Synthetic Biology and Molecular Genetics in Non-Conventional Yeasts:

Current Tools and Future Advances

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ABSTRACT

Coupling the tools of synthetic biology with traditional molecular genetic techniques can enable the rapid prototyping and optimization of yeast strains. While the era of yeast synthetic biology began in the well-characterized model organism *Saccharomyces cerevisiae*, it is swiftly expanding to include non-conventional yeast production systems such as *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Yarrowia lipolytica*. These yeasts already have roles in the manufacture of vaccines, therapeutic proteins, food additives, and biorenewable chemicals, but recent synthetic biology advances have the potential to greatly expand and diversify their impact on biotechnology. In this review, we summarize the development of synthetic biological tools (including promoters and terminators) and enabling molecular genetics approaches that have been applied in these four promising alternative biomanufacturing platforms. An emphasis is placed on synthetic parts and genome editing tools. Finally, we discuss examples of synthetic tools developed in other organisms that can be adapted or optimized for these hosts in the near future.

ABBREVIATIONS

ARS - Autonomously Replicating Sequence

CEN - Centromere

CRISPR – Clustered, Regularly Interspaced, Short Palindromic Repeats

DSB – Double Strand Break

GFP – Green Fluorescent Protein

GRAS – Generally Regarded As Safe

gRNA – Guide RNA (for CRISPR)

HR – Homologous Recombination

IRES – Internal Ribosomal Entry Site

FDA – Food and Drug Administration

NHEJ – Non-Homologous End-Joining

RNAi – RNA Interference

RNAP II / III - RNA Polymerase II / III

TALEN – Transcription Activator-like Effector Nuclease

TF – Transcription Factor

UAS – Upstream Activation Sequence

UTR – Untranslated Region

WT – Wild Type

ZeoR – Zeocin Resistance Cassette

ZFN – Zinc Finger Nuclease

INTRODUCTION

The scope and prospect of synthetic biology is continuously evolving and expanding (Andrianantoandro et al., 2006; Cameron et al., 2014; Purnick and Weiss, 2009), yet fundamentally relies on the premise that biology can be built from a collection of well-characterized parts and subsystems. For more conventional, model organisms (such as *Escherichia coli* and *Saccharomyces cerevisiae*), this approach is aided by existing and ongoing efforts to catalogue synthetic parts and genome editing tools (Lee et al., 2015; Purnick and Weiss, 2009). The general tractable nature of genetic modifications and basic molecular biology and protein expression tools in these organisms aids such efforts (Ferrer-Miralles et al., 2009). In recent years, one of the major breakthrough areas for synthetic biology is expediting the often long and costly design-build-test cycle for industrial biotechnology. With the aid of modern genome editing tools, panels of production strains can now be created at unprecedented rates to support high throughput studies of genotype to phenotype relationships (Bao et al., 2015). The emergence of this capacity promises to spark a renaissance in the complex rewiring and engineering of organisms for production purposes. These synthetic biological capabilities have enabled impressive engineering efforts in model organisms, but there are many other non-conventional organisms of great interest to industrial biotechnology for which synthetic biology approaches are far more immature. The promise of synthetic biology in these organisms could significantly impact the metabolic engineering and recombinant protein production fields by establishing unique and robust expression and production platforms for biomanufacturing.

The potential of synthetic biology for rewiring organisms for production begets the question of which organism to choose, especially when not restricted to model organisms (Alper and Stephanopoulos, 2009). The ideal expression system would be a universal platform capable

of efficiently upgrading a variety of low-cost feedstock into value-added, renewable products. At the same time, the choice of host system imparts a substantial effect on industrial production (Westfall et al., 2012), as production-relevant phenotypes are usually multifactorial and difficult to unravel (Mateos et al., 2006; Stahmann et al., 2001). The innate advantages of a particular host or strain may include tolerance for high concentrations of product or precursor (Ling et al., 2014; Peralta-Yahya et al., 2012), moderate to high *de novo* synthesis of precursors or product (Blazeck et al., 2014a), or robustness to processing conditions (Dijkstra et al., 2014; Hahn-Hägerdal et al., 2005). These complex traits are often difficult to characterize and reductively define, and thus they are not easily transferred into another preferred strain or organism.

Despite their higher relative complexity, yeasts tend to have many beneficial, industrially attractive traits relative to commonly used bacteria such as *E. coli* (Chen and Nielsen, 2013; Kim et al., 2014; Liu et al., 2013; Wildt and Gerngross, 2005). A few of these attributes include: growth to high cell densities on a wide variety of carbon sources, ability to perform a variety of post-translational modifications, potential to compartmentalize reactions in organelles, high secretion capacity, and a lack of susceptibility to infectious agents like bacteriophage (Blount et al., 2012). While filamentous fungi also share many of these advantageous characteristics, they are often more difficult to transform with exogenous DNA (Kawai et al., 2010) and less amenable to simple bioreactor cultivation. The process engineering complexity of large-scale growth of filamentous fungi is due in part to variations in hyphal morphology that can lead to rheological mixing challenges and mass transfer limitations (Gibbs et al., 2000).

The vast majority of yeast synthetic biology tools have been developed in *S. cerevisiae* due to its well-annotated genome, genetic tractability, and overall ease of use (Nielsen et al., 2013). Likewise, the model yeast *S. cerevisiae* has historically dominated the arena of modified

yeasts for industrial processing. However, there are many other non-conventional yeast hosts with favorable traits (Buckholz and Gleeson, 1991; Gellissen et al., 2005), some of which are also used for industrial bioprocesses. In particular, with advances in synthetic biology, non-conventional yeasts such as *Hansenula polymorpha* (syn. *Ogataea polymorpha*), *Kluyveromyces lactis*, *Pichia pastoris* (syn. *Komagataella pastoris*), and *Yarrowia lipolytica* may take on expanded roles as industrial hosts.

In this review, we highlight the advances of synthetic biology in light of these four promising non-conventional yeast strains. Specifically, we describe the importance and status of basic synthetic parts, enabling molecular biology techniques, and genome-editing technologies in these organisms. Initially, we establish the importance and current uses of these four non-conventional yeasts. Next, we evaluate the state of synthetic parts including promoters, terminators, and episomal replication elements. Subsequently, we review techniques for site-specific cassette integrations, controlled and scarless excisions, and related genome editing methods including CRISPR-Cas. Ultimately, we provide a prospective for the future with respect to the application of synthetic biology in these promising non-conventional yeasts.

NON-CONVENTIONAL YEAST SYSTEMS

Non-conventional yeasts can provide many potential advantages over *S. cerevisiae* with respect to pathway requirements, desired product profile, and gross physiology (Gellissen and Hollenberg, 1997; Wolf, 2012). While some non-conventional yeasts have served as long-standing industrial protein production platforms, their utility in the small molecule chemical space is sometimes limited to native metabolite overproduction phenotypes. However, the increasing availability of high-quality yeast genome sequences (De Schutter et al., 2009; Dujon

et al., 2004; Ramezani-Rad et al., 2003; Sherman et al., 2009), effective vectors and transformation methods (Bianchi et al., 1987; Cregg et al., 1985; Das and Hollenberg, 1982; Davidow and Dezeuw, 1984; Faber et al., 1994; Gaillardin et al., 1985; Roggenkamp et al., 1986), and emerging synthetic tools are beginning to change this picture. This review focuses on four non-conventional yeasts of significant biotechnological interest: *H. polymorpha*, *K. lactis*, *P. pastoris*, and *Y. lipolytica*. Each of these organisms presents with different advantages, commonalities, and differences when compared with *S. cerevisiae*. A short survey of basic characteristics for these yeasts is illustrated in **Figure 1**, and a selection of significant products currently produced from these hosts is provided in **Table 1**. A brief summary of several unique and promising traits is also presented in the text that follows. For more detailed descriptions of these individual yeast systems, the authors refer the reader to organism-specific reviews for *H. polymorpha* (Saraya et al., 2012), *K. lactis* (Rodicio and Heinisch, 2013), *P. pastoris* (Vogl et al., 2013), and *Yarrowia lipolytica* (Madzak, 2015), and to several previous non-conventional yeast reviews (Dominguez et al., 1998; Gellissen et al., 2005; Kim et al., 2014; Radecka et al., 2015; Wolf, 2012).

Products derived from all four of these systems have been assigned Generally Regarded as Safe (GRAS) status by the United States FDA (Dominguez et al., 1998; Saraya et al., 2014; Vogl et al., 2013), which eases potential regulatory concerns when selecting an expression system for a new product. Whereas *S. cerevisiae* is a poor host for some human therapeutic proteins due to its tendency to hyperglycosylate (Bretthauer and Castellino, 1999) and produce potentially allergenic glycan structures, *H. polymorpha* and *P. pastoris* have both been extensively glycoengineered to produce strains with humanized N-glycosylation (Cheon et al., 2012; Wildt and Gerngross, 2005). *K. lactis* has also been engineered to reduce the

hypermannosylation of expressed proteins (Liu et al., 2009). *Y. lipolytica* is an oleaginous, alkane assimilating yeast with high potential for use as a platform for the production of lipid and fatty acid-derived products (Blazeck et al., 2014b; Tai and Stephanopoulos, 2013) and has been used for recombinant carotenoid and polyunsaturated fatty acid production industrially (Xue et al., 2013). *H. polymorpha* is a highly thermotolerant yeast capable of converting glucose and xylose to ethanol at temperatures up to 45-50°C (Suwannarangsee et al., 2010).

All of these hosts possess a broader or diversified catabolic repertoire compared to *S. cerevisiae*, thus enabling lower cost bioprocesses (Gellissen et al., 2005; Rymowicz et al., 2010). High level recombinant protein expression has also been achieved using all four of these systems, and production titers similar to or greater than *S. cerevisiae* are possible (Dominguez et al., 1998). Additionally, each of these organisms has substantial protein secretion capacity that can exceed that of *S. cerevisiae* by several orders of magnitude (Muller et al., 1998). Finally, all of these yeast species are ‘Crabtree-negative’ and as such, do not undergo aerobic alcoholic fermentation (Kiers et al., 1998). This can be an advantageous phenotype for increasing biomass accumulation in aerated cultures without losing overflow carbon to undesired alcohol production.

Despite the advantages described above, each of these hosts is considered non-conventional and suffers from some molecular genetic intractability compared with *S. cerevisiae*. In particular, one significant characteristic that has historically hampered modification of non-conventional yeast systems relative to *S. cerevisiae* is DNA repair pathway preference. *S. cerevisiae* strongly favors homologous recombination (HR), whereas all four non-conventional yeasts covered by this review display a stronger preference for non-homologous end-joining (NHEJ) when exogenous DNA is introduced (Vogl et al., 2013). NHEJ results in random, spontaneous integrations of all or part of a cassette or vector instead of the precise integration

that is possible with highly active HR and short homology targeting regions. Thus, strain development often requires extensive, time-consuming phenotypic or PCR-based screening to select the desired HR-mediated transformant from large library sizes. This challenge has previously been addressed by modifying the host strain background to minimize NHEJ and by flanking the desired integration cassette with longer homology sequences to direct HR at the desired locus. Deletion of the NHEJ-promoting proteins Ku70 (Näätsaari et al., 2012; Verbeke et al., 2013) or Ku80 (Kooistra et al., 2004; Saraya et al., 2012) significantly increased gene targeting efficiency, but differences in growth rate, overall transformation rate, phenotypic stability, and sensitivity to ultraviolet light have also been observed in several of these strains. While HR-mediated gene targeting can be effectively achieved in *S. cerevisiae* by including homology arms of at least 40 base pairs on the 5' end of primers when amplifying a gene of interest (Baudin et al., 1993; Longtine et al., 1998), 500 to 3000 base pairs of flanking homology may be required to isolate a non-conventional yeast transformant harboring the desired gene replacement (Blazeck et al., 2014b; Horwitz et al., 2015). Generation of targeted integration cassettes with such long locus-specific flanking regions requires additional PCR reactions, molecular cloning steps, and time, thus limiting the throughput of genetic manipulations. Nevertheless, advances in synthetic biology and genome editing tools are beginning to alleviate these limitations, as described later. Overall, non-conventional yeasts have many distinguishing characteristics and can serve as potent hosts for chemical and biologics production through the use of enhancing synthetic biology tools.

