MINIREVIEW

Heterologous protein production in the yeast *Kluyveromyces lactis*

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Abstract

*Kluyveromyces lacti*s is both scientifically and biotechnologically one of the most important non-*Saccharomyces* yeasts. Its biotechnological significance builds on its history of safe use in the food industry and its well-known ability to produce enzymes like lactase and bovine chymosin on an industrial scale. In this article, we review the various strains, genetic techniques and molecular tools currently available for the use of *K. lactis* as a host for protein expression. Additionally, we present data illustrating the recent use of proteomics studies to identify cellular bottlenecks that impede heterologous protein expression.

Introduction

The yeast *Kluyveromyces lactis* has been studied for decades and has a well-established track record of safe use in various food industry applications (Bonekamp & Oosterom, 1994). Since the 1950s, *K. lactis* has been used as a source of lactase (β-galactosidase), an enzyme that degrades milk sugar (lactose) and is necessary for production of lactose-free dairy products. Dried and inactivated *K. lactis* was also used as a protein supplement in food in the 1960s. In the 1980s, *K. lactis* was one of the first yeasts for which a transformation system was established (Das & Hollenberg, 1982), leading to its development as an efficient host for heterologous protein expression. As an expression host, *K. lactis* is best known for its use in commercial production of the milk clotting enzyme bovine chymosin (Van den Berg *et al*., 1990). This protein was the first heterologous enzyme originating from a higher eukaryote that was produced at low cost in a microorganism, and the process developed for its industrial-scale production was widely recognized as a major biotechnological achievement. In 1993, (Swinkels *et al*., 1993) first reviewed the use of *K. lactis* as a host for heterologous protein expression. At that time, eight proteins had been successfully secreted from *K. lactis*. Today, over 40 proteins have been produced with *K. lactis*, illustrating its utility as an alternative yeast expression system (Table 1). These proteins originate from bacteria, fungi, viruses, plants, and mammals, emphasizing the ability of *K. lactis* to efficiently produce a diverse range of heterologous proteins.

As a host for heterologous protein production, *K. lactis* has a number of advantages over other yeast expression systems. These include easy genetic manipulation, the ability to use both integrative and episomal expression vectors, and the availablility of a fully sequenced genome (Dujon, 2004). Growth of *K. lactis* can be performed in standard yeast medium and does not require the explosion-proof fermentation equipment necessary for large-scale growth of methylotrophic yeasts such as *Pichia pastoris*. In addition, enzymes from *K. lactis* have GRAS (generally regarded as safe) FDA status, permitting their use in various food and feed applications. Finally, an easy-to-use reagent kit for *K. lactis* protein expression is now offered by New England Biolabs and was developed in collaboration with DSM.

In this article, we review the protein production track record of *K. lactis* and summarize advances in the development of genetically modified host strains, molecular tools and methods for protein expression in *K. lactis* made over the past decade. Where possible, we provide a perspective on

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Table 1. Recombinant proteins secreted from *Kluyveromyces lactis*

Protein	Source	Method(s)	Promoter(s)	Leader(s)	Yield		Reference(s)
					Flask	Fermentor	
D-amino acid oxidase	*Trigonopsis variabilis*	pKD1	Sc GAL1	NA	150 U g^{-1}		Gonzalez *et al*. (1997)
α-amylase	Mouse	pKD1	Sc PGK	KT	0.527 U mL^{-1}		Tokunaga *et al*. (1997)
α-amylase	*Schwanniomyces occidentalis*	pKARS	Native	Native	30 mU mL^{-1}		Strasser *et al*. (1989)
Cellulase	*Teredinibacter turnerae*	LAC4 integration	Kl LAC4-PBI	Kl α-factor			D. Distel, *et al*. (unpublished)
Cellulase	*Aspergillus aculeatus*	pKD1	Kl LAC4	Native			Müller *et al*. (1998)
Cellulase	*Humicola insolens*	pKD1	Kl LAC4	Native			Müller *et al*. (1998)
Endoxylanase	*Neocallimastix frontalis*	pKD1	Sc PGK	KT	110 U mL^{-1}		Durand *et al*. (1999)
Enterokinase	Cow	LAC4 integration	Kl LAC4-PBI	Kl α-factor	2 mg L^{-1}		C. Taron, *et al*. (unpublished)
Galactanase	*Aspergillus aculeatus*	pKD1	Kl LAC4	Native			Müller *et al*. (1998)
α-galactosidase	*Cyamopsis tetragonoloba*	pKARS	Sc GAL7	Sc pre-SUC2	2 mg L^{-1}		Bergkamp *et al*. (1992)
		pKD1	Sc GAL7	Sc pre-SUC2	90 mg L^{-1}		
		rDNA integration	Sc GAL7	Sc pre-SUC2	250 mg L^{-1}		
α-galactosidase	Coffee bean	LAC4 integration	Kl LAC4	Kl α-factor			C. Taron, *et al*. (unpublished)
â-galactosidase (lactase)	*Kluyveromyces lactis*	pKD1	Sc PGK	KT			Becerra *et al*. (2001)
Glucoamylase	*Arxula adeninivorans*	pKD1	Sc GAP	Native	101 nkat mL^{-1}		Bui *et al*. (1996)
Glycoprotein E2	*Hepatitis C virus*	TRP1 integration	Kl AAC	KT	1 mg L^{-1}		Mustilli *et al*. (1999)
Growth hormone	Trout	pKD1	Kl PHO5	Kl PHO5			Ferminan & Dominguez (1998)
Insulin precursor	Pig	pKD1	Sc ADH2	Sc α-factor	30 mg L^{-1}		Feng *et al*. (1997)
Interferon alpha A	Human	pKD1	Sc PGK	Sc α-factor	2 mg L^{-1}		Chen *et al*. (1992)
Interleukin-1 beta	Human	pKD1	Sc PGK	KT	80 mg L^{-1}		Fleer *et al*. (1991a)
			Sc PHO5				
Invertase	*Saccharomyces cerevisiae*	rDNA integration	Sc α-factor	Sc α-factor			Hsieh & Da Silva (1998)
Laccase	*Pleurotus ostreatus*	pKD1	Kl ADH4	Native	2.03 U mL^{-1}		Piscitelli *et al*. (2005)
β-lactoglobulin	Sheep	pKD1	Sc PGK	Native	50 mg L^{-1}		Rocha *et al*. (1996)
Luciferase	*Gaussia princeps*	LAC4 integration	Kl LAC4-PBI	Kl α-factor	1 mg L^{-1}		C. Taron, *et al*. (unpublished)
Lipase	*Thermomyces lanuginosus*	pKD1	Kl LAC4	Native			Müller *et al*. (1998)
Lysozyme (HEW)	Chicken	pKD1	Kl LAC4	Native	0.5 mg L^{-1}		Tanaka *et al*. (2000)
			Sc PHO5				
			Sc PGK				
Lysozyme	Human	pKD1	PGK	Pre-HEW	100 mg L^{-1}		Iwata *et al*. (2004)
		URA3 integration	Kl LAC4				
	Human	rDNA integration	Kl GAL7	KT	2.4 mg L^{-1}		Rossolini *et al*. (1992)
	Human	rDNA integration	Kl GAL7	KT		130 mgL^{-1}	Maullu *et al*. (1999)
Macrophage colony stimulating factor	Human						Hua *et al*. (1994)
Maltose binding protein	*Escherichia coli*	LAC4 integration	Kl LAC4-PBI	Kl α-factor	65 mg L^{-1}		C. Taron, *et al*. (unpublished)
Ovalbumin	Chicken	LAC4 integration	Kl LAC4-PBI	Kl α-factor		1 mgL^{-1}	C. Taron, *et al*. (unpublished)
Pancreatic trypsin inhibitor	Cow	pKD1	Kl LAC4	Sc α-factor	0.32 mg L^{-1}		Panuwatsuk & Da Silva (2003)
Phospholipase A2 (PLA)	Pig	LAC4 integration	Kl LAC4	Prepro-PLA	40 mg L^{-1}		Swinkels et al. (1993)
Polygalacturonase	*Aspergillus aculeatus*	pKD1	Kl LAC4	Native			Müller *et al*. (1998)
Prochymosin	Cow	LAC4 integration	Kl LAC4	Prepro-chymosin	333 U mL^{-1}		Van den Berg et al. (1990)
				Synthetic			
				Sc α-factor			
				A. niger pre-glaA			

