MINIREVIEW

What makes *Komagataella phaffii* non-conventional?

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One sentence summary: Non-conventional features render the yeast *K. phaffii* an attractive model organism and an efficient host for biotechnology applications.

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ABSTRACT

The important industrial protein production host *Komagataella phaffii* (syn Pichia pastoris) is classified as a non-conventional yeast. But what exactly makes *K. phaffii* non-conventional? In this review, we set out to address the main differences to the ‘conventional’ yeast *Saccharomyces cerevisiae*, but also pinpoint differences to other non-conventional yeasts used in biotechnology. Apart from its methylotrophic lifestyle, *K. phaffii* is a Crabtree-negative yeast species. But even within the methylotrophs, *K. phaffii* possesses distinct regulatory features such as glycerol-repression of the methanol-utilization pathway or the lack of nitrate assimilation. Rewiring of the transcriptional networks regulating carbon (and nitrogen) source utilization clearly contributes to our understanding of genetic events occurring during evolution of yeast species. The mechanisms of mating-type switching and the triggers of morphogenic phenotypes represent further examples for how *K. phaffii* is distinguished from the model yeast *S. cerevisiae*. With respect to heterologous protein production, *K. phaffii* features high secretory capacity but secretes only low amounts of endogenous proteins. Different to *S. cerevisiae*, the Golgi apparatus of *K. phaffii* is stacked like in mammals. While it is tempting to speculate that Golgi architecture is correlated to the high secretion levels or the different N-glycan structures observed in *K. phaffii*, there is recent evidence against this. We
conclude that K. phaffii is a yeast with unique features that has a lot of potential to explore both fundamental research questions and industrial applications.

**Keywords:** Komagataella phaffii; non-conventional yeast; biotechnology; protein production; carbon metabolism; methylotrophy

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**INTRODUCTION**

During the course of evolution, many different lifestyles emerged among yeasts. Thanks to their diversity, the world is surrounded by distinct yeast species inhabiting many different environments, metabolizing several carbon sources and producing a variety of metabolites (Kurtzman, Fell and Boekhout 2011). Komagataella phaffii (formerly known as Pichia pastoris) is among the Ascomycota yeasts from the Saccharomycetes class (Heitinger, Gasser and Mattanovich 2020). Approximately, 5200 genes are encoded on four rather large chromosomes, with a total genome size of 9.4 Mb. Komagataella phaffii is a methylotrophic yeast that can utilize methanol as the sole carbon and energy source. Besides methanol, it can grow on a number of carbon sources including glucose, glycerol, ethanol, trehalose, L-rhamnose, mannitol, sorbitol, D-glucitol, lactic acid, succinic acid, acetic acid and citric acid (varies among different strains; Sreekrishna et al. 1997; Kurtzman, Fell and Boekhout 2011; Sahu and Rangarajan 2016).

Since the 1990s, K. phaffii is among the preferred hosts for recombinant protein production, and more recently, K. phaffii has also been employed for non-protein products (Peña et al. 2018; Werten et al. 2019; Zhu et al. 2019; Karbalaei, Rezaee and Farsiani 2020; Duman-Özdamar and Binay 2021; Gao, Jiang and Lian 2021). Furthermore, K. phaffii serves as a model organism in biomedical research and basic cell biology (Bernauer et al. 2020). Its attractiveness as a biotechnological host and model organism is strongly connected to features that distinguish K. phaffii from other yeasts and classify it as a non-conventional yeast.

**METHYLOTROPHY**

The most obvious non-conventional feature of K. phaffii is its ability to metabolize methanol. Recent phylogenetic analyses of the budding yeasts show that all methylotrophic yeasts cluster in one clade (Shen et al. 2018), indicating that methylotrophy has probably evolved only once. The first enzymatic step of methanol utilization (MUT), the oxidation of methanol to formaldehyde, is catalyzed by an alcohol oxidase (Aox1/2). Sequence similarity proposes that this enzyme belongs to formaldehyde, is catalyzed by an alcohol oxidase (Aox1/2). Sequence similarity proposes that this enzyme belongs to formaldehyde, is catalyzed by an alcohol oxidase (Aox1/2).

