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Minireview

MINIREVIEW

Yeast synthetic biology for the production of recombinant therapeutic proteins

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ABSTRACT

The production of recombinant therapeutic proteins is one of the fast-growing areas of molecular medicine and currently plays an important role in treatment of several diseases. Yeasts are unicellular eukaryotic microbial host cells that offer unique advantages in producing biopharmaceutical proteins. Yeasts are capable of robust growth on simple media, readily accommodate genetic modifications, and incorporate typical eukaryotic post-translational modifications. *Saccharomyces cerevisiae* is a traditional baker's yeast that has been used as a major host for the production of biopharmaceuticals; however, several nonconventional yeast species including *Hansenula polymorpha*, *Pichia pastoris*, and *Yarrowia lipolytica* have gained increasing attention as alternative hosts for the industrial production of recombinant proteins. In this review, we address the established and emerging genetic tools and host strains suitable for recombinant protein production in various yeast expression systems, particularly focusing on current efforts toward synthetic biology approaches in developing yeast cell factories for the production of therapeutic recombinant proteins.

Key words: biopharmaceuticals; glycoengineering; recombinant protein; secretory pathway engineering; yeast cell factory

INTRODUCTION

Yeasts have been used for thousands of years in food and fermentation processes to produce alcoholic beverages and breads. In recent times, the industrial importance of yeasts has extended beyond its traditional use in fermentation into various healthcare sectors, such as in the production of therapeutic recombinant proteins. Yeast cells are particularly advantageous as hosts for biopharmaceutical production in that they are generally recognized as safe (GRAS) organisms. The traditional baker's yeast *Saccharomyces cerevisiae* is one of the best-characterized eukaryotes and most widely used host systems for biopharmaceutical production since the early days of genetic engineering and recombinant protein production (Martinez *et al.*, 2012). During last decade, nonconventional yeast species including *Hansenula polymorpha*, *Pichia pastoris*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe*, and *Kluyveromyces lactis* have been developed as alternative hosts for the production of recombinant proteins (Table 1).

Since the complete genomic sequence of *S. cerevisiae* was published as the first-sequenced eukaryotic genome in 1996 (Goffeau *et al.*, 1996), the whole-genome sequences of several nonconventional yeast species are also currently available in public databases (Wood *et al.*, 2002; Dujon *et al.*, 2004; De Schutter *et al.*, 2009; Ravin *et al.*, 2013), facilitating functional genomics analysis based on various 'omics' techniques. Systems biology-based integrated functional genomics could provide a deeper, more holistic understanding of yeast biology, which can help identify potential limitations in protein production on a global scale. Furthermore, novel methods and tools in synthetic biology are under active development to facilitate rapid and efficient engineering of yeast by rational design, based on the information obtained from systems-level analyses. Thus, synthetic biology approaches are expected to accelerate the construction of 'yeast factories' designed for the production of recombinant proteins with improved yield and quality (Vogl *et al.*, 2013). In

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Table 1. General characteristics and genomic information of various yeasts developed as hosts for recombinant protein production.

Yeast species	Attributes	References
Saccharomyces cerevisiae	Favorable public acceptance	Nevoigt (2008)
	GRAS status	
	The most well studied of simple eukaryotes	
	Amenable to both classical genetics and modern recombinant DNA techniques	
	Versatile vector systems (episomal, integrative, copy-number regulated) are available (Invitrogen)	
	A wide range of mutant strains	
	Well-established fermentation and downstream processing	
	Hypermannosylation with immunogenic terminal α-1,3-linked mannose residues	Camirand *et al.* (1991)
	Genome sequencing: Reference strain S288C; 12 157 Kb (6273 ORFs); Accession number PRJNA128	Goffeau *et al.* (1996)
Pichia pastoris	GRAS status	Ahmad *et al.* (2014)
	Tightly regulated, methanol-inducible *AOX* promoters	
	A Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes	
	Can grow rapidly on inexpensive media at high cell densities (up to 150 g DCW L^{-1})	
	Integrated vectors developed that help genetic stability of the recombinant elements, even in continuous and large-scale fermentation processes	
	Well-established commercial vector systems and host strains (Invitrogen)	
	A lesser extent of hypermannosylation compared to *S. cerevisiae;* No terminal α-1,3-linked mannose residues	Bretthauer (2003)
	Genome sequencing: Reference strain GS115; 9216 Kb (5040 ORFs); Accession number PRJNA39439, PRJEA37871	De Schutter *et al.* (2009)
Hansenula polymorpha	GRAS status	Gellissen *et al.* (2005)
	Stringently regulated strong promoters (*MOX*, *FMDH*, etc.)	
	A Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes	
	Stable, multicopy integration of foreign DNA into chromosomal locations	
	Thermotolerant (growth up to 45 °C), resistant to heavy metals and oxidative stress	
	Can assimilate nitrates	
	A lesser extent of hypermannosylation compared to *S. cerevisiae*; No terminal α-1,3-linked mannose residues	Kang *et al.* (1998) and Kim *et al.* (2004)
	Genome sequencing: Reference strain DL1; 9056 Kb (5325 ORFs); Accession number PRJNA60503	Ravin *et al.* (2013)
Yarrowia lipolytica	An oleaginous yeast, based on its ability to accumulate large amounts of lipids	Madzak *et al.* (2004)
	GRAS status	
	Can grow in hydrophobic environments, that is able to metabolize triglycerides, fatty acids, *n*-alkanes, and *n*-paraffins as carbon sources for the bioremediation of environments contaminated with oil spills	
	Can secrete a variety of proteins via cotranslational translocation and efficient secretion signal recognition similar to higher eukaryotes	
	Availability of a commercial expression kit (YEASTERN BIOTECH CO., LTD.)	
	Salt tolerance	
	A lesser extent of hypermannosylation compared to *S. cerevisiae*; a lack of the immunogenic terminal α-1,3-mannose linkages	Song *et al.* (2007)
	Genome sequencing: Reference strain CLIB122; 20 503 Kb (7042 ORFs); Accession number PRJNA12414	Dujon *et al.* (2004)
Schizosaccharomyces pombe	A fission yeast, reflecting proliferation of higher eukaryotic cells	Takegawa *et al.* (2009)
	Many cellular processes similar to those of higher eukaryotes, such as mRNA splicing, post-translational modification (including protein galactosylation), cell cycle control, etc.	
	Transcription start site similar to that in higher eukaryotes	
	Expression vectors for high-level expression developed	
	Presence of galactose in both *O*- and *N*-linked glycans	Ballou *et al.* (1994)
	Genome sequencing: Reference strain 972h-; 12 554 Kb (5364 ORFs); Accession number PRJNA127	Wood *et al.* (2002)

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Table 1. Continued.

Yeast species	Attributes	References
Kluyveromyces lactis	GRAS status A Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes Lactose-fermenting present in milk and whey, and the strong, lactose-inducible *LAC4* promoter Very high cell density (> 100 g DCW L^{-1}) Able to use both integrative and episomal expression vectors An available easy-to-use reagent kit for *K. lactis* protein expression (New England Biolabs)	van Ooyen *et al.* (2006)
	Terminal *N*-acetylglucosamine and no mannose phosphate	Lopez-Avalos *et al.* (2001)
	Genome sequencing: Reference strain NRRL Y-1140; 10 689 Kb (5502 ORFs); Accession number PRJNA12377	Dujon *et al.* (2004)

GRAS, generally regarded as safe.

this review, we address recent advances in yeast synthetic biology with respect to therapeutic recombinant protein production, particularly focusing on genetic tools and host strains suitable for secretory protein production in several yeast expression systems.