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Prochymosin	Goat	pKD1	Sc PGK	KT	12 mU mg^{-1}		Vega-Hernandez (2004)
Rennin	*Mucor pusillus*	LAC4 integration	Kl LAC4	Sc α-factor	20 mg L^{-1}		Ferbeyre *et al.* (1991)
Serum albumin (HSA)	Human	Plasmid/Integrated	PGK	Pre-HSA		3 gL^{-1}	Fleer *et al.* (1991b)
				Prepro-HSA			
		pKD1	Kl ADH4	Prepro-HSA	300 mg L^{-1}		Saliola *et al.* (1999)
		LAC4 integration	Kl LAC4	Prepro-HSA		3.3 gL^{-1}	Swinkels *et al.* (1993)
		LAC4 integration	Kl LAC4-PBI	Kl α-factor	150 mg L^{-1}		Colussi & Taron (2005)
scFv antibody anti-RAS	Rat	pKD1	Kl LAC4	GA fusion	10 mg L^{-1}		Swennen *et al.* (2002)
				Pre-GA			
scFv antibody 4B2	Mouse	pKD1	Kl LAC4	Sc α-factor	1.3 mg L^{-1}		Robin *et al.* (2003)
Transthyretin	Mouse	LAC4 integration	Kl LAC4-PBI	Kl α-factor	50 mg L^{-1}		B. Slatko, *et al.* (unpublished)
V8 protease	*Staphylococcus aureus*	LAC4 integration	Kl LAC4-PBI	Kl α-factor	2 mg L^{-1}		C. Taron, *et al.* (unpublished)
Xylanase	*Dictyoglomus thermophilum*	pKD1	Kl LAC4	KT	130 mg mL^{-1}		Walsh & Bergquist (1997)
			Sc PGK				
Xylanase	*Humicola insolens*	pKD1	Kl LAC4	Native			Müller *et al.* (1998)
Xylanase	*Thermotoga* species	pKD1	Kl LAC4	KT	120 mg mL^{-1}		Walsh *et al.* (1998)
D-amino acid oxidase	*Trigonopsis variabilis*	pKD1	Sc GAL1	NA	150 U g^{-1}		Gonzalez *et al.* (1997)
α-amylase	Mouse	pKD1	Sc PGK	KT	0.527 U mL^{-1}		Tokunaga *et al.* (1997)
			Sc PHO5				
α-amylase	*Schwanniomyces occidentalis*	pKARS	Native	Native	30 mU mL^{-1}		Strasser *et al.* (1989)

the utility of these advancements for industrial production of proteins from *K. lactis*. Finally, we present a novel proteomics-based approach for identifying cellular bottlenecks to heterologous protein expression that may lead to development of improved industrial production strains.

Industrial use of *Kluyveromyces lactis*

Most proteins that are produced from yeast in industry are secreted from cells. In general, companies are often reluctant to produce intracellularly expressed enzymes in yeast due to the complicated downstream processing required to lyse cells and isolate the target protein on a large scale. However, *Kluyveromyces lactis* has successfully been used to produce both secreted and intracellular enzymes on an industrial scale at DSM. The milk clotting enzyme bovine prochymosin is produced in secreted form from *K. lactis* cells (Van den Berg *et al.*, 1990). In addition, the native intracellularly expressed *K. lactis* enzyme lactase is produced on an industrial-scale and is sold under the trade name Maxilact™ (DSM Food Specialties, Delft, The Netherlands). This enzyme has obtained GRAS status and is mainly used in milk products to degrade milk sugar (lactose), thereby making it possible for individuals who are lactose-intolerant to consume milk-based products. Most importantly, industrial-scale fermentation and downstream processing protocols have been developed for isolation of the enzyme directly from *K. lactis* cells.

The success of *K. lactis* as a host for protein expression in the food industry suggests that this yeast could also be used for large-scale therapeutic protein production in the pharmaceutical industry. In fact, numerous proteins of pharmaceutical relevance have been produced in *K. lactis*. The mammalian proteins interleukin 1-β (Fleer *et al.*, 1991a), interferon α A (Chen *et al.*, 1992), β-lactoglobulin (Rocha *et al.*, 1996), lysozyme (Iwata *et al.*, 2004), macrophage colony stimulating factor (Hua *et al.*, 1994), serum albumin (Fleer *et al.*, 1991b; Saliola *et al.*, 1999; Colussi & Taron, 2005) and insulin precursor (Feng *et al.*, 1997) have been secreted from *K. lactis* to high titer. *Kluyveromyces lactis* is also proficient in the production of single-chain Fv antibodies (Swennen *et al.*, 2002; Robin *et al.*, 2003; van Ooyen *et al.*, unpublished). One drawback to the use of *K. lactis* (and other yeast and fungal expression systems) for the production of therapeutics is that secreted proteins that become glycosylated will bear a non-human glycosylation pattern. This can adversely affect the half-life, tissue distribution and immunogenicity of a therapeutic protein. However, advances in the area of humanizing yeast protein glycosylation pathways by expressing human glycosylation enzymes in yeast (Gerngross, 2004) may prove to be a promising technology for yeast therapeutic protein production, and may be applicable to *K. lactis*.

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Finally, the biotechnological potential of *K. lactis* is not limited to its excellent protein synthesizing capabilities. *K. lactis* was used as a baby food component during the Biafra war in the 1960s (Bonekamp & Oosterom, 1994). More recently, *K. lactis* has aroused interest as a possible source of single-cell protein (Ghaly *et al.*, 2005) with putative probiotic properties (Kumura *et al.*, 2004), as well as for oligonucleotide-derived flavor enhancers (Belem & Lee, 1998) and lactic acid (Porro *et al.*, 1999). Additionally, *K. lactis* produces commercially interesting native enzymes like inulinase (Laloux *et al.*, 1991), phospholipase B (Oishi *et al.*, 1999) and chitinase (Colussi *et al.*, 2005).

Tools for genetic modification of *Kluyveromyces lactis*

The basic genetic tools for modification of *K. lactis* for heterologous protein expression (e.g. vectors, promoters, selectable markers, etc.) were first reviewed over a decade ago. Here we summarize these concepts and highlight important technological advances that pertain to heterologous protein production in *K. lactis*. Where appropriate, we provide a perspective on the utility of these advances for industrial-scale protein production.

Host strains

A wide variety of *K. lactis* strains are available from various sources such as CBS (www.cbs.knaw.nl) and ATCC (www.atcc.org). For basic research purposes, the *K. lactis* research community has chosen to work with strain CBS 2359. The genome of this strain has been sequenced by a French consortium (Dujon *et al.*, 2004). For heterologous protein expression, the choice of strain background is of paramount importance to achieve maximum yield. For example, in the early 1990s Fleer *et al.* (1991b), Fleer (1992) and Swinkels *et al.* (1993) examined over 50 wild-type *K. lactis* isolates for their ability to secrete recombinant human serum albumin (HSA) and found great variability in the yield of HSA from strain to strain. For this reason, successful industrial strain backgrounds are highly sought after. The commercial kit *K. lactis* strain GG799 is a wild-type haploid food industry isolate that has a track record of excellent protein synthesizing and secretory capabilities. In addition, there is little glucose repression of the lactase (*LAC4*) promoter in GG799, so *LAC4*-based expression is high in standard yeast culture medium with glucose as a carbon source.

In industry, production strains are often further improved for their ability to secrete a desired protein by various mutagenesis techniques, or by methods that stably increase the copy number of an expression construct. Furthermore, various mutations have been identified that cause cells to have a 'super-secreting' phenotype and these strains can be exploited in heterologous protein secretion strategies. These concepts will be further explored in subsequent sections of this text.

Selectable markers

Common auxotrophies (e.g. *ura3*, *leu2*, *trp1*) that are often found in *Saccharomyces cerevisiae* strain backgrounds have been widely applied to *K. lactis* strains. Such markers permit a wide range of genetic and molecular techniques for manipulating yeast. However, auxotrophic markers have some drawbacks for industrial protein production applications. For example, many industrial production strains have been subjected to mutagenesis to improve their ability to produce desired proteins. These strains are often diploid or aneuploid, or have certain chromosomal regions amplified. The use of auxotrophic mutations in these backgrounds can be complicated by their unconventional genome organization. Strain auxotrophies can also have a detrimental effect on the yield of heterologously produced proteins in yeast. For example, in *S. cerevisiae*, secretion of a heterologous xylanase was dramatically reduced in an auxotrophic strain background (Gorgens *et al.*, 2004). This was overcome by maintaining an excess of the auxotrophic amino acid in the growth medium, but this remedy also reduced the growth rate of the strain compared to a prototrophic parent strain. A similar effect was observed in *K. lactis* where introduction of a uracil auxotrophy (*ura3*) into a strain that produces bovine enterokinase reduced yield over 10-fold even when grown in the presence of exogenous uracil or uridine (C.H. Taron, unpublished). It is likely that cells that are already burdened by abundant production of a heterologous protein are further stressed by having to scavenge the auxotrophic nucleotide from the growth medium. Thus, while auxotrophic markers can be beneficial for initially creating and testing strains, prototrophic backgrounds are often preferred for large industrial production fermentations to achieve maximum cell growth and ultimately maximum protein yield.