**CRABTREE PHENOTYPE AND DIFFERENT PATHWAYS FOR CARBON UTILIZATION**

Glucose is one of the carbon sources on which K. phaffii can readily grow and form biomass (Kurtzman, Fell and Boekhout 2011). However, glucose uptake is limited compared to Crabtree-positive yeasts such as Saccharomycoses cerevisiae \((q_{\text{max}} \approx 0.35–0.60 \text{ g} / \text{g} / \text{h})\) for K. phaffii and 2.88–2.16 \text{ g} / \text{g} / \text{h} for S. cerevisiae in aerobic cultures (Diderich et al. 1999; Otterstedt et al. 2004; Maurer et al. 2006; Ata et al. 2018). It is argued that the high number of hexose transporters might be one of the reasons for the superior glucose uptake metabolism of Crabtree-positive (respiro-fermenting) yeasts. Accordingly, S. cerevisiae possesses more than 15 hexose transporters which allows it to transport glucose at a high rate (Boles and Hollenberg 1997; Elbing et al. 2004). Eventually, the high glucose flux exceeds the respiration capacity and leads to overflow metabolism of glycolysis which results in reduced biomass yields and ethanol production even at aerobic conditions (Fig. 1). In contrast, K. phaffii has a reduced number of hexose transporters similar to other respiratory yeasts such as Klyveromyces lactis, Hansenula polymorpha.
Figure 1. Regulation of the Crabtree phenotype in yeasts. (Left) Crabtree negative yeasts have a limited glucose uptake capacity and exclusively ferment under aerobic conditions. (Middle) Due to the overflow metabolism, Crabtree positive yeasts have a respiro-fermentative metabolism which is controlled at the metabolite level. (Right) Overexpression of CRA1 upregulates the glycolytic genes leading to overflow metabolism, and demonstrating that glycolysis in K. phaffii is controlled at the transcriptional level.

(syn Ogataea polymorpha and Ogataea parapolymorpha) and Schef tersomyces (Pichia) stipitis (Mattanovich et al. 2009). It possesses one high affinity transporter (GTH1) and three HXT isozymes (HXT1, HXT2 and HXT400; Valli et al. 2016). This prevents the overflow metabolism of glycolysis so that K. phaffii exclusively performs respiration under fully aerobic conditions (Ata et al. 2018). Therefore, it is classified as canonical Crabtree-negative yeast. Additionally, the fraction of carbon entering the PPP is higher in K. phaffii, in contrast to S. cerevisiae where the main flux is through glycolysis (see below). Recently, a transcription factor (TF), CRA1 (a Gal4-like TF, homolog of ScGAL4) was identified to be controlling glycolysis and fermentation metabolism of K. phaffii (Ata et al. 2018). Constitutive overexpression of this TF resulted in increased glucose uptake and ethanol production rates, as well as upregulation of glycolytic genes, which switched the Crabtree-negative phenotype of K. phaffii to Crabtree-positive (Fig. 1). Interestingly, the hexose transporters were not regulated by CRA1 overexpression suggesting that hexose transporters are not responsible for the increased glycolytic flux in K. phaffii.

Komagataella phaffii cannot metabolize galactose but according to sequence homology it contains a GAL10 gene in addition to CRA1 (homolog of ScGAL4; Valli et al. 2016). In a comparative genomics analysis, it was shown that the ability of galactose utilization has been lost at least seven times during yeast evolution (Riley et al. 2016). It might be expected that a few GAL homologs in K. phaffii might have been inherited from an ancestral yeast that could consume galactose and eventually were lost during its evolution. The function of GAL10 in K. phaffii is not known, but the ScGAL4 homolog CRA1 is apparently controlling glycolysis in K. phaffii, in contrast to S. cerevisiae where Gal4 regulates the GAL genes (Ata et al. 2018). The whole genome duplication (WGD) marks a transition in the role of Gal4 where its function switched from a generalist TF controlling glycolysis to a specialist TF regulating galactose metabolism (Choudhury and Whittewy 2018). In yeasts which originate prior to this transition like Candida albicans, Debaryomyces Hansenii, or Schizosaccharomyces pombe, the Leloir pathway for galactose utilization is controlled by Cph1. In post-WGD yeasts, Cph1 was eventually lost, Mig1 and Gal4 (along with other regulatory proteins) have been recruited to co-regulate the Leloir pathway, while in pre-WGD yeasts like K. phaffii and C. albicans, Gal4 has a regulatory function in the central carbon metabolism (Marchenko, Levitin and Whittewy 2007; Askew et al. 2009; Ata et al. 2018).

There is a high diversity among different yeast species on the ability of growing on glycerol as the sole carbon source (Kurtzman, Fell and Boