank all our colleagues in the EU project for helpful discussions.

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