YEAST SPECIES AS HOSTS FOR THE PRODUCTION OF THERAPEUTIC RECOMBINANT PROTEINS

Pharmaceutical proteins comprise one of the fastest growing groups of molecular medicines and currently play important roles in the treatment of many diseases. The estimated market value of biopharmaceuticals, including recombinant therapeutic proteins, nucleic acid-based products, and engineered cell-based products, is *c.* 70–80 billion USD, with an annual growth rate of *c.* 7–15% (Goodman 2009; Walsh 2010). *Saccharomyces cerevisiae*, *Escherichia coli*, and mammalian cells are the most widely used host systems for biopharmaceutical protein production, accounting for 15%, 31%, and 43% of biopharmaceutical products developed, respectively (Berlec and Strukelj 2013). The main strength of *E. coli* is its capacity for fast and hardy growth in bioreactors using simple media, although producing eukaryotic proteins in *E. coli* often results in inclusion body formation and/or low specific yields. Mammalian cells are ideal for incorporating typical eukaryotic post-translation modifications, such as glycosylation (Demain and Vaishnav 2009; Walsh 2010b; Berlec and Strukelj 2013); however, the culture of mammalian cells is relatively slow, requires complex media, and is vulnerable to viral contaminations. As unicellular eukaryotic microbial host cells, yeast offers unique advantages in biopharmaceutical protein production. The use of yeasts enables an ideal combination of hardy growth on simple media in large-scale bioreactors with the capacity of the desired post-translational modifications and feasibility in genetic manipulations (Mattanovich *et al.*, 2012). Dozens of pharmaceutical products produced in *S. cerevisiae* including vaccines and blood factors have been marketed since the first industrial production of recombinant human insulin in *S. cerevisiae* in 1987, several of which are blockbusters (Walsh 2010a, b).

Some physiological characteristics of non-*Saccharomyces* yeast species, such as faster growth on cheap carbon sources and higher secretion capacity than *S. cerevisiae*, make them practical alternative hosts for biotechnological purposes. The biopharmaceutical products produced in various yeast species, which are on the market or in the final stages of development, are summarized in Table 2. The recombinant hepatitis B vaccine produced in the nonconventional yeast *H. polymorpha* has been commercialized (Seo *et al.*, 2008). The *H. polymorpha*-produced hepatitis B virus S antigen (HbsAg) was found to be assembled into yeast-derived lipid membranes. Previous studies have indicated that this lipoprotein particle structure is essential for the antigenicity of the HBsAg (Rutgers *et al.*, 1988). The methanol induction condition, which is routinely used for high-level production of recombinant proteins in the methylotrophic yeast *H. polymorpha*, is favorable condition for lipid membrane formation and thus advantageous to produce the recombinant hepatitis B vaccine with more desirable antigenicity. Another methylotrophic yeast *P. pastoris* was recently approved as a host for biopharmaceutical production. The first recombinant biopharmaceutical, a Kallikrein inhibitor (Kalbitor), developed in *P. pastoris* was approved by the FDA in 2009, and several others are undergoing evaluation in clinical trials (Walsh 2010a). Traditional approaches for the development of toolboxes for genetically engineering host strains have been applied in nonconventional yeasts to extend protein production capacity, improve product quality, and ease downstream processing (Krainer *et al.*, 2012; Mattanovich *et al.*, 2012; Saraya *et al.*, 2014). Combining recent advances in molecular and genetic resources with the scale-up processes of high cell-density cultivation in various yeast species increases the diversity of choices for yeast expression systems in which recombinant protein production and optimization can be performed quickly and efficiently.

TOOLBOXES FOR THE OPTIMIZED EXPRESSION OF RECOMBINANT PROTEINS

A goal of synthetic biology is to use modular, well-characterized biological parts to construct novel genetic devices and complex cell-based systems following engineering principles. Thus, successful synthetic biological approaches for protein production require the development of synthetic promoters, codon-optimized synthetic genes, artificial transcription factors, and

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Table 2. Commercial biopharmaceuticals produced by yeast.

System	Protein	Brand name	Therapeutic area	Company
S. cerevisiae	Hepatitis (or plus other infectious disease) vaccines (I)	Comvax	*H. influenzae* type B and hepatitis B infection in infants	Merck
		Recombivax	Hepatitis B	
		Euvax B		Sanofi Pasteur (France)
		Engerix-B		GlaxoSmithKline (GSK)
		Fendrix		
		Ambirix	Hepatitis A and B	
		Twinrix		
		Pediarix8	Various conditions inducing hepatitis B in children	
		Tritanrix-HB	Diphtheria, tetanus, pertussis, and hepatitis B	
		Infanrix Hep B		
		Infanrix-Penta	Diphtheria, tetanus, pertussis, polio, and hepatitis B	
		Infanrix-Hexa	Diphtheria, tetanus, pertussis, hepatitis B, polio, and *H. influenzae* type B	
		Hexavac		Aventis Pasteur
		Procomvax	*H. influenzae* type B and hepatitis B	
		Primavax	Diphtheria, tetanus, and hepatitis B	
		HBVaxPro	Hepatitis B in children and adolescents	Aventis Pharma
	Lepirudin (S)	Refludan	Heparin-induced thrombocytopenia type II	Hoechst Marion Rousse (USA), Behringwerke AG (Germany)
	Desirudin (S)	Revasc	Venous thrombosis	Canyon Pharmaceuticals (UK)
	Insulin (S)	Actrapid, Velosulin, Monotard, Insulatard, Protaphane, Mixtard, Actraphane, Ultratard	Diabetes mellitus	Novo Nordisk
	Insulin aspart (S)	Novolog, Novolog FlexPen, Novolog Penfill, NovoRapid, NovoRapid Penfill, Novomix 30, Novolog mix 70/30		
	Insulin detemir (S)	Levemir, Levemir FlexPen		
	GLP-1 (S)	Victoza	Type 2 diabetes	
	Glucagon (S)	GlucaGen	Hypoglycemia	
		Glucagon		Eli Lilly
	GM-CSF (S)	Leukine	Cancer, bone marrow transplant	Berlex Laboratories
		Leucomax		Novartis
	HGH (S)	Valtropin	Dwarfism, pituitary turner syndrome	Biopartners
	PDGF (I)	Regranex	Lower extremity diabetic neuropathic ulcers	Ortho-McNeil Pharmaceutical (USA), Janssen-Cilag
		GEM 125	Periodontal defects	Luitpold Pharmaceuticals (USA) BioMimetic Pharmaceuticals (USA)
	HPV vaccine (I)	Gardasil	Cervical cancer caused by human papillomavirus (HPV)	Merck, Sanofi Pasteur, Merck Sharp and Dohme
	Rasburicase (I)	Fasturtec, Elitex	Hyperuricemia	Sanofi-Synthelabo (France), Sanofi-Aventis (France)
P. pastoris	Ecallantide (I)	Kalbitor	Hereditary angioedema	Dyax (USA)
	Insulin (S)	Insugen	Type 2 diabetes	Biocon (India)
	Human serum albumin (S)	Medway	Blood volume expansion	Mitsubishi Tanabe Pharma (Japan)
	Hepatitis vaccine (I)	Shanvac	Hepatitis B	Shantha/Sanofi (India)
	IFN-α 2b (S)	Shanferon	Hepatitis C, cancer	Shantha/Sanofi (India)
	Ocriplasmin (I)	Jetrea	Vitreomacular adhesion (VMA)	ThromboGenics (Belgium)

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Table 2. Continued.

System	Protein	Brand name	Therapeutic area	Company
	Anti-IL-6R Ab (I)	Nanobody ALX-0061	Rheumatoid arthritis	Ablynx (Belgium)
	Anti-RSV Ab (S)	Nanobody ALX00171	Respiratory syncytial virus (RSV) infection	Ablynx (Belgium)
	HB-EGF (I)	-	Treatment of interstitial cystitis/bladder pain syndrome (IC/BPS)	Trillium (Canada)
	Collagen (I)	-	Medical research reagents/dermal filler	Fibrogen (USA)
H. polymorpha	HBV vaccine (I)	Hepavax-Gene	Hepatitis B	Rhein Biotech (Germany), Green Cross Vaccine Corp (Korea)
Y. lipolytica	Pancrelipase (S)	Creon, Ultresa, Viokase	Exocrine pancreatic insufficiency	Aptalis Pharma

(I), intracellular expression; (S), secretory expression.

insertion expression cassettes as typical biological components and devices. These tools can target different levels in biosynthetic processes and allow multilevel modifications of yeast host strains to improve the quality and yield of recombinant proteins.