Dominant bacterial selectable marker genes that confer resistance to the antibiotics geneticin (G418; Sreekrishna *et al.*, 1984) or hygromycin B have also been widely used for transformation of *K. lactis*. Dominant selections are useful because they allow for genetic modification of prototrophic strains. While this method of selection is extremely useful for small-scale research applications, it is not practical in the industrial production setting where costs preclude the large-scale growth of cells in the presence of antibiotics, and where the use of antibiotic resistance genes in production strains may be undesirable for regulatory reasons.

An interesting advance in the area of selectable markers is a dominant nitrogen source selection strategy developed at DSM (Selten *et al.*, 1997). This method exploits the ability of *Aspergillus nidulans* acetamidase (encoded by the *amdS*

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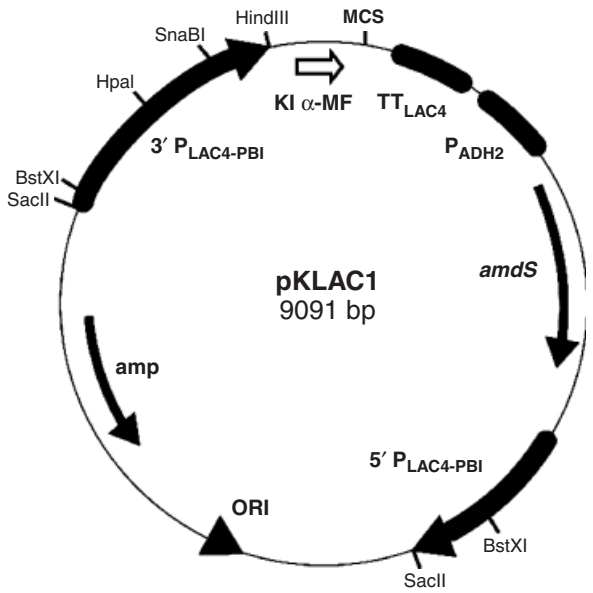

Fig. 1. The pKLAC1 integrative expression vector. pKLAC1 (9091 bp) contains the 5′- and 3′-ends of the *LAC4* promoter ($P_{LAC4\text{-}PBI}$) separated by DNA encoding β-lactamase (Ap^R) and the pMB1 origin (ori) to allow for its propagation in *Escherichia coli*. The *Kluyveromyces lactis* β-mating factor pre-pro secretion leader sequence (αMF), multiple cloning site (MCS), and the *LAC4* transcription terminator (TT) lie immediately downstream of 3′-$P_{LAC4\text{-}PBI}$. The yeast *ADH2* promoter (P_{ADH2}) drives expression of an acetamidase selectable marker gene (*AmdS*). The vector can be linearized by digestion with *Sac*II or *Bst*XI to create a linear DNA fragment capable of inserting into the native *LAC4* promoter region of the *K. lactis* genome.

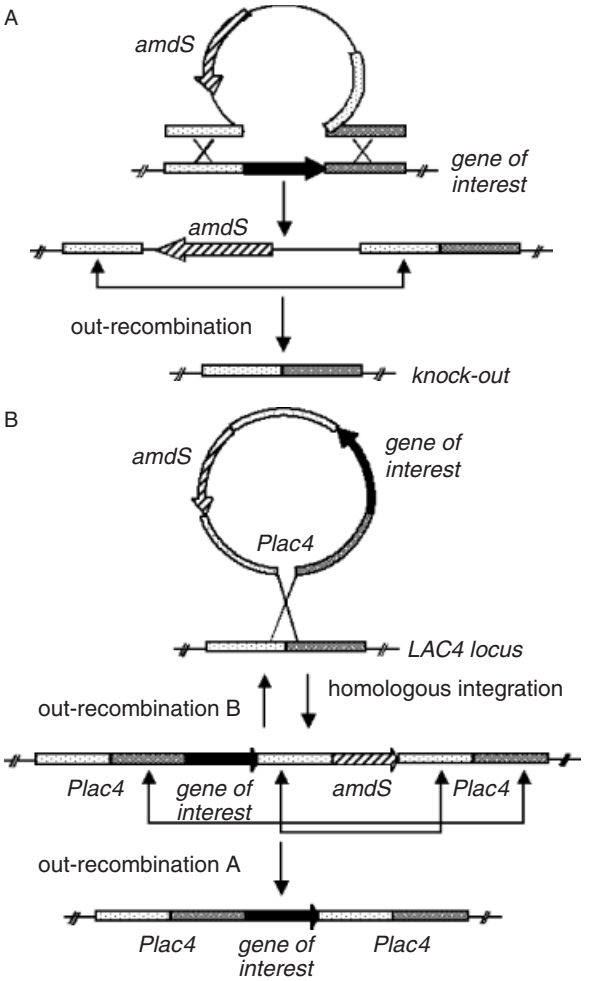

Fig. 2. Use of amdS selection and counterselection to obtain marker-free *Kluyveromyces lactis* production strains. Introduction of repeated sequences lead to complete removal of marker sequences and *amdS* can be re-used for transformation. (a) Use of the *amdS* marker in the production of knock-out strains. (b) Use of the *amdS* marker in the production of protein expression strains.

gene) to break down acetamide to ammonia for use as a source of nitrogen. Because *K. lactis* cells have a limited ability to process acetamide, only transformed cells overexpressing acetamidase from a vector can form colonies on agar medium containing acetamide as the only source of this nitrogen. This method has successfully been used to create *K. lactis* GG799 strains that produce bovine enterokinase, maltose binding protein, ovalbumin, cellulase and mouse transthyretin, using the integrative expression vector pKLAC1 supplied with the commercial kit (Fig. 1; Colussi & Taron, 2005, P. Colussi and C.H. Taron, unpublished).