SURVEYING THE AVAILABLE SYNTHETIC BIOLOGY PARTS

Promoters

Promoters are responsible for driving gene expression and are one of the first key synthetic parts required in a host system. Due to their critical role in expression cassette design, promoters are likely the most characterized and engineered genetic part in many yeast systems. In this vein, promoters that span high strength and a range of expression are necessary to provide coarse, quantitative, and temporal control of expression. Such a range is exemplified by the frequent use of a series of *S. cerevisiae* constitutive promoters such as pCYC1, pTEF1, pADHI, pGPD/pTDH3 (Mumberg et al., 1995) combined with the strong, inducible pGALI. In the case where *E. coli* shuttle vectors are used, these fungal promoters should ideally have minimal to no activity in the cloning host to prevent toxic gene expression in *E. coli*.

The field of yeast synthetic promoter engineering has been growing quickly, especially in the model yeast *S. cerevisiae*. Certainly more advanced, engineered promoter elements can enable sophisticated control and/or coordination of a pathway, network, or circuit (Liang et al., 2013; Teo and Chang, 2014) in a minimal space. However, most of the field is dominated by the use of native yeast promoter scaffolds, resulting in relatively large promoter elements with low overall sequence diversity (Alper et al., 2005; Nevoigt et al., 2006) when compared to bacterial counterparts. This lack of diversity can deleteriously affect the stability of expression cassettes in *S. cerevisiae* by promoting homologous recombination between similar elements.

Methodologies to increase promoter activity through a hybrid promoter approach offer a valuable advancement for tuning expression performance in yeast (Blazeck et al., 2012a; Khalil et al., 2012), but the resultant promoters are also typically large and rely to some extent on endogenous sequences such as upstream activation sequences (UAS) and core elements. To address some of these concerns, it may be useful to generate a synthetic or recoded toolbox of non-native orthogonal promoters with tunable activity and regulation over a wide dynamic range (Blount et

al., 2012). This endeavor has recently been advanced in *S. cerevisiae* by sorting large random nucleotide libraries for active, modular core promoters and UAS elements that create very short but highly functional promoters (Redden and Alper, 2015). However, as described below, there are still many limitations in most of the non-conventional yeast hosts. Nevertheless, advances and paradigms from *S. cerevisiae* have promise for transferability to these non-conventional fungal systems.

H. polymorpha expression cassettes are commonly constructed using the strong inducible methanol oxidase promoter (p*MOX*) (Krasovska et al., 2007), but constitutive elements such as the glyceraldehyde-3-phosphate dehydrogenase promoter (p*GAP*) have also been utilized (Heo et al., 2003). The promoters typically used in this system are endogenous promoters and have not been engineered to enhance or alter performance, although a *H. polymorpha* glucose repression-deficient strain background has been identified that allows for induction of p*MOX* with methanol in the presence of glucose (Krasovska et al., 2007). This lack of glucose repression can be exploited in bioprocessing by utilizing an inexpensive glucose-based medium containing a minimal inducing concentration of flammable methanol, instead of an induction medium with high-concentration methanol as the primary carbon source.

Heterologous protein production strategies in *K. lactis* have frequently relied on *S. cerevisiae* promoters such as p*GAL1* or p*PGK* (Van Ooyen et al., 2006). The acceptable performance of these promoters in *K. lactis*, including glucose-repression and galactose-induction for p*GAL1* (Horwitz et al., 2015), suggests a high level of promoter element transferability between the two platforms, which increases the potential that promoter engineering efforts in *S. cerevisiae* can be beneficially applied to the *K. lactis* expression system. Protein expression efforts have also routinely used the *K. lactis* *LAC4* promoter (p*LAC4*) for

protein expression due to its native strength and 100-fold induction by lactose/galactose (Dickson et al., 1981; Swinkels et al., 1993). However, this element is complicated by a Pribnow box-like sequence that drives constitutive expression of the gene of interest in *E. coli* during vector construction (Dickson and Riley, 1989). To bypass this undesirable trait, targeted mutagenesis of the Pribnow box-like element was used to develop a promoter variant (pLAC4-PBI) that displayed significantly reduced activity in *E. coli* and retained full activity in *K. lactis* (Colussi and Taron, 2005). This semi-synthetic promoter construct can be used to clone and express gene products that are toxic to *E. coli*, such as bovine enterokinase. This promoter has increasingly been used for recombinant protein production in *K. lactis* due to the desirable characteristics discussed above and its commercialization in the form of a *K. lactis* protein expression kit currently marketed by New England Biolabs (NEB, Accessed 08/21/2015).

The most common *P. pastoris* promoter is derived from the strong, inducible alcohol oxidase 1 gene (pAOXI) (Tschopp et al., 1987). Several semi-rational promoter engineering efforts have been used to improve this element. For example, by using *in silico* transcription factor (TF) binding site prediction, researchers were able to identify seven cis-acting elements that contribute to the tight glucose repression and strong methanol induction of pAOXI (Hartner et al., 2008). Following this, a panel of pAOXI mutants was assembled that had broad activity (ranging from 6% to 160% activity of wild type pAOXI) and varied regulation, including a glucose-repressed variant that no longer requires methanol for induction (Hartner et al., 2008). The utility of these promoter variants was demonstrated by a 1.3-fold increase in recombinant porcine trypsinogen activity relative to wild-type (WT) pAOXI (Ruth et al., 2010). Moreover, the methanol-independent promoter variant has potential bioprocessing advantages, including the elimination of flammable methanol and avoidance of costs associated with increased cooling-

and oxygen-demand from large scale methanol fed-batch processes (Jungo et al., 2007). Promoter engineering through random mutagenesis has been attempted in this organism as well. In one study, a random mutagenesis library of *pAOX1* resulted in promoter variants with significantly reduced glucose-repression and up to 2-fold increased activity over WT *pAOX1* based on a luciferase reporter assay (Berg et al., 2013). In another study, the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (*pGAP*) was used as a scaffold and resulted in a set of promoters with a range of activity from 0.01 to 19.6-fold of WT *pGAP* activity based on GFP fluorescence intensity (Qin et al., 2011). Finally, a series of synthetic promoters have been explored in this host. Synthetic hybrid promoters generated by fusing cis-acting elements with a minimal truncated *AOX1* core showed a reduced size (Hartner et al., 2008), but only 10% activity compared to that of WT *pAOX1*. In another report, the development of a synthetic core promoter linked with the upstream region of *pAOX1* resulted in a range of expression (from 10% to 117% of full-length *pAOX1*) along with retained glucose-repression and methanol induction (Vogl et al., 2014). In aggregate, many promoter engineering approaches have been attempted in this non-conventional yeast.

Finally, the host *Y. lipolytica* has been the subject of several promoter engineering efforts, beginning with the strong *XPR2* alkaline extracellular protease promoter (*pXPR2*). *pXPR2* has historically been used to express a variety of heterologous proteins, but its functional use is limited to pH above 5.5-6.0 and requires high concentrations of peptones for full induction (Barth, 2013; Madzak et al., 1999; Ogrydziak et al., 1977). Deletion analysis of the upstream regions of *pXPR2* revealed two UAS that resulted in a significant reduction in promoter activity upon simultaneous deletion (Blanchin-Roland et al., 1994). However, complex regulation was still observed in this UAS-deleted *pXPR2*. Subsequent functional analysis identified a sequence

(UAS1B) that enhanced promoter activity and was comparably insensitive to the complex regulation displayed by pXPR2 (Madzak et al., 1999). Insertion of 1-4 direct repeats of UAS1B upstream of a minimal *LEU2* promoter (Leum) yielded a series of semi-synthetic hybrid promoters with increasing strength, as measured by beta-galactosidase (LacZ) activity (Madzak et al., 2000). The constructs with 4 direct repeats (hp4d) or inverted repeats (hp4i) displayed activities similar to fully induced pXPR2 without requiring peptones, a strict pH range, or the omission of preferred carbon and nitrogen sources from the media. Synthetic removal of these constraints greatly simplified the expression of recombinant proteins in *Y. lipolytica*. Next, an extended series of UAS1B(n)-Leum hybrid promoters was developed (Blazeck et al., 2011) to further demonstrate the utility of hybrid promoters. Substantial increases in LacZ activity, hrGFP fluorescence, and mRNA levels were observed over the range of 8 to 32 copies of UAS1B, and no growth defects were detected. Maximum strength was observed with 24-28 copies, and the whole series spanned an mRNA dynamic range of approximately 400-fold without signs of TF pool depletion. Similar and proportional increases in expression strength were also demonstrated using 8 or 16 copies of UAS1B coupled to the constitutive *TEF1* promoter (pTEF1). These results suggest that even relatively strong WT *Y. lipolytica* promoters such as pTEF1 are enhancer-element limited, and that UAS elements can serve as modular transcriptional amplifiers of native promoters. A follow-up study then established similarly strong promoters in a reduced sequence space by combining several alternative UAS elements with a shortened UAS1B-based scaffold (Blazeck et al., 2012b). Ultimately, these hybrid promoters are the strongest constitutive promoters currently available for protein expression in *Y. lipolytica*.

Terminators

Transcriptional terminators serve a mechanistic role in transcription (i.e. modulating disengagement of the transcriptional machinery), but also influence mRNA stability (Geisberg et al., 2014; Mischo and Proudfoot, 2013). However, the impact of terminators on mRNA abundance and protein output is often underappreciated relative to promoters. Most commonly used *S. cerevisiae* expression vectors use a small set of previously identified, non-optimal native terminators such as *CYC1t* or *ADH1t* (Mumberg et al., 1995). However, these terminators are often longer than necessary and may not provide optimal mRNA half-life. To address these limitations in *S. cerevisiae*, extensive surveys of native terminators have been conducted (Curran et al., 2013; Yamanishi et al., 2013). More recently, a series of short, fully synthetic terminators was found to be capable of increasing fluorescent protein production by up to 3.7-fold and mRNA levels by up to 4.4-fold over *S. cerevisiae* *CYC1t* (Curran et al., 2015). These synthetic sequences were fully distinct from endogenous terminators and ranged from 35-75 base pairs long, which enables assembly via short and inexpensive oligonucleotides. As these highly useful tools are just emerging in *S. cerevisiae*, it is not surprising that the area is still fertile for non-conventional yeasts.

H. polymorpha expression cassettes commonly include the endogenous methanol oxidase terminator (*MOXt*), amine oxidase terminator (*AMOt*) (Saraya et al., 2012) or heterologous *S. cerevisiae* terminators such as *ADH1t* (Cox et al., 2000). *K. lactis* vectors frequently make use of the endogenous beta-galactosidase terminator (*LAC4t*), while *S. cerevisiae* terminators such as *GPD1t*, *ADH1t*, *ADH2t* are also functional and commonly used (Rosa et al., 2013). The endogenous *AOX1* terminator is generally used in *P. pastoris* (Sreekrishna et al., 1997) expression cassettes, although *S. cerevisiae* terminators such as *CYC1t* can also be used

(Tschopp et al., 1987). *S. cerevisiae* *CYCIt* has also been used in *Y. lipolytica* (Blazeck et al., 2011). The functionality of *S. cerevisiae* terminators across all four of these non-conventional yeast hosts suggests a high degree of transferability for this synthetic part across yeast species and strains. This fact bodes well for quickly expanding the synthetic terminator toolbox, but few studies have systematically compared terminator performance across yeast species.

Additionally, the reliance on *CYCIt* for most of these hosts points toward an immediate area for improvement as this terminator performs poorly (with respect to mRNA half-life) relative to many of the higher-capacity terminators studied in *S. cerevisiae* (Curran et al., 2013).

To demonstrate transferability of synthetic terminators across yeast strains, a subset of the previously referenced short synthetic *S. cerevisiae* terminators developed by Curran *et al* were evaluated in *Y. lipolytica* (Curran et al., 2015). In this experiment, up to 60% improvements in GFP fluorescence over wild-type *CYCIt* were achieved in plasmid expression systems. While it remains unclear why the observed improvement in fluorescent protein and mRNA output was substantially higher in *S. cerevisiae* relative to *Y. lipolytica*, the consistent rank order and general transferability of these synthetic parts is encouraging. Nevertheless, there may still be some unidentified organism-specific factors involved in termination that influence the ultimate performance of these parts. Regardless, it appears that synthetic terminator advances achieved in *S. cerevisiae* can be rapidly imported into other non-conventional yeasts with some likelihood for success.

Vector Replication Elements

Episomal plasmids provide a convenient test bed for genetic constructs by serving as a quick, intermediate proof of concept step between initial prototype construction and full

chromosomal integration. Moreover, a replicative vector can provide additional workflow flexibility to an expression system. Transformation efficiencies for plasmids tend to be much higher than for genomic integration, and plasmids often can afford a higher copy number depending on the selection marker used (Karim et al., 2013; Orr-Weaver et al., 1981). *S. cerevisiae* plasmids containing a centromere sequence (CEN) and autonomously replicating sequence (ARS) are often referred to as CEN/ARS vectors and are stably maintained at low copy numbers (Clarke and Carbon, 1980; Sikorski and Hieter, 1989). Vectors based on the native *S. cerevisiae* 2 μ circular plasmid are maintained at significantly higher copy numbers (Broach and Hicks, 1980). Both types of replicating vectors can be stably propagated (provided selection pressure is maintained) and have been used for both metabolic engineering and recombinant protein production in *S. cerevisiae*. In non-conventional yeasts, these elements are not as broadly reliable and available (or even as well understood mechanistically), although replicative vectors have been described for all four systems.

H. polymorpha plasmids containing ARS elements have been isolated and demonstrate high transformation efficiency and episomal replication (Bogdanova et al., 1995). One of these ARS is a heterologous sequence harbored within the *S. cerevisiae* *LEU2* marker (Faber et al., 1992). However, none of these extrachromosomally replicating plasmids are stable enough for general use in an industrial expression system (Saraya et al., 2012) due to frequent loss and/or chromosomal integration.

Plasmids containing the commonly used *S. cerevisiae* 2 μ elements are capable of replicating in *K. lactis*, but are much less stable than in *S. cerevisiae* and expression is frequently lost over relatively few generations. Incorporation of a heterologous stabilizing element from the *Kluyveromyces drosophilarum* plasmid pKD1 can significantly improve this instability (Bianchi

et al., 1987). While this advance has enabled broader use of plasmids in *K. lactis*, genomic integration is still preferred due to remaining plasmid instability and relatively frequent marker integration from plasmids in strains that express Ku80 (Iwata et al., 2004; Swinkels et al., 1993).

Multiple ARS elements have been identified for use in *P. pastoris* that support high transformation efficiencies and autonomous replication (Cregg et al., 1985). While vectors based on these ARS elements are stable enough to be used for screening gene libraries (Lee et al., 2005), long-term strain stability also typically requires chromosomal integration of a linearized expression cassette (Cereghino and Cregg, 2000).

Likewise, plasmid maintenance and expression is rather poor in the host *Y. lipolytica*, with plasmids often showing bimodal expression profiles and very low copy numbers. To improve these vectors, the synthetic fusion of various promoters upstream of a centromeric region increased copy number, GFP fluorescence, and LacZ activity by up to 80% (Liu et al., 2014) in an expression cassette independent manner. While autonomously replicating and episomal vectors have been described for all four of these non-conventional yeasts, the preferred mode of delivering a stable, highly expressed cassette in all of these hosts is still chromosomal integration. This reality also necessitates a robust suite of molecular genetic and genome editing tools, as discussed in the next section.

THE EXPANDING FRONTIER OF GENOME EDITING METHODS

Targeted Recombination

As discussed previously, NHEJ facilitates frequent, random integration of exogenous DNA in most non-conventional yeasts. In contrast, most industrial strain engineering efforts require the construction of a metabolic pathway or a desirable strain background using well-

controlled integrations, disruptions, and excisions. The canonical approach of sequential integration of cassettes followed by marker recycling has historically been more difficult in non-conventional yeasts. The original tools and cassette designs for facilitating this process use either heterologous site-specific recombinases or native, cellular homologous recombination directed by long flanking homology arms. In some cases, accessory nucleases may be used to induce double-strand breaks (DSBs) that stimulate and direct native HR at the desired locus (Krogh and Symington, 2004), but this step is not always required. When prokaryotic or synthetic nucleases are utilized in yeast, it is essential to validate that the protein is properly localized to the nucleus for cleavage of the genomic DNA. The heterologous nuclear localization signal (NLS) from the Simian Virus 40 T antigen (commonly referred to as the SV40 NLS) has long been used for this purpose in *S. cerevisiae* (Benton et al., 1990), and a toolbox of ten endogenous and heterologous NLS sequences was recently curated and characterized for use in *P. pastoris* (Weninger et al., 2015).

A variety of heterologous recombinases have been explored across host systems, with one of the more historically popular choices being Cre-*lox* from P1 bacteriophage (Sternberg and Hamilton, 1981). The Cre-*lox* recombination system has been used in all yeast expression systems discussed in this review, including *S. cerevisiae* (Sauer, 1987) and the non-conventional yeasts *H. polymorpha* (Krappmann et al., 2000), *K. lactis* (Steensma and Ter Linde, 2001), *P. pastoris* (Marx et al., 2008), and *Y. lipolytica* (Fickers et al., 2003), often for selection marker recycling. The general Cre-*lox* system has been covered extensively elsewhere (Hoess and Abremski, 1990), so this article will focus only on more recent advances in yeasts, such as self-excising Cre-*lox* cassettes (Figure 2A). Design of a self-excising Cre-*lox* module in *P. pastoris* has been achieved using the inducible pAOXI to drive tightly-regulated Cre expression (Pan et

al., 2011). After introducing a *lox71*-p*AOX1*-Cre-ZeoR-*lox66* cassette, double crossover HR transformants were selected on Zeocin plates. Addition of methanol subsequently induced Cre expression, causing recombination between the *lox71* and *lox66* sites. This site-specific recombination event yielded a double mutant *lox72* site at the target locus and resulted in the removal of the p*AOX1*-Cre-ZeoR fragment. Since this mutant *lox72* site is minimally recognized by Cre recombinase (Albert et al., 1995; Arakawa et al., 2001), retargeting by subsequent rounds of Cre expression is mitigated. However, the successive accumulation of *lox72* scar sites across the genome may still occasionally be subject to HR-driven rearrangements and deletions, as even a 33 base pair homology tract can occasionally trigger HR in a *P. pastoris* cassette (Yang et al., 2009).