Synthetic promoters for protein expression

Efficient transcription is a critical step in controlling gene expression at the initial stage. Thus, well-characterized constitutive or inducible promoters with strong transcriptional activity are generally used to achieve overproduction of recombinant proteins. The well-characterized and strong constitutive *TEF1* and *GPD* (*TDH3*) promoters have long been used to predominately direct high-level expression of heterologous genes in *S. cerevisiae* (Partow *et al.*, 2010). It is noteworthy that in the case of secretory protein expression, strong overexpression might rather lead to lower secretion efficiency due to aggregation of misfolded proteins in the endoplasmic reticulum (ER), as reported in the expression of single-chain antibody fragments, insulin precursor, and α-amylase in *S. cerevisiae* (Shusta *et al.*, 1998; Liu *et al.*, 2012). Thus, a set of promoters with different extent of transcriptional activity would be useful to achieve optimal secretion expression. However, native promoters may not offer continual gradients of transcriptional strength, thereby limiting the ability to fine-tune gene expression.

In recent years, significant efforts have been made to develop constitutive promoters exhibiting a wide range of transcriptional activities. For example, synthetic promoter libraries have been created through the application of randomized oligonucleotides (Jeppsson *et al.*, 2003) and the random mutagenesis of promoter regions via error-prone PCR (Alper *et al.*, 2005), extending the choice of available constitutive promoters in *S. cerevisiae*. More recently, a study on converting a constitutive promoter to a set of synthetic regulatable promoters with a tailor-made regulatory profile was reported (Blount *et al.*, 2012). On the other hand, the constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene of *P. pastoris* has been also engineered by random mutagenesis to generate additional promoters with the potential for finely tuned gene expression or the generation of new regulatory circuits (Qin *et al.*, 2011). Such diverse sets of constitutive promoters with varied activities and sequences will facilitate the investigation of optimal expression levels of heterologous genes in constructing yeast host strains with heterologous biosynthesis pathway, such as the humanized glycosylation pathway.

Inducible promoter systems offer the advantage of controlling gene expression levels in response to the presence of specific inducer or repressor molecules (Shen *et al.*, 2012). The most popular inducible promoters employed for recombinant protein production in *S. cerevisiae* are the endogenous *GAL1* or *GAL10* promoters. Although such native promoter systems provide well-defined, predictable expression profiles, a disadvantage of these promoters is that the inducer molecule, galactose, is consumed as a carbon source by yeast cells, which further complicates the control of gene expression. Moreover, galactose is a relatively expensive carbon source, further limiting the benefits of *GAL* promoters when producing recombinant therapeutic proteins on an industrial scale. A recent study using a modified *GAL1* promoter with six Zif268 binding sites showed that this synthetic promoter, in the presence of an agonist β-estradiol that binds to and activates the transcriptional activity of estrogen receptor, was able to control gene expression levels with greater sensitivity in *S. cerevisiae* with an artificial transcription factor Z_3EV. The Z_3EV factor was constructed by fusion of the Zif268 DNA-binding domain containing three zinc fingers, the ligand binding domain of the human estrogen receptor, and viral protein 16 from herpes simplex virus (McIsaac *et al.*, 2014). The synthetic promoter was shown to be induced by a nonhormonal small molecule recognized by the modified human estrogen receptor. The industrial potential of such synthetic *GAL* promoters should be evaluated in further application studies.

In the methylotrophic yeast *P. pastoris*, the methanol-inducible *AOX1* promoter (P_{AOX1}) is the most frequently utilized strong and tightly controlled promoter. Because the use of toxic and inflammable methanol can pose a considerable safety risk in industrial settings, this promoter has been extensively studied to create synthetic variants with increased promoter strength and altered methanol-free regulation (Vogl and Glieder 2013). Based on an *in silico* analysis for putative conserved eukaryotic transcription factor binding sites within the P_{AOX1}, the transcriptional activity of this promoter was rationally optimized by mutagenesis (Hartner *et al.*, 2008; Xuan *et al.*, 2009). In addition, it was shown that short semi-synthetic variants of P_{AOX1} constructed by fusing natural core promoter fragments with *cis*-acting elements had greater transcriptional activity than the full-length wild-type promoter (Ruth *et al.*, 2010).

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Optimizing synthetic gene sequence for yeast expression

All living organisms use synonymous codons with variable frequencies that encode specific amino acid residues, although different organisms use alternative redundant codons for a given amino acid with differing efficacy. Codon usage is important because target genes may possess codons that the desired host rarely uses due to a codon usage bias or that originate from organisms using noncanonical code, causing inefficient translation. The translational efficiency of heterologous genes can be improved by optimizing synonymous codon usage to better match that of the host organism. Such codon optimization has been established as a standardized tool for improving heterologous gene expression, particularly in the disciplines of synthetic biology and metabolic engineering (Gustafsson *et al.*, 2004; Young and Alper 2010). While most commonly applied in prokaryotic systems, codon optimization has also been of considerable benefit to yeast expression systems. For example, codon optimization of the human papillomavirus type 58 L1 gene enhanced its expression in *S. cerevisiae*, increasing the yield of soluble L1 protein and the amount of purified product attainable for vaccine production (Kim and Kwag 2013).

With improvements in the speed and cost of gene synthesis, codon-optimized target genes may be readily redesigned and chemically synthesized. A variety of additional tools and approaches have been developed for the redesign of target genes, including modification of translation initiation regions, alteration of mRNA structural elements, and the use of different codon biases (Gustafsson *et al.*, 2004). In addition to codon optimization, the balance of A+T/G+C content has also been proposed as a major factor affecting translation efficiency. The expression of diphtheria toxin and human glucocerebrosidase in *P. pastoris* was improved by codon optimization and removal of an AT-rich region that caused early termination of transcription (Woo *et al.*, 2002). A very recent study in *P. pastoris* further demonstrated the importance of codon optimization and balanced A+T/G+C content for the production of lysostaphin, a promising therapeutic agent for treating staphylococcal infections, particularly those caused by the methicillin-resistant *Staphylococcus aureus* (Zhao *et al.*, 2014).

The effect of the codon bias and consensus sequence (CACA) at the translation initiation site on the expression of human interferon alpha 2b (hIFN-α2b) was investigated in *Y. lipolytica*. Whereas codon optimization increased hIFN-α2b production by 11-fold, the additional insertion of a CACA sequence upstream of the initiation codon of the codon-optimized hIFN-α2b construct increased production by 16.5-fold, indicating that translational efficiency should be also considered as a key factor in heterologous protein expression levels (Gasmi *et al.*, 2011). A recent study reported the use of a condition-specific codon optimization approach, based on codon usage bias generated using only genes induced under a given condition. The application of this delicate approach in *S. cerevisiae* showed that the synthetic *CatA* gene encoding *Acinetobacter baylyi* catechol 1,2-dioxygenase, optimized for stationary phase production, generated an approximate threefold increase in yield over a commercially optimized gene variant (Lanza *et al.*, 2014).