Acetamide selection is also useful for making multiple genetic modifications to prototrophic *K. lactis* strains because the marker can be recycled using a counterselection with fluoroacetamide. In this technique, cells that express acetamidase convert fluoroacetamide to the toxic compound fluoroacetate and subsequently die. This strategy can be exploited for recycling the *amdS* marker gene by providing flanking direct repeat sequences upstream and downstream of the *amdS* gene so that it can be recombined out of the genome and reused in a subsequent transformation (Fig. 2). This is reminiscent of the ura-blaster technique often used in *Candida albicans* genetics (Alani *et al*., 1987) but with the advantage that *amdS* can be used as marker in prototrophic, diploid and aneuploid industrial strains. Additionally, no exogenous DNA fragments are left after out-recombination. Selection and counterselection with the *amdS* marker can be useful for both repeated gene knock-out (Fig. 2a), or repeated directed integration of expression cassettes (Fig. 2b).

Promoters for gene expression

High-level heterologous gene expression in *K. lactis* has been achieved using promoters native to *K. lactis* and promoters originating from *S. cerevisiae* (Table 1). To date, the most frequently used promoters have been the constitutive *S. cerevisiae* phosphoglycerate kinase (*PGK*) promoter and the

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regulatable *S. cerevisiae* acid phosphatase (*PHO5*) and *K. lactis* lactase (*LAC4*; β-galactosidase) promoters. These promoters are induced by the presence of phosphate or galactose/lactose in the growth medium, respectively. However, none of the promoters is fully repressed in the absence of inducer. The *K. lactis* alcohol dehydrogenase promoter (*KlADH4*) is induced in response to exogenous ethanol in *K. lactis* strains that cannot produce ethanol from fermentable carbon sources (Rag strains; Saliola *et al.*, 1999). An ethanol-dependent response element was also identified within the *KlADH4* promoter and confers ethanol-dependent induction upon genes like *PGK* and *LAC4* when inserted into their promoters (Saliola *et al.*, 1999).

The *K. lactis LAC4* promoter was one of the first employed to direct heterologous gene expression in *K. lactis* and it has been used for industrial-scale expression of the heterologous protein bovine prochymosin at DSM (Van den Berg *et al.*, 1990). The *LAC4* gene and its promoter were originally cloned by the yeast promoter's ability to function in *Escherichia coli* and produce active lactase (Dickson & Markin, 1978). However, its transcriptional capabilities in *E. coli* have often been problematic to the process of assembling expression vectors in bacteria prior to their introduction into yeast for expression, particularly if the desired gene encodes a protein that is deleterious in *E. coli*. Recently, a lactase promoter variant ($P_{LAC4\text{-}PBI}$) has been constructed by deleting sequences within the promoter that resemble the bacterial Pribnow box transcriptional element (Colussi & Taron, 2005). The variant exhibits a nearly complete inhibition of bacterial expression without affecting the level of expression achieved in *K. lactis*. Moreover, it has been used to clone and express various deleterious proteins (e.g. bovine enterokinase and mouse transthyretin) that were unattainable using the wild-type *LAC4* promoter (Colussi & Taron, 2005).

Episomal and integrative vectors

An attractive feature of *K. lactis* as a host system is that vectors that replicate extrachromosomally (episomal vectors) or that integrate into the *K. lactis* genome (integrative vectors) can be used to transform cells. Both vector systems have been used to direct expression of heterologous proteins (Table 1) and both have their inherent advantages and disadvantages. For example, episomal vectors provide higher copy numbers of expression constructs but can be unstable in the absence of selection. Conversely, integrative vectors typically provide higher stability in the absence of selection but are usually maintained at lower copy numbers.

The episomal vectors most widely used in *K. lactis* are derived from the pKD1-plasmid of *Kluyveromyces drosophilarum* (Falcone *et al.*, 1986; Chen *et al.*, 1986). The pKD1 plasmid is similar to the 2 μm plasmid of *S. cerevisiae* with respect to its stability and structure and is stably maintained at about 60–80 copies per cell (Falcone *et al.*, 1986). Copy number is often reduced to about 20 for plasmids containing heterologous genes (Morlino *et al.*, 1999). However, it has been shown that overexpression of chromosomally integrated recombinase A gene simultaneously with pKD1-based expression of human interleukin 1B (IL-1B) elevated plasmid copy number and improved IL-1B secretion fourfold (Morlino *et al.*, 1999). In addition to full pKD1 vectors, smaller vectors containing the pKD1 origin and a *cis*-acting stability locus (CSL) have been constructed and used for heterologous protein expression in *K. lactis* (Hsieh & Da Silva, 1998). Other episomal vectors also replicate in *K. lactis*. A plasmid carrying both a centromere (cen) and autonomously replicating sequence (ars) has been used for expression cloning of fungal cDNAs in *K. lactis* (Van der Vlugt *et al.*, 1999). Vectors containing a *K. lactis* ARS sequence (KARS) have been used to express fungal α-galactosidase (Bergkamp *et al.*, 1992) and a plant α-amylase (Strasser *et al.*, 1989). Finally, the pKL1 and pKL2 linear cytoplasmic killer plasmids replicate in *K. lactis* but are not used for heterologous protein expression due to their poor stability.