HR methods are often expedited through the use of counter-selectable enzyme systems such as *URA3/5*-fluoroorotic acid or *TRP1/5*-fluoroanthranilic acid. These modules are frequently referred to as blasters or pop/loop-outs and have long been used in fungal genetics (Alani et al., 1987). A functional URA blaster has been demonstrated in *P. pastoris* (Nett and Gerngross, 2003), and a TRP blaster has been used in *Y. lipolytica* (Cheon et al., 2003). A TRP blaster has also been constructed for use in *H. polymorpha* (Cheon et al., 2009), but was ineffective at counterselection based cassette excision. A blaster or pop-out sequence is typically constructed by inserting a counterselection marker into a direct repeat that is flanked by homologous upstream and downstream regions for targeting HR. The direct repeats can be based on a coding sequence such as *lacZ* (Nett and Gerngross, 2003), but they are often a UTR sequence (Cheon et al., 2003) or a common terminator such as *CYC1t*. After initial integration into the genome and selection on dropout plates, the cognate antimetabolite is then added to select for cells that have undergone HR and marker gene excision. Reliance on classical

counterselectable markers often requires many serial rounds of integration and excision due to the small number of available enzyme-antimetabolite substrate pairs. These biosynthetic counterselectable markers also depend on the availability or derivation of an auxotrophic strain, which further complicates their use in industrial strain engineering where auxotrophy may not be desirable.

In systems with very tightly regulated promoters, more sophisticated and broadly applicable synthetic systems can be imported. For example, a toxic protein such as *E. coli* MazF can be used to select for single crossover events resulting in loss of an integrated cassette under inducing conditions (Yang et al., 2009). In *P. pastoris*, integration of a MazF-based cassette (comprised of *CYCIt*-*pAOXI-mazF-AOXIt*-*ZeoR-CYCIt*) was achieved using Zeocin selection in the absence of methanol. Subsequent growth on methanol-containing medium induced MazF production and allowed for the isolation of colonies that had undergone HR to remove the *pAOXI-mazF-AOXIt-ZeoR-CYCIt* fragment. As with the *Cre-lox* based approach, the *CYCIt* scar left over in this approach could lead to long term recombination-based instabilities within the strain. Importantly, the MazF and *Cre-lox* schemes can be applied in prototrophic strains and thus require less manipulation of the production strain genome outside of the target locus.

To reduce the impact of genomic scars, a variation has been tried in *H. polymorpha* to allow for scarless editing by placing MazF production under control of the methanol-inducible *MOX* promoter (*pMOX*) (Song et al., 2015). In this case, a *pMOX-mazF-ZeoR* cassette was flanked by two identical sequence blocks containing homology to the region upstream (US) of the target locus. At the terminal 3' end, a flanking arm homologous to the region downstream (DS) of the target locus was added to target the entire linear fragment for initial HR-mediated integration. Sequential steps of selection on Zeocin followed by induction with methanol

resulted in HR events that fused the selected US homology region directly to the DS one, thus creating a scarless integration event (Figure 2B). A similar scarless technique has also been utilized in *K. lactis* by combining the AmdS (acetamidase) marker with fluoroacetamide counterselection (Van Ooyen et al., 2006). In this approach, a tightly regulated promoter was not required. While each of these scarless approaches requires relatively large homology blocks for use in non-conventional yeast, advances in seamless multi-fragment cloning such as Gibson assembly (Gibson et al., 2009) can help to significantly reduce the burden of creating these constructs for multiple target loci.

CRISPR-Cas

The field of synthetic biology and genome editing has recently undergone a transformation with the heterologous implementation of the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR-Cas) system across a multitude of host organisms (Cong et al., 2013; Jinek et al., 2012). In *S. cerevisiae* (DiCarlo et al., 2013), this approach has greatly facilitated genome editing by enabling easily programmable, multiplex induction of DSBs. Since the initial demonstration in yeast, an increasing number of simultaneous, multiplexed integrations and deletions have been achieved with relatively short timelines and high frequencies of success (Bao et al., 2015; Mans et al., 2015). The advantages of CRISPR-Cas have recently been extended to an industrially relevant *K. lactis* strain, where six genes were integrated at three targeted loci, thus enabling rapid pathway construction for the biopolymer precursor muconic acid (Horwitz et al., 2015). Significantly, this strain was constructed in less than one month using multiplexed markerless integrations. Traditional approaches within this prototrophic *K. lactis* strain would have been much more cumbersome due to the lack of

auxotrophic markers and resultant need for repeated dominant marker recycling. While triple unmarked integrations were only observed at a rate of 2.1%, the speed and ability to screen strains greatly reduced the design-built-test cycle for this non-conventional host. However, these unmarked integrations were facilitated by a previous deletion of *YKU80* to minimize NHEJ, suggesting that further improvements in targeting efficiency are still needed to enhance genome editing in wild-type or industrial strains of non-conventional yeasts.