On the other hand, there are other challenges to optimize gene sequences by incorporating orthogonal pathways and potential synthetic biology solutions. For example, Hancock *et al.*, reported the expansion of the genetic code of *S. cerevisiae* for incorporation of diverse unnatural amino acids via a pyrrolysyl-tRNA synthetase/$tRNA_{CUA}^{Pyl}$ pair. The functional and orthogonal pyrrolysyl-tRNA synthetase/$tRNA_{CUA}^{Pyl}$ pairs from *Methanosarcina barkeri* and *M. mazei* were evolved in *E. coli* and used to allow the site-specific incorporation of a variety of unnatural amino acids in *S. cerevisiae*. By this approach, an alkyne-containing amino acid, an important post-translationally modified amino acid and one of its analogs, a photocaged amino acid, and a photo-cross-linking amino acid were successfully incorporated into proteins in yeast (Hancock *et al.*, 2010).

Copy-number control tools

Another important approach for manipulating heterologous gene expression in yeast involves controlling the gene copy number of a desired expression cassette. In *S. cerevisiae*, 2 μ-based plasmids are maintained at c. 5–30 copies per cell, while yeast centromeric plasmids containing an autonomously replicating origin paired with a yeast centromere are present at c. 1 copy per cell (Fang *et al.*, 2011). High-copy plasmids facilitate strong gene expression, but can impose a heavy burden on cells resulting in increased instability of the constructs. Conversely, low-copy plasmids are more stable, but their use is limited by lower gene expression levels. Inserting an expression cassette in a target locus on a native yeast chromosome by homologous recombination is beneficial because it allows an expression cassette to be stably maintained even in the absence of selective pressure. High-level expression of heterologous proteins may be achieved by introducing DNA cassettes into multiple sites in yeast chromosomes, such as the yeast repeating ribosomal (Lopes *et al.*, 1996), delta (Oliveira *et al.*, 2007), and sigma element sequences (Kudla and Nicolas 1992). These multiple integration approaches coupled with the use of a deficient selection marker have been advanced as helpful technologies to modulate the copy number of an integrated DNA construct in *S. cerevisiae* and *Y. lipolytica* (Nicaud *et al.*, 2002). Recently, 'Reiterative Recombination' was developed as an efficient platform technology in *S. cerevisiae* for the assembly of combinatorial libraries for simultaneous expression of several different proteins through continuous targeted integration and the use of exchangeable, replaceable selection markers (Wingler and Cornish 2011).

However, it has become apparent that there is not always a linear correlation between expression cassette copy number and protein yield, particularly for the secretory production of recombinant proteins (Aw and Polizzi 2013). For example, a study involving secretory production of human serum albumin in *H. polymorpha* showed that the effect of gene dosage effect on expression was abolished using an optimized expression cassette. Thus, a single copy of the expression vector integrated into the *MOX* locus was sufficient for maximal expression of human serum albumin (Kang *et al.*, 2001). Similarly, expression of human serum albumin in *P. pastoris* showed that increasing gene copy number did not result in higher production levels due to secretion saturation (Whyteside *et al.*, 2011). Thus, using controllable multiple integration vectors would be a good strategy to introduce optimal copy numbers for high-level expression of recombinant proteins.

Gene deletion and integration tools for host strain construction

Efficient strategies for gene replacements and marker recycling are essential for synthetic biology applications involving knocking out genes and introducing multiple heterologous genes besides the expression cassettes. In addition to the

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counter-selection strategy using the *URA3* marker that has been conventionally applied in yeast, more advanced approaches such as the Cre/*loxP* and Flp/*FRT* recombinase-based systems have been developed. These systems proceed via active excision of marker gene fragments to recycle markers for sequential rounds of deletions in several yeast species including *S. cerevisiae* (Park *et al.*, 2011), *H. polymorpha* (Qian *et al.*, 2009), *P. pastoris* (Pan *et al.*, 2011; Naatsaari *et al.*, 2012), and *Y. lipolytica* (Fickers *et al.*, 2003). These strategies have been applied to the development of a conditional gene deletion system, in which the Cre or Flp recombinases are placed under the strict regulation of galactose- or methanol-inducible promoters to control target gene deletion processes.

In eukaryotes, double-strand breaks in DNA can be repaired by two mechanisms that work independently but competitively: one is homologous recombination and the other is nonhomologous end-joining (NHEJ). Site-specific knockout and integration for construction of yeast strains rely on endogenous homologous recombination. Whereas in *S. cerevisiae,* homologous recombination is highly efficient, nonhomologous end-joining is the preferred pathway for repairing double-strand breaks in most other non-*Saccharomyces* yeasts. After deletion of *KU70* or *KU80* homolog-encoding proteins involved in nonhomologous end-joining, the frequency of homologous recombination was significantly enhanced in several yeast species including *H. polymorpha*, *P. pastoris*, and *Y. lipolytica* (de Jong *et al.*, 2010; Naatsaari *et al.*, 2012; Verbeke *et al.*, 2013). Thus, these *ku*-deletion mutant strains are useful recipients for targeted gene manipulation when constructing useful host strains for the production of recombinant proteins in nonconventional yeast systems.

In very recent, several cutting-edge technologies based on bacterial nucleic acid modification systems have made dramatic leaps in genome engineering in eukaryotic systems. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems originate from immune system of bacteria and archaea by RNA-guided nuclease activity (Barrangou *et al.*, 2007). The type II bacterial CRISPR/Cas9 system has been applied to *S. cerevisiae* for genome engineering. The increased homologous recombination efficiency and feasibility for site-specific mutation and allelic replacement were observed in *S. cerevisiae*, indicating that the CRISPR-Cas system has a great potential to become a valuable genome engineering tool in yeast (DiCarlo *et al.*, 2013). Transcription activator-like effectors fused to the catalytic domain of endonucleases (TALENs) have been widely used for genome editing or engineering by generating site-specific double-strand breaks, which can be repaired through DNA repair mechanisms including NHEJ and homology-directed repair (HDR) (Gaj *et al.*, 2013). A recent study presented the potential of homodimeric TALENs as a useful tool to simultaneously modify the yeast genome at multiple genomic loci in a site-specific manner (Aouida *et al.*, 2014).

HOST STRAIN ENGINEERING FOR SECRETORY PRODUCTION OF RECOMBINANT PROTEINS

Engineering of protein secretion pathway

Due to the similarities between yeast and animal cell secretion pathways, yeasts have been a preferred microbial host system for the secretory production of recombinant proteins. Particularly, yeasts secrete only a few endogenous proteins, so it is easy to purify the secreted recombinant protein. Secretory protein production requires multiple steps in the conversion of protein-coding DNA sequences to mature proteins, which is complex and involves many different stages in processing, including transcription, translation, translocation, post-translational modifications and protein folding, peptide cleavage and additional glycosylation, sorting, and secretion (Hou *et al.*, 2012). Although yeast is a favorable platform for secretory production of recombinant proteins, several limiting steps, such as different glycosylation processes and proteolytic degradation, are often encountered during secretory production of heterologous proteins in yeast. Each step of the protein secretion pathway may be an engineered target for improving protein expression levels and quality (Fig. 1). Therefore, synthetic biology approaches represent excellent strategies for engineering yeast secretion pathways to satisfy commercial production requirements.

The initial step of secretion is the transfer of a protein through the ER membrane into the secretion pathway. Secretion signal sequences are required to translocate proteins into the ER and thus the efficiency of signal sequences is one of critical factors in determining the final yield of secretory proteins. In general, the native prepro-leader sequences derived from yeast secretory proteins, such as *S. cerevisiae* alpha-mating factor, and their modified versions are exploited to facilitate secretory expression of heterologous proteins in yeast. It was reported that the synthetic prepro leaders of alpha-mating factor lacking consensus *N*-linked glycosylation sites directed secretion of correctly folded insulin precursor with a yield comparable to, or better than, the native leader in *S. cerevisiae* (Kjeldsen *et al.*, 1998).