The potential instability of episomal vectors can pose problems for expression of some heterologous proteins, especially in large industrial applications where cells are often subjected to prolonged growth in the absence of selection. For example, after growth of a *K. lactis* strain secreting human lysozyme expressed from a pKD1 vector in rich medium, only 17.3% of cells retained the vector (Iwata *et al.*, 2004). This is in contrast to $>91.5\%$ of cells retaining an integrated expression vector producing the same enzyme under the same growth conditions (Iwata *et al.*, 2004). Autoselection strategies have been devised to improve plasmid stability (Fleer, 1992; Hsieh & Da Silva, 1998). For example, a strain carrying a chromosomal null mutation of the essential *PGK* gene is forced to maintain a pKD1 expression vector that carries a functional copy of the *PGK* gene in addition to a desired heterologous gene. This method has been used to produce HSA with prolonged growth in nonselective medium without significant plasmid loss (Fleer, 1992).

An alternative to episomal plasmid-based protein expression is the use of integrative expression vectors. Integrative vectors carry sequences homologous to regions of the *K. lactis* chromosome that direct insertion of the vector into the genome by homologous recombination. A common expression method utilizes the strong *LAC4* promoter to drive gene expression and to direct insertion of the vector into the genome at the *LAC4* locus (Van den Berg *et al.*, 1990; Swinkels *et al.*, 1993). A major benefit of the use of integrative vectors is the increased genetic stability of the transformed strain compared to episomal vectors. Thus, integration is often a preferred method for strains that are to be used in large-scale industrial processes.

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A drawback of integrative expression is the reduction in vector copy number compared to episomal vectors. The majority of transformants that form colonies using G418 selection and *LAC4* as the locus for vector integration contain a single copy of the integrated vector. However, strains containing 2–10 tandemly integrated vectors can be found in roughly 2–5% of the transformant population (Swinkels *et al*., 1993). A similar transformation using expression vector pKLAC1 (that allows for acetamide selection in place of G418) routinely yields strains bearing two to six integrated copies in $>$ 90% of the transformant population (Colussi & Taron, 2005; P. Colussi and C. Taron, unpublished). This is likely due to the growth advantage of multiple integrants (that also contain multiple integrated copies of the *amdS* marker gene) on medium containing acetamide compared to single integrants.

Various methods have been devised to increase the copy number of integrative vectors. One method involves integration into the ribosomal DNA (rDNA) locus, a region of tandemly repeating DNA (up to about 60 repeats) in the *K. lactis* chromosome, which frequently leads to multicopy integration (Bergkamp *et al*., 1992; Rossolini *et al*., 1992). To obtain stable integration, it is advantageous to couple the foreign gene to a selectable marker gene that is expressed from a suboptimal promoter. For instance, the *TRP1* gene preceded by an attenuated form of its own promoter may lead to selection of cells containing over 15 integrated copies of the desired gene on medium lacking tryptophan (Bergkamp *et al*., 1992). Another method exploits chromosomal recombination events. For example, it has been found at DSM that some *K. lactis* strains harbor two copies of the lactase (*LAC4*) locus, especially diploid or aneuploid industrial strains. In these strains, the process of gene conversion (or mitotic recombination) can be exploited to double the copy number of an integrated expression vector after multicopy integration has already been achieved at one locus (Fig. 3). This results in a more stable chromosomal arrangement than direct integration of the same number of copies at one locus.

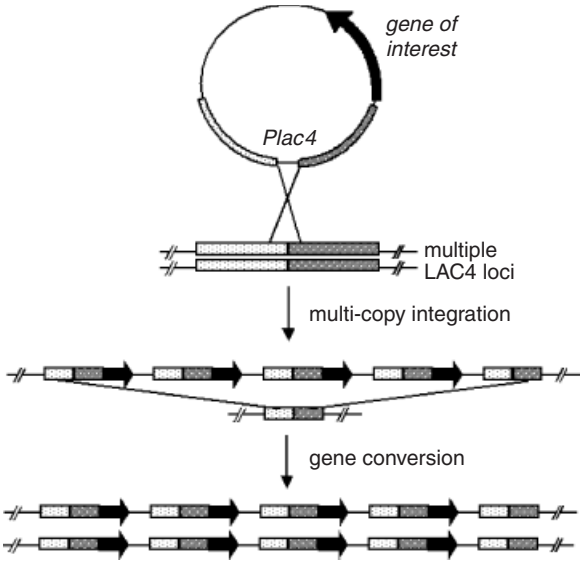

Fig. 3. Building a production strain. Gene copy number of the gene of interest is amplified by selection for multi-copy integration in one locus, and gene-conversion to a second locus.

Genetic strain enhancements

Over the past 15 years, enormous strides have been made in developing techniques for the genetic modification of *K. lactis* strains. These efforts have been enhanced by the completion of the *K. lactis* genome sequencing project and annotation of the finished sequence (Dujon, 2004). Researchers can now rapidly modify the *K. lactis* genome to construct novel strains with properties beneficial to heterologous protein production.

Protein expression and secretion in yeast is a complex, multi-step process. Improvements to protein production have been explored at many major points of protein synthesis, folding and secretion. For example, as described in earlier sections, strong promoters and multi-copy expression techniques can be applied to raise mRNA levels of the gene of interest. Additionally, mRNA stability can be improved through mutation or protein synthesis can be improved by optimizing the target gene's codon usage. Finally, host strains can be genetically modified to produce more of the desired protein. For example, protein folding can be enhanced by increasing the expression of certain chaperone proteins or by deleting genes encoding proteins detrimental to protein expression. Classical methods involving random mutagenesis and screening for cells with improved protein production are still widely used techniques for industrial strain improvement. Finally, novel proteomic analyses can be used to identify bottlenecks in cellular pathways that affect protein production.