While CRISPR-Cas systems such as this have rapidly become the most widely-adopted tool for inducing DSBs for the purpose of genome editing, it is also possible to use engineered meganucleases (Epinat et al., 2003), zinc-finger nucleases (ZFNs) (Doyon et al., 2011), or TAL effector nucleases (TALENs) (Li et al., 2011) in yeast. While construction of these programmable nucleases is typically more difficult and costly than the equivalent CRISPR-Cas solution, the lack of extensive genome editing tools in non-conventional yeast systems may yield opportunities where these tools can still be beneficial. In contrast to CRISPR, the target sequence for engineered nucleases is programmed by directly altering the amino acid sequence of the DNA-binding domain (Carroll, 2011). If *in vivo* production and processing of sequence-specific RNA is limiting, then protein encoded recognition may enable more effective genome editing after a suitable nuclease has been engineered. For example, the implementation of CRISPR-Cas9 in the fission yeast *Schizosaccharomyces pombe* required the development of a novel promoter/leader-ribozyme method to produce mature guide RNA for targeting (Jacobs et al., 2014). The CRISPR system can now be utilized in *S. pombe*, but this host-specific solution was not obvious, thus suggesting a potential role for RNA-independent genome editing methods in scenarios where RNA expression tools are not well-developed or understood. Regardless of the nuclease used, the ability to induce targeted DSBs with relatively high specificity for genome

editing has the potential to significantly reduce barriers to efficient strain engineering in non-conventional yeast systems.

FUTURE OUTLOOK

Non-conventional yeast systems require an expanded synthetic biology toolbox to compete with more established hosts as production platforms. For example, highly controllable and tunable expression cassettes must be developed and implemented more widely. The *H. polymorpha*, *K. lactis*, and *P. pastoris* systems already have tightly repressed, strongly induced promoters available, but *Y. lipolytica* lags slightly behind in this regard. In this host, several weak and moderate strength promoters are inducible and the FBA1-IN promoter is relatively strongly induced by glucose (Hong et al., 2012), but a promoter with tight repression and induction performance comparable to *H. polymorpha* pMOX, *K. lactis* pLAC4, *P. pastoris* pAOX1, or *S. cerevisiae* pGAL1 would be a highly desirable development. A synthetic combination of enhancing UAS and regulatory/repressor elements may be capable of achieving this objective. While tunable, hybrid multi-copy UAS promoters have been shown to be relatively stable in *Y. lipolytica* (Blazeck et al., 2011), their repeat-based design is fundamentally susceptible to recombination events. Targeted recombination events are much less common in WT *Y. lipolytica* than in *S. cerevisiae* due to a preference for NHEJ instead of HR, but interventions affecting the DNA repair and recombination pathways (e.g., deletion of Ku70/80) may adversely affect the stability of repeat-based promoters. To this end, the development of synthetic promoters such as those available in *S. cerevisiae* would be preferred for reducing homology while increasing the diversity and orthogonality of available promoter sequences in all of the non-conventional yeasts (Curran et al., 2014; Redden and Alper, 2015).

In addition to the development of parts that allow for programmable expression of a gene, pathway, or circuit of interest, synthetic biology and metabolic engineering efforts in non-conventional yeast systems could also greatly benefit from the design or adaptation of tools for the perturbation of endogenous gene expression. For example, RNA interference (RNAi) has yet to be implemented in the non-conventional yeasts covered in this review, despite successes in importing a synthetic RNAi system in *S. cerevisiae* through heterologous expression of either the *Saccharomyces castelli* Argonaute and Dicer proteins (Drinnenberg et al., 2009) or human machinery (Suk et al., 2011). Optimization of RNAi in *S. cerevisiae* has already enabled its use as an additional tool for rapid biological prototyping for metabolic engineering (Crook et al., 2014). Likewise, a similar approach via CRISPR interference (Larson et al., 2013) shows promise as a complementary tool that can be readily imported into these non-conventional host systems once the CRISPR-Cas system is demonstrated. Extension of these perturbation methods to non-conventional yeast systems would enable more rapid testing of deletion targets for strain engineering, especially when HR-mediated genetic manipulations remain slow and laborious for many strains.

Markerless, multiplexed integration and deletion has begun to be realized using CRISPR-Cas in the non-conventional yeast *K. lactis* (Horwitz et al., 2015), the fission yeast *Schizosaccharomyces pombe* (Jacobs et al., 2014), and the pathogenic yeast *Candida albicans* (Vyas et al., 2015). The widespread use of this approach in many diverse organisms (Hsu et al., 2014) provides promise for other non-conventional yeast expression platforms and could obviate the need for cumbersome auxotrophic markers (van den Berg and Maruthachalam, 2014) and antibiotics (Herrero et al., 1990). The precise and efficient generation of DSBs using CRISPR-

Cas may also help to alleviate some of the challenges stemming from inefficient HR, potentially without resorting to deletion of the Ku proteins involved in non-homologous DNA repair.

However, one challenge with CRISPR-Cas portability has been the production of sufficient quantities of properly processed guide RNA (gRNA) for targeting. In most organisms, including *S. cerevisiae* (DiCarlo et al., 2013), gRNA expression is accomplished using RNA polymerase III (RNAP III) promoters. However, RNAP III promoters are not as well-characterized as RNAP II promoters, especially for non-conventional hosts. As a result, the development of effective CRISPR-Cas systems for non-conventional yeasts may require optimization of a heterologous RNAP III promoter (e.g., pSNR52 from *S. cerevisiae*) or the screening of endogenous RNAP III promoters. Alternatively, self-cleaving ribozymes could be used to process a RNAP II transcribed mRNA and generate mature gRNA (Gao and Zhao, 2014). Once the CRISPR-Cas technology is implemented more widely in the non-conventional yeasts, it should immediately become possible to substitute a catalytically inactive (also called ‘dead’, or ‘nuclease- null’) dCas9 into the system to establish similar transcriptional control strategies, circuits, and networks to those that are being explored in *S. cerevisiae* (Zalatan et al., 2015).

Finally, although some internal ribosomal entry sites (IRES) are claimed to function in yeast (Edwards and Wandless, 2010; Liang et al., 2012; Thompson et al., 2001), efficient viral IRES sequences commonly used in mammalian expression vectors are not functional in *S. cerevisiae* (Thompson et al., 2001) and multicistronic IRES cassettes are rarely used in yeast systems. Alternatively, co-translationally cleaved 2A sites based on viral peptide sequences have been used to design multicistronic vectors for many eukaryotic expression systems (Kim et al., 2011; Szymczak and Vignali, 2005), including *S. cerevisiae* (Beekwilder et al., 2014; de Felipe

et al., 2003; Park et al., 2008; Unkles et al., 2014) and the non-conventional yeast *P. pastoris* (Geier et al., 2015). The conserved mechanism of 2A cleavage at the ribosome suggests the potential for diversification of expression cassette designs in non-conventional yeasts. Such an approach can be useful for hosts that still lack a diverse set of tunable promoter sequences (i.e. *H. polymorpha*, *K. lactis*, *Y. lipolytica*).