Correct protein folding in the yeast ER is very important as it determines whether the protein enters the secretory pathway or whether it is targeted for ER-associated degradation (ERAD). Misfolding of nascent peptides leads to a luminal burden that results in ER stress, which regulates the unfolded protein response (UPR) (Patil and Walter 2001; Payne *et al.*, 2008). A recent study showed that imbalance of heterologous protein folding and disulfide bond formation rates generated runaway oxidative stress, which also leads to UPR activation (Tyo *et al.*, 2012). Both UPR and ERAD govern the flux of proteins through the ER-to-Golgi apparatus for secretion. Several studies to manipulate the ER luminal environments have shown that enhanced secretion capacities of target proteins can be obtained through overexpression of protein-folding factors (chaperones) or redox enzymes in the ER, including *SIL1*, *LHS1*, *JEM1*, *SCJ1*, *KAR2*, *ERO1,* and *PDI1* in *S. cerevisiae* (Smith *et al.*, 2004; Payne *et al.*, 2008), *P. pastoris* (Zhang *et al.*, 2006; Gasser *et al.*, 2007), and *K. lactis* (Lodi *et al.*, 2005). The overexpression of Hac1p, a transcriptional activator in the UPR response, led to expression of a set of ER chaperones, improving secretory production of some heterologous proteins in *S. cerevisiae* (Valkonen *et al.*, 2003) and *P. pastoris* (Guerfal *et al.*, 2010). However, depending on the target protein, coexpression of Hac1p could instead decrease heterologous expression or exert no effect on expression levels, indicating the effect of Hac1 overexpression needs to be evaluated on a case-by-case basis.

Overexpression of Sly1p and Sec1p, involved in vesicle trafficking from ER-to-Golgi and Golgi-to-plasma membrane, respectively, improved the secretion of heterologous human insulin precursor and α-amylase proteins in *S. cerevisiae*, along with the secretion of an endogenous invertase protein (Hou *et al.*, 2012). These results strongly suggest that upregulating protein trafficking can improve protein secretion. On the other hand, activation of heat shock factor 1, a primary heat shock response (HSR) transcription factor, can also induce upregulated expression of many genes encoding cytoplasmic or secretory protein, folding chaperones that facilitate protein

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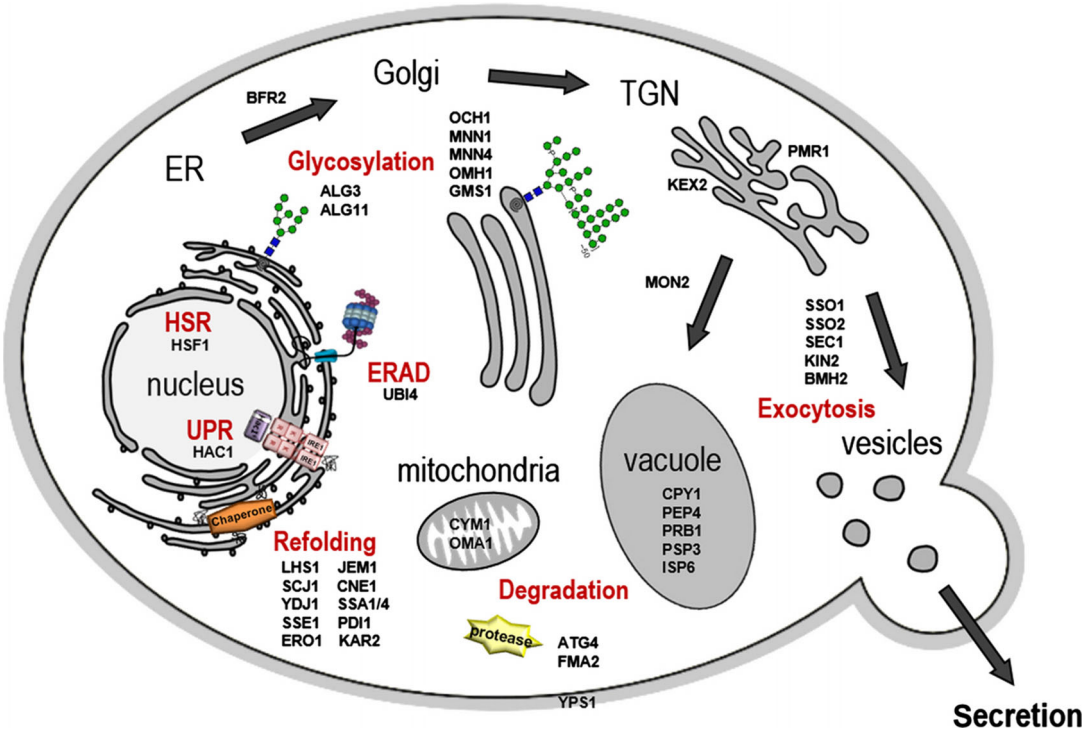

Figure 1. Schematic representation of the yeast secretory pathway together with target genes for host strain engineering and enhanced production of heterologous proteins. Secretory proteins enter the endoplasmic reticulum (ER). The accumulation of misfolded proteins leads to the induction of the unfolded protein response (UPR) and ER-associated degradation (ERAD). The heat shock response (HSR) also induces upregulated expression of many genes encoding cytoplasmic or secretory protein-folding chaperones. Correctly folded proteins are transported to the Golgi for further processing including additional glycosylation. Proteins exiting the Golgi may be secreted extracellularly or targeted to vacuoles for storage or degradation. Alternatively, recombinant products are subjected to proteolytic degradation by several proteases located in cell compartments such as the Golgi, cytosol, mitochondria, and the cell wall. The functions of target genes are described in Table 3.

folding and prevent the accumulation of misfolded or aggregated proteins. A very recent study on the overexpression of a constitutively activated mutant of heat shock factor 1 in *S. cerevisiae* showed improved heterologous protein secretion by constitutively activating HSR (Hou *et al.*, 2013).

Yeasts express numerous proteases, many of which are either localized to the secretory pathway or pass through it en route to the cell surface. Some recombinant proteins are subjected to aberrant proteolytic cleavage by proteases during the secretion process, which not only reduces the final yield of proteins but also affects the quality of recombinant proteins. In this case, construction of protease-deficient strains with multiple gene deletions could be employed as an efficient strategy to solve the protein cleavage problem. For example, a multiple-yapsin-deficient mutant strain of *S. cerevisiae* lacking the *YPS1*, *YPS2*, *YPS3*, *YPS6*, and *YPS7* genes showed diminished cleavage of the recombinant parathyroid hormone protein at high cell-density cultivation during fed-batch fermentation (Cho *et al.*, 2010). Yapsin-deficient strains have been developed in other yeast species, including *H. polymorpha* and *P. pastoris*, demonstrating that aspartyl proteases localized at the cell surface are principally responsible for aberrant proteolytic cleavage of secretory recombinant proteins (Sohn *et al.*, 2010; Wu *et al.*, 2013). In addition, reducing major intracellular protease activities by the disruption of vacuolar protease genes, such as *PEP4* and *PRB1*, also enhanced the secretory production of human interferon-β by c. 10-fold in *S. cerevisiae* (Tomimoto *et al.*, 2013).

Yeast strain engineering strategies have been extended from single target gene manipulation to the deletion and/or overexpression of multiple genes involved in various processes. As a representative example, the simultaneous modification of several targets through the deletion of *YPS1* (previously named *YAP3*) and *HSP150* and overexpression of *PDI1* was applied to engineer-enhanced production of recombinant human transferrin (rTf) with improved yield and quality in *S. cerevisiae* (Finnis *et al.*, 2010). Overexpression of *PDI1* improves correct protein production by assisting protein folding, and deletion of *YPS1* diminishes target protein degradation. A secretory protein Hsp150p is generally copurified with other recombinant proteins. Therefore, deletion of *HSP150* facilitates purification in downstream processing by eliminating a potential contaminant. Indeed, the final production strain, which was chemically mutagenized and further modified with the *YAP3* and *HSP150* deletions and *PDI1* overexpression, showed nearly 50-fold increased production of rTf compared to the original progenitor strain. A similar approach involving multiple combined genetic manipulations has also been applied toward the improved production of human growth hormone (hGH) in the fission yeast *S. pombe*. Approximately 30-fold enhanced secretion of hGH was achieved through the deletion of seven obstructive proteases. However, intracellular retention of secretory hGH was found in the resultant multiprotease-deficient strains. As a solution to increase hGH secretion efficiency, further deletions of vacuolar protein sorting genes were made and the deletion of *VPS10*, involved in vacuolar protein sorting, was found to lead to an approximately twofold increase in hGH secretion (Idiris *et al.*, 2010). These studies clearly demonstrate the possible application of secretory-pathway modification and multiple protease deletion for improving heterologous protein secretion from yeast. Some other case studies on secretion pathway engineering, which are not mentioned in this review, are summarized in Table 3.