Gene targeting

Genetic modification of *K. lactis* strains can be performed using a number of standard approaches. Strain genomes can be altered by random mutagenesis followed by screening for a desired trait. Additionally, host strains can be modified by integration of transforming DNA constructs, such as integrative expression vectors or gene deletion cassettes, into the genome. In these cases, it is desirable to target the transforming DNA to integrate at a specific chromosomal locus. This is accomplished by including DNA sequence homologous to the targeted integration site on the transforming DNA construct to facilitate its integration by homologous recombination. Periodically, transforming DNA integrates off-target

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(ectopic integration). The rate of ectopic integration varies from organism to organism. For example, targeted integration into the *S. cerevisiae* genome is virtually 100% efficient, with as little as 40 bp of homologous DNA. However, in *K. lactis,* targeted integration at many loci has often been difficult, with as little as 1–2% efficiency in some cases (Stark & Milner, 1989; Zeeman *et al*., 1998; Zeeman & Steensma, 2003; Colussi *et al*., 2005). Integration efficiency in *K. lactis* also often varies from locus to locus. Integration of expression vectors at the *LAC4* locus is $>$ 90% efficient, typically.

Various approaches have been employed to increase the gene targeting efficiency in *K. lactis*. For example, gene targeting at various loci can be improved by using T-DNA from the tumor-inducing (Ti) plasmid from *Agrobacterium tumefaciens* (Bundock *et al*., 1999). By employing the T-DNA fragments, targeted integration in the *KlTRP1* locus increased from 18 to 93%. Additionally, targeted integration into the *K. lactis* genome can be dramatically improved by deleting the *KlKu80* recombination gene (Kooistra *et al*., 2004). In a Δ*Klku80* background, targeted integration at the *KlADE*2 locus was increased to 97% and was independent of the length of the flanking homologous sequences. DNA constructs having only 50–100 bp of homologous flanking sequences can be correctly targeted in Δ*Klku80* mutant strains. As these constructs can be easily constructed using PCR, this technique will likely be very useful for functional analysis of the *K. lactis* genome and for rapid modification of industrial expression strains. Short exogenous double-stranded DNA fragments added to cell transformation reactions can also enhance accurate gene targeting (Kooistra *et al*., 2004). This simple technique might also be useful for construction of defined strains, although the presence of additional foreign DNA fragments might increase the chance for contamination of the host genome by their ectopic integration.

Supersecreting strains and mutants

A large number of studies describe isolation of *K. lactis* mutants with improved protein secretion characteristics. Bartkeviciute & Sasnauskas (2003) isolated both dominant and recessive *K. lactis* mutants conferring a supersecretion phenotype for *Bacillus amyloliquefaciens* alpha amylase. Among the suppressors of this phenotype was a gene *(KlMNN10*) that encodes a putative mannosyltransferase involved in glycoprotein synthesis. Deletion of *KlMNN10* resulted in a supersecreting phenotype as well (Barteviciute & Saskaunas, 2004). In this context, the vanadate mutants of (Uccelletti *et al*., 1999) that also show protein glycosylation defects may be interesting hosts for protein secretion. Donnini *et al*. (2004) isolated a glucose repression-defective mutant of *K. lactis* which showed improved production of several heterologous proteins, such as glucoamylase from the yeast *Arxula* and mammalian proteins like HSA and interleukin-1. Inactivation of the *K. lactis PMR1* gene, a calcium-dependent ATPase that functions in the Golgi, had a positive effect on secretion of several proteins as well (Uccelletti *et al*., 2004). Finally, overexpression of *K. lactis* genes involved in exocytosis (*SS01*) and protein translocation into the endoplasmic reticulum (*SEB1*/*SBH1*) enhanced protein secretion in *S. cerevisiae* (Toikkänen *et al*., 2004) and one could speculate that these proteins may do the same in *K. lactis*.

The summary given above indicates that it is relatively easy to obtain mutants with a supersecreting phenotype from *K. lactis*. Some seem to be specific for a limited number of proteins, whereas others have a rather general effect. It may be possible to combine several of these mutations in a single strain background to create a '*K. lactis* superbug' for enhanced expression of secreted proteins.

Other mutant strain backgrounds

Improved strains of *K. lactis* are not limited to supersecretion mutants. It may also be advantageous to overexpress chaperones that aid in protein folding or to delete unwanted host activities. For example, overexpression of protein disulfide isomerase has a positive effect on expression of human lysozyme (Iwata *et al*., 2004) and of human serum albumin in *K. lactis* (Bao & Fukuhara, 2001), but not of interleukin-1. It was also recently shown that secretion of HSA could be improved 15-fold by duplicating *KlERO1*, a gene encoding the Ero protein that is involved in oxidative protein folding in the endoplasmic reticulum (Lodi *et al*., 2005).

Another interesting *K. lactis* strain is a chitinase-deficient mutant (Colussi *et al*., 2005). This strain harbors a null mutation of a gene (*KlCTS1*) encoding an abundantly secreted *K. lactis* extracellular chitinase and produces no proteins that can bind to the polysaccharide chitin. Thus, it is a suitable host for the secretion of proteins carrying a chitin-binding affinity tag (Colussi *et al*., 2005). Secreted proteins bearing such a domain can be rapidly immobilized to various inexpensive chitin matrices like beads or chitin-coated microtiter dishes. Another interesting strain contains a mutation in *KlGAL1*, a gene encoding a bifunctional protein involved in galactokinase activity and induction of the lac/gal regulon that ultimately controls expression of the *LAC4* promoter (Meyer *et al*., 1991). Thus, the strain is dramatically reduced in its ability to consume galactose as a carbon source, but retains its ability to induce *LAC4* expression in the presence of galactose or lactose. Utilizing this strain background, heterologous genes under control of the *LAC4* promoter can be expressed in response to galactose provided as a gratuitous inducer during growth of cultures using glucose as a carbon source (Hsieh & Da Silva, 2000; Panuwatsuk & Da Silva, 2003).

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A proteomics approach to strain enhancement

Microbial gene expression is being revolutionized by the use of genomics and proteomics approaches to study sequenced microbial genomes. DNA micro-array technology has been employed to study the simultaneous expression of all the genes present in a microorganism by measuring their mRNA levels in response to a given stimulus. Additionally, methods for studying a microorganism's proteome are developing. Although currently not as easy as measuring RNA expression, proteomic analyses enable assessment of protein production. This is important because increased or decreased mRNA levels do not always accurately predict a change in protein production. Methods involving genomics and proteomics are currently being widely used in industry to discover new and interesting products, to define bottlenecks in protein and metabolite production, and to improve fermentation protocols.