CONCLUSION

The field of synthetic biology is growing rapidly, especially in model host systems such as *Saccharomyces cerevisiae*. This growth has led to the development of many intricate and highly engineered pathways, circuits, and control schemes, and it holds enormous potential for promising non-conventional yeasts such as *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Yarrowia lipolytica*. As improved synthetic vector and cassette components such as promoters, terminators, and replication elements become increasingly available for these systems, complex rewiring and engineering efforts will become more feasible and efficient. Improved genome editing methods for targeted integrations are already enabling researchers to bypass some of the genetic challenges associated with NHEJ-dominated DNA repair pathways in these organisms. In addition, many recently developed and powerful synthetic biology tools have not yet been imported into these host systems or have not yet been exploited to their full potential. In the near future, these tools will be used to overcome the remaining native limitations of non-conventional yeasts and enable engineering strategies that have previously been reserved for use in tractable model systems. This ultimately will enable industrial biotechnology to fully leverage the innate advantages of these organisms for the economical production of small molecules and proteins.

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Table and Figure Legends

Table 1

Examples of industrially relevant products made using non-conventional yeast systems. A wide variety of industrial biotechnology and biopharmaceutical products are either currently manufactured in non-conventional yeast hosts or have reached production levels that may allow for industrial production in the near future. A set of selected examples are presented for each of the four non-conventional yeast species covered by this review.

Figure 1

Summary of basic characteristics of the non-conventional yeasts addressed by this review.

A selection of notable or advantageous similarities and divergences between the yeast hosts covered by this review are presented, along with an image of each species. Images have been reprinted with permission from the publishers: *H. polymorpha* (Gellissen et al., 2005), *K. lactis* (Iyer et al., 2005), *P. pastoris* (Krainer et al., 2013), and *Y. lipolytica* (Dulermo et al., 2014).

*Indicates phylogenetic similarity.

Figure 2

Diagram of synthetic marker excision approaches using tightly regulated inducible promoters.

A) After HR-mediated integration of a Cre-containing disruption cassette and dominant selection using Zeocin, the addition of methanol induces Cre expression from *pAOX1*. Cre promotes site-specific recombination between *lox71* and *lox66*, excising the Cre and ZeoR cassettes and leaving an inactive *lox72* scar at the disruption locus.

B) After HR-mediated integration of a MazF-containing disruption cassette and dominant selection using Zeocin, the addition of methanol induces MazF expression from *pMOX*. Cells that have undergone HR between the 5' US repeats have excised the MazF cassette and survive, while high level MazF expression is lethal to cells that still contain the disruption cassette. Note that the initial double-crossover integration of the disruption cassette may be accomplished using a 2-fragment / split marker approach (not shown in diagram).

Tables

Table 1.

Host	Product	Product Type	Product Use	Reference
<i>H. polymorpha</i>	Hepatitis B Surface Antigen (HBsAg)	Protein/Peptide	Vaccine (Hepatitis B Virus)	(Janowicz et al., 1991), (Kulkarni et al., 2006)
<i>H. polymorpha</i>	Hirudin	Protein/Peptide	Biopharmaceutical (Anticoagulant)	(Weydemann et al., 1995)
<i>H. polymorpha</i>	Insulin-like Growth Factor 1 (IGF-1)	Protein/Peptide	Biopharmaceutical (Growth hormone)	(Brierley et al., 1997)
<i>H. polymorpha</i>	Phytase	Enzyme	Food/Beverage Additive, Agricultural Supplement	(Mayer et al., 1999)
<i>H. polymorpha</i>	Hexose Oxidase	Enzyme	Food/Beverage Additive	(Fraatz et al.,

			(Bread dough modifier)	2014)
<i>H. polymorpha</i>	Triacylglycerol Lipase	Enzyme	Food/Beverage Additive	(Fraatz et al., 2014)
<i>K. lactis</i>	Chymosin	Enzyme	Food/Beverage Additive (Rennet component)	(Van den Berg et al., 1990)
<i>K. lactis</i>	Lactase	Enzyme	Food/Beverage Additive (Lactose depletion)	(Van Ooyen et al., 2006)
<i>K. lactis</i>	Glycolic Acid	Organic Acid	Renewable Chemical Precursor	(Bianchi et al., 2001)
<i>K. lactis</i>	Lactic Acid	Organic Acid	Food/Beverage Additive, Renewable Chemical Precursor	(Bianchi et al., 2001)
<i>P. pastoris</i>	Ecallantide	Protein/Peptide	Biopharmaceutical (Hereditary angioedema)	(Markland et al., 1996), (Cicardi et al., 2010)
<i>P. pastoris</i>	Insulin Glargine	Protein/Peptide	Biopharmaceutical (Insulin analog)	(Kannan et al., 2009)
<i>P. pastoris</i>	Phospholipase C	Enzyme	Plant Oil Processing	(Farr and Proctor, 2013)
<i>Y. lipolytica</i>	Citric Acid	Organic Acid	Food/Beverage Additive, Renewable Chemical Precursor	(Forster et al., 2007)
<i>Y. lipolytica</i>	Erythritol	Sugar Alcohol	Food/Beverage Additive	(Mironczuk et al., 2014)
<i>Y. lipolytica</i>	α -Ketoglutaric Acid	Organic Acid	Renewable Chemical Precursor	(Yovkova et al., 2014)
<i>Y. lipolytica</i>	Lipids (Triacylglycerol)	Fatty Acid	Renewable Chemical Precursor	(Blazek et al., 2014a), (Tai and Stephanopoulos, 2013)
<i>Y. lipolytica</i>	Lycopene	Carotenoid	Food/Beverage Additive, Supplement	(Matthäus et al., 2014)
<i>Y. lipolytica</i>	Omega-3 Eicosapentaenoic Acid	Fatty Acid	Biopharmaceutical, Supplement	(Xue et al., 2013)

Figure 1

Figure 2