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Table 3. Target genes modified in strain engineering for enhancement of secretory protein production.

Category	System	Gene	Protein	Biological function	References
Protein folding, quality control	*Sc*	*SIL1*	Cochaperone, NEF	Protein translocation into the ER	Payne *et al.* (2008)
		LHS1	Cochaperone	Polypeptide translocation and folding	
		JEM1	DnaJ-like cochaperone	Chaperone binding	
		SCJ1	HSP70-type cochaperone	Chaperone binding	
		HSF1	Heat shock transcription factor	Response to highly diverse stresses	Hou *et al.* (2012a, b, 2013)
	Hp	*CNE1*	ER chaperone calnexin	Folding and quality control of glycoproteins	Klabunde *et al.* (2007)
	Pp	*YDJ1*	Type I HSP40 cochaperone	Regulation of HSP90 and HSP70 functions	Zhang *et al.* (2001, 2006)
		SSA1	Cochaperone, HSP70 family	Protein folding	Zhang *et al.* (2001, 2006)
		SSA4		SRP-dependent protein-membrane targeting and translocation	Gasser *et al.* (2007)
		SSE1		Unfolded protein binding	
	Sc, Pp	*HAC1*	bZIP transcription factor	UPR regulation	Valkonen *et al.* (2003) and Gasser *et al.* (2007)
		KAR2	HSP70 chaperones, BiP	Protein folding, UPR regulation	Harmsen *et al.* (1996) and Zhang *et al.* (2001, 2006)
	Kl, Pp	*ERO1*	Thiol oxidase	Oxidative protein folding	Lodi *et al.* (2005) and Gasser *et al.* (2007)
	Sc, Kl, Pp	*PDI1*	Protein disulfide isomerase	Disulfide bond formation of proteins	Smith *et al.* (2004), Lodi *et al.* (2005) and Zhang *et al.* (2001, 2006)
Degradation	*Sc*	*CYM1*	Cytosolic metalloprotease	Mitochondrial lysine-specific metalloprotease	Jonson *et al.* (2004)
		CPY1	Carboxypeptidase Y	Vacuolar serine-type C-terminal exopeptidase	Van Den Hazel *et al.* (1996)
	Sc, Pp	*KEX2*	Proprotein convertase	Serine-type calcium-dependent serine protease	Zhang *et al.* (2001, 2006) and Werten and de Wolf (2005)
	Pp, Hp	*YPS1*	Yapsin-1	Aspartic-type endopeptidase	Werten and de Wolf (2005), Cho *et al.* (2010) and Choi *et al.* (2003)
	Sc, Kl	*UBI4*	Ubiquitin	Ubiquitin-26S proteasome system	Chen *et al.* (1994) and Bao and Fukuhara (2001)
	Sc, Pp, Cb	*PEP4*	Proteinase A	Vacuolar aspartyl protease	Kang *et al.* (1998, 2000, 2001), Komeda *et al.* (2002) and Wu *et al.* (2013)
	Sc, Sp, Cb	*PRB1*	Proteinase B	Vacuolar serine-type endopeptidase	Kang *et al.* (1998, 2000, 2001), Komeda *et al.* (2002) and Idiris *et al.* (2010)
	Sp	*ATG4*	Cysteine protease	Cytosolic cysteine-type endopeptidase	Idiris *et al.* (2010)
		OMA1	Metalloendopeptidase	Mitochondrial membrane-bound metalloendopeptidase	
		FMA2	Methionine aminopeptidase	Cytosolic aminopeptidase specific to N-terminal methionine	
		PPP20	Aminopeptidase	Vacuolar/cytosolic aminopeptidase	

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Table 3. Continued.

Category	System	Gene	Protein	Biological function	References
Trafficking	*Sc*	*SEC1*	Sm-like protein	SNARE binding, docking of exocytic vesicles	Hou *et al.* (2012, b, 2013)
		MON2	SEC7 family, GEF	Endocytosis, Golgi-to-endosome transport, vacuole integrity	Kanjou *et al.* (2007)
		PMR1	Ca^{2+}/Mn^{2+} ATPase	Calcium-dependent protein sorting and processing	Harmsen *et al.* (1996)
		SSO1, SSO2	Plasma membrane t-SNARE	Fusion of secretory vesicles at the plasma membrane	Ruohonen *et al.* (1997)
	Pp	*BFR2*	90S preribosome	ER-to-Golgi transport, rRNA processing	Gasser *et al.* (2007)
		BMH2	14-3-3 protein	Exocytosis, vesicle transport, and Ras/MAPK signaling	
		CUP5	Vacuolar membrane ATPase	Hydrogen ion transmembrane transport	
		KIN2	Serine/threonine protein kinase	Exocytosis regulation	
		SEC63	Sec63 complex subunit	Cytosol-to-ER transport	Zhang *et al.* (2001, 2006)
	Sc, Sp	*Vsp10*	Vacuolar protein sorting receptor	Protein targeting to vacuole, vacuolar transport	Zhang *et al.* (2001, 2006) and Idiris *et al.* (2010)
Glycosylation	*Sc, Kl*	*MNN1*	α-1,3-mannosyltransferase	Addition of α-1,3-mannose to *N*-/*O*-linked oligosaccharides	Nakanishi-Shindo *et al.* (1993) and Liu *et al.* (2009, 2012)
	Sc	*MNN4*	Positive regulator of Mnn6p	Mannosylphosphorylation of *N*-linked oligosaccharides	Chiba *et al.* (1998)
		ALG11	α-1,2-mannosyltransferase	*N*-linked oligosaccharide assembly	Parsaie Nasab *et al.* (2013)
	Sp	*GMS1*	UDP-galactose transporter	Transport of UDP-galactose into the lumen of Golgi apparatus	Ohashi and Takegawa (2010)
		OMH1	*O*-glycoside α-1,2-mannosyltransferase	Extending α-1,2-linked mannose in the *O*-glycan pathway	Ikeda *et al.* (2009)
	Sc, Pp, Hp	*ALG3*	α-1,3-mannosyltransferase	Synthesis of dolichol-linked oligosaccharide for *N*-glycosylation	Nakanishi-Shindo *et al.* (1993), Hamilton and Gerngross (2007) and Cheon *et al.* (2012)
	Sc, Pp, Kl Sp, Hp, Yl	*OCH1*	α-1,6-mannosyltransferase	Outer chain elongation of *N*-linked oligosaccharides	Nakanishi-Shindo *et al.* (1993), Hamilton and Gerngross (2007), Song *et al.* (2007), Liu *et al.* (2009, 2012), Ohashi and Takegawa (2010) and Cheon *et al.* (2012)

Sc, S. cerevisiae; *Pp, P. pastoris*; *Sp, S. pombe*; *Kl, K. lactis*; *Cb, C. boidinii*; *Hp, H. polymorpha*; *Yl, Y. lipolytica*.