An experiment demonstrating the use of proteomics to identify bottlenecks in *K. lactis* protein secretion was performed at the Wageningen University in collaboration with DSM and is shown in Fig. 4. To gain insight into the cellular response of *K. lactis* to overexpression of heterologous proteins, cellular proteins produced by a chymosin-producing strain were compared with those produced by its wild-type parent strain (CBS 2359). After fermentation, intracellular proteins were extracted from cells and proteins were separated by two-dimensional (2D) gel electrophoresis in triplicate. The resulting gel profiles were compared using 2D gel-imaging software. New and differentially expressed protein 'spots' were excised from the gels, digested with trypsin and sequenced using mass spectrometry (nanoLC-MS/MS). The sequences of each peptide were used to query the *K. lactis* genome database to identify the protein species present in each spot. In Fig. 4, seven differentially expressed proteins and various reference proteins are shown. Interestingly, production of proteins such as heat shock protein (Hsp26p) and superoxide dismutase (Sod2p) were significantly upregulated in the chymosin-producing strain, indicating that the cells experienced stress. Three different isoforms of Hsp26p were overexpressed in the chymosin-producing strain. They originate from the same gene but differ in their co- or post-translational modifications. Lower production of the Gal10 protein suggests that the extra copies of the *LAC4* promoter used to drive synthesis of the chymosin cDNA might affect the regulation of the *GAL* genes.

This experiment indicates the usefulness of proteomic analysis in obtaining leads for the identification of obstacles to protein production in expression strains that may not be obvious or detectable by other means. Once identified, various genetic techniques can be applied to follow up those leads, which may alleviate the bottleneck and further enhance the production capabilities of the cell.

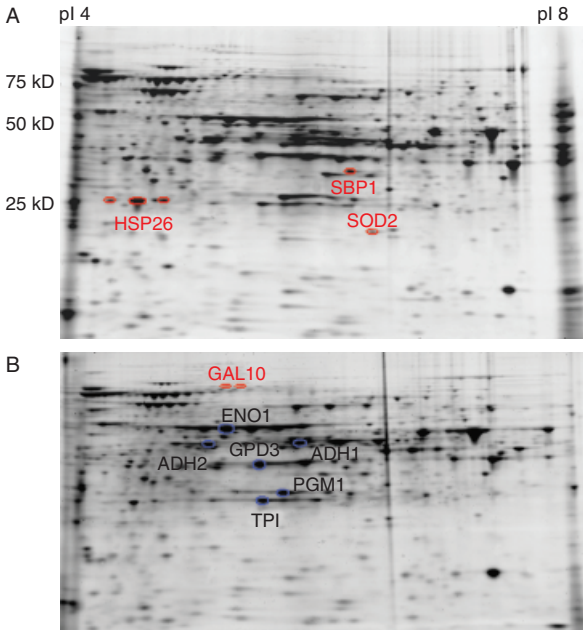

Fig. 4. 2D gels of (a) chymosin-producing strain and (b) corresponding wild-type strain CBS 2359. Some of the differentially expressed proteins are shown: HSP26 (KLLA0F18909g, similar to YBR072w HSP26 heat shock protein singleton from *Saccharomyces cerevisiae*), SBP1 (KLLA0E00484g, similar to YHL034c SBP1 single-strand nucleic acid binding protein singleton from *S. cerevisiae*), SOD2 (KLLA0E03509g, highly similar to YHR008c SOD2 superoxide dismutase (Mn), mitochondrial singleton from *S. cerevisiae*), GAL10 (KLLA0F08415g, GAL10 bifunctional protein), and ENO1 (KLLA0A09185g, highly similar to YGR254w ENO1 enolase I from *S. cerevisiae*). In addition, some of the reference spots are shown: ENO1 (KLLA09185g, highly similar to YGR254w ENO1 enolase I from *S. cerevisiae*), ADH2 (KLLA0F18260g, alcohol dehydrogenase II), ADH1 (KLLA0F21010g, alcohol dehydrogenase I), GPD3 (KLLA0A11858g, highly similar to YGR192c glyceraldehyde-3-phosphate dehydrogenase 3 from *S. cerevisiae*), PGM1 (KLLA0E11891g, highly similar to YKL152c GPM1 phosphoglycerate mutase I from *S. cerevisiae*), TPI (KLLA0F18832g, triosephosphate isomerase).

Fermentation and production methods

In the previous sections, we examined the capacity of *K. lactis* to produce certain products, with an emphasis on its ability to express enzymes and other proteins. However, successful expression of a desired protein is just one aspect of commercialization. Methods for large-scale fermentation, downstream processing, and product formulation must also be developed. Additionally, it is often necessary to modify these processes for each new product. For example, factors like the product's method of expression (e.g. intra- or extracellular expression), the product's stability, or the need to remove specific side-products need to be optimized for each production strain. In addition, the production and regulatory requirements for food and pharmaceutical products also differ vastly.

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Some studies have addressed the optimization of fermentation conditions to achieve high yields of heterologous proteins (Fleer *et al.*, 1991; Blondeau *et al.*, 1994; Walsh *et al.*, 1998; Maullu *et al.*, 1999; Saliola *et al.*, 1999; Panuwatsuk & Da Silva, 2003). Additionally, optimized growth of *K. lactis* as a source of single-cell protein has been examined (Ghaly *et al.*, 2005). However, for industrial-scale applications, a combination of genetic strain enhancement, and optimization of fermentation conditions and downstream processing protocols will ultimately dictate whether a sufficient expression level and product quality can be obtained for commercial production.

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