Synthetic biology for yeast glycoengineering

Most proteins with therapeutic applications are produced with post-translational modifications, which affect their function and stability. Among various post-translational modifications, glycosylation is the most common and at the same time the most complex modification (Walsh 2010b). Although yeasts can perform *N*- and *O*-glycosylation of proteins, the final glycosylation patterns of yeasts significantly differ from those of humans. High-mannose-type glycans and terminal α-1,3-mannose linkages associated with glycoproteins produced from *S. cerevisiae* are known to cause short serum half-lives or even immunogenicity of therapeutic proteins (De Pourcq *et al.*, 2010; Walsh 2010b). Thus, several efforts have been made toward the development of intelligent yeast strains devoid of yeast-specific glycans and the ability to synthesize human-type glycans (Fig. 2). The pioneering work by Dr. Jigami's group demonstrated the high potential of yeast glycoengineering to produce recombinant proteins with human-type glycans. A glycoengineered *S. cerevisiae* strain was constructed by deletion of the *OCH1* gene that is responsible for the initiation of yeast-specific outer chain biosynthesis and subsequent introduction of an *Aspergillus saitoi* α-1,2 mannosidase in the ER for mannose trimming. This yeast strain was shown to produce human compatible $Man_5GlcNAc_2$ glycan (Chiba *et al.*, 1998). Intensive attempts have been continued to implement humanized glycosylation pathways in various nonconventional yeast species, in which most *N*-linked glycans

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are much less hypermannosylated and lack hyperimmunogenic terminal α-1,3-linked mannose residues compared to those of *S. cerevisiae* (Hamilton and Gerngross 2007; De Pourcq *et al.*, 2010; Cheon *et al.*, 2012). Deletion of the *OCH1* gene has been carried out in various nonconventional yeast species, such as *H. polymorpha, P. pastoris, S. pombe, K. lactis,* and *Y. lipolytica*, and is considered as an initiation step required for humanized glycoengineering (Hamilton and Gerngross 2007; Song *et al.*, 2007; Liu *et al.*, 2009; Ohashi and Takegawa 2010; Cheon *et al.*, 2012).

To achieve complex-type humanized glycosylation, further introductions of additional glycosyltransferases and glycosidases involved in human glycosylation pathways should

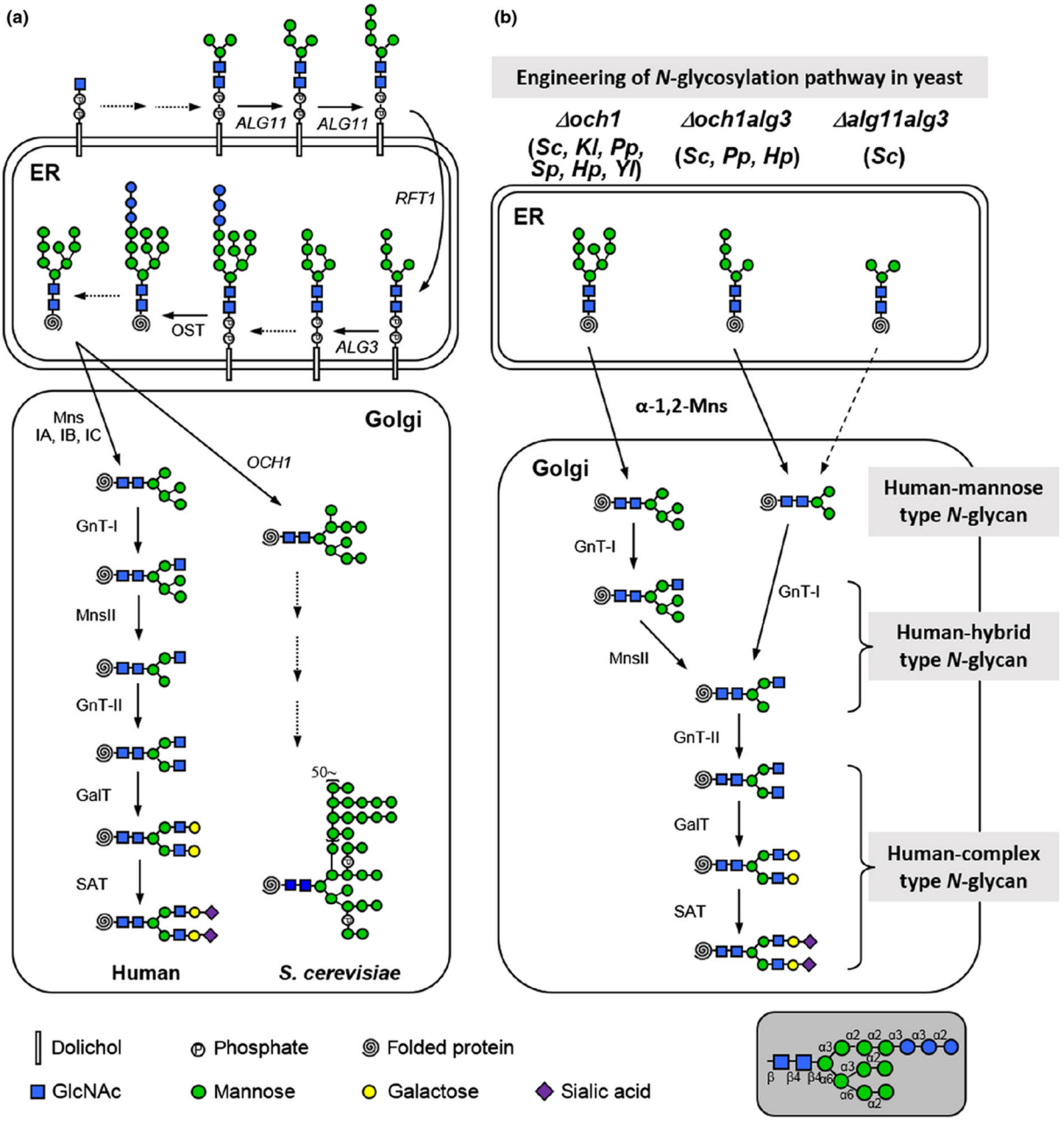

Figure 2. Schematic representation of engineering strategies of N-glycosylation pathways in yeasts. (a) N-glycosylation pathways in mammals and *S. cerevisiae*. Lipid-linked glycan assembly in the ER is a highly conserved process. The core oligosaccharide $Man_8GlcNAc_2$ is made in the ER and transferred to the Golgi, where further glycan processing diverges between humans and yeast. In humans, mannosidase (Mns) enzymes trim three mannose residues, leaving a $Man_5GlcNAc_2$ structure, which is modified to complex glycans through β-1,2-*N*-acetylglucosaminyltransferase I (GnT-I), MnsII, GnT-II, galactosyltransferase (GalT), and sialyltransferase (SAT). In contrast, *N*-glycans of *S. cerevisiae* are further extended by attachment of only mannose residues in the Golgi apparatus. (b) Engineering of the N-glycosylation pathway for the production of human complex-type *N*-glycans in several yeast species. The deletion of the *OCH1* gene blocks outer chain elongation in Golgi apparatus. Introduction of heterologous α-1,2-mannosidase (α-1,2-Mns) into the *och1* deletion mutant is required for mannose trimming to generate the $Man_5GlcNAc_2$ structure, which can be further utilized as the substrate for the GnT-I, MnsII, and GnT-II. On the other hand, a $Man_3GlcNAc_2$ structure is generated in the *och1alg3* double-mutant-expressing α-1,2-Mns or directly in alg3alg11 double-mutant strains without heterologous expression of an α-1,2-Mns. The *N*-linked trimannosyl core glycan can serve as a substrate for GnT-I and GnT-II, giving rise to the human-hybrid/complex-type *N*-glycan in yeast cells. *Sc, S. cerevisiae*; *Pp, P. pastoris*; *Sp, S. pombe*; *Kl, K. lactis*; *Hp, H. polymorpha*; *Yl, Y. lipolytica*.

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be made in yeast. Besides simple expression of these genes, precise spatial positioning along the ER-Golgi secretory pathway is crucial for a successful assembly of the foreign glycosylation pathway. For functional targeted expression at the yeast Golgi membrane, semi-synthetic glycosyltransferase and glycosidase libraries composed of several combinations of C-terminal catalytic domains and transmembrane fragments were constructed, and these combinatorial libraries combined with high-throughput screening were used to screen *P. pastoris*-engineered strains harboring optimal foreign glycosylation pathways (Choi *et al.*, 2003). Finally, the production of humanized glycoproteins with terminal sialic acids in *P. pastoris* was achieved by the introduction of an additional nine synthetic genes enabling sialic acid biosynthesis in yeast (Hamilton *et al.*, 2006).

An alternative strategy for reconstructing the biosynthetic pathway of initial lipid-linked oligosaccharide synthesis, which removes the requirement of mannosidase II, was developed based on deletion of the *ALG3* gene coding for dolichyl-phosphate-mannose (Dol-P-Man)-dependent α-1,3 mannosyltransferase in *P. pastoris* and *H. polymorpha* (Davidson *et al.*, 2004; Cheon *et al.*, 2012). In a recent study, a novel synthetic *N*-glycosylation pathway generated by the *ALG3* and *ALG11* double deletion in *S. cerevisiae* was reported to produce glycoproteins containing a trimannosyl core glycan as a major portion of humanized *N*-glycan structures (Parsaie Nasab *et al.*, 2013). Moreover, the overexpression of protozoan oligosaccharyltransferases, which possess altered specificities for the oligosaccharide and also for the protein acceptor site, was proposed as a promising tool to improve glycosylation site occupancy in the glycoengineered yeast strains, which ensures the production of glycoproteins fully glycosylated with homogeneous *N*-glycans (Piirainen *et al.*, 2014).

In addition to the *N*-glycosylation engineering, the glycoengineering of yeasts has been targeted toward *O*-glycosylation, which is characterized by relatively shorter glycan structures. Unlike the *O*-glycan of yeasts, the *O*-linked glycan structure of mammalian systems is elongated by mucin-type glycosylation, which includes an *N*-acetylgalactosamine (GalNAc) conjugated to the Ser/Thr residue (Hanisch 2001). This sugar is subsequently extended with other sugars, including *N*-acetylglucosamine (GlcNAc), galactose, fucose, and sialic acid. The other mammalian *O*-glycosylation is described as α-dystroglycan type (Lommel and Strahl 2009), which initiates with a mannose conjugated to the Ser/Thr residue and is elongated with GlcNAc, galactose, and sialic acid residues. Some higher eukaryotic glycan structures also have glucuronic acid or xylose residue, which is particularly important to linking proteoglycans to proteins or the biosynthesis of various types of glycosaminoglycans (Wilson 2004), respectively. Modified *O*-glycosylation pathways have been established in *S. cerevisiae* to produce mammalian-type glycosylation patterns by heterologous expression of several foreign genes involved in *O*-glycosylation of higher eukaryotes. The human GDP-fucose transporter and human *O-FucT-1* genes were expressed in *S. cerevisiae* to produce GDP-fucose from intrinsic GDP-mannose in the yeast cytosol, resulting in production of an *O*-fucosylated epidermal growth factor domain (Chigira *et al.*, 2008). The artificial *S. cerevisiae* strain was constructed by introducing UDP-glucose dehydrogenase and UDP-glucuronic acid decarboxylase derived from *Arabidopsis thaliana*, which is capable of converting UDP-glucose to UDP-xylose through the two-step process (Oka and Jigami 2006). Recently, production of mucin-type glycoproteins in this baker's yeast was succeeded through genetic engineering to introduce various heterologous enzymes, absent in yeast, such as ppGalNAc-T1, core1 β1–3GalT, UDP-Gal/GalNAc transporter, and UDP-Gal/GalNAc 4-epimerase (Amano *et al.*, 2008). Moreover, human-like sialylated glycans, similar to α-dystroglycan-type glycoforms, were created in *P. pastoris* by the introduction of the enzymes α-1,2-mannosidase and β-1,2-*N*-acetylglucosaminyltransferase 1 into the engineered *P. pastoris* strain harboring a heterologous sialic biosynthesis pathway (Hamilton *et al.*, 2013).

Perspectives of yeast factories for recombinant protein production

Yeasts are particularly amenable to studying the mechanistic basis of high-yielding recombinant protein production experiments using synthetic biology tools. In the postgenome era, significant advances in the yeast toolbox of genetic elements have been made toward synthetic biology approaches from classical genetic engineering. Genome-scale metabolic models based on whole-genome sequence information are available not only for *S. cerevisiae* (Osterlund *et al.*, 2012), but also for other nonconventional yeasts such as *P. pastoris* and *Y. lipolytica* (De Schutter *et al.*, 2009; Sherman *et al.*, 2009). Moreover, genome-scale engineering of *S. pombe* by large-scale gene deletion was shown to be applicable for the construction of host strains designed for enhanced production of recombinant proteins (Sasaki *et al.*, 2013).

A genome-scale model consisting of core components in the secretory machinery and reactions for the protein secretory machinery was recently constructed using a bottom-up approach in *S. cerevisiae* (Feizi *et al.*, 2013). This model offers knowledge of the protein secretory machinery in other eukaryotic organisms as well as in yeast. More importantly, it could be utilized to estimate the metabolic and energy demands on the secretory machinery and thereby finally assess targets of metabolic engineering for improving protein secretion. Importantly, new platform strains and molecular cloning tools as well as *in vivo* glycoengineering to produce humanized glycoforms have made yeast a more practical host for therapeutic recombinant protein production. The synthetic biology approach of targeting glycosylation engineering should facilitate implementation of the humanized glycosylation pathway as well as assembly of synthetic glycosylation processes to attach several kinds of artificial oligosaccharide chains. The creation of glycoproteins with tailor-made glycoforms and differing pharmacodynamics using glycoengineered yeast strains will surely pave the way for the production of new glycoprotein pharmaceuticals with better properties than natural variants. Therefore, it is predicted that synthetic biology approach would further expand the use of various yeasts as a major workhorse for production of next-generation biopharmaceutical proteins.

In the respect of industrial applications, high cell-density fermentation of yeast is one of the important strategies to increase the ultimate yield of recombinant proteins. However, the move from low-density cultivation at shake flasks to high-density cultivation in bioreactors requires considerable re-optimization of several culturing conditions, such as pH, aeration, and carbon source feed rate (Cereghino *et al.*, 2002). Moreover, high cell-density cultivation of yeast not only produces more contaminating proteins but also causes an accumulation of lipid-like substances in high-salt medium. In the case of the production of recombinant human interleukin-6 (rhIL-6) in *P. pastoris*, PEG-8000 precipitation was shown to be an effective approach to eliminate unnecessary contaminating proteins, thus facilitating the downstream purification process (Li *et al.*, 2011). On the other hand, it has been reported that specific productivity of antibodies produced by a

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glycoengineered *P. pastoris* in high cell-density cultivation was improved through oxygen-limited cultivation. Moreover, the oxygen-limited process was successfully scaled to 1200 L-manufacturing pilot scale, which showed not only increased antibody concentration (up to 1.9 g L^{-1}) but also improved *N*-glycosylation pattern and reduced antibody fragmentation (Berdichevsky *et al.*, 2011). Thus, it is apparent that intensive efforts are needed to develop physiologically rational and suitable cultivation process with protein-specific adjustments to upgrade yield and quality of recombinant proteins. As systems and synthetic biology approaches would provide more comprehensive information on host physiology, along with more rationally designed host engineering suitable for large-scale cultivation, yeast cell factories will become increasingly more efficient and economic platforms for the production of recombinant therapeutic proteins, which are equipped with robust and commercially viable processes.

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AUTHORS' CONTRIBUTION

K.H. and Y.S.J. contributed equally to this work.

Conflict of interest statement. None declared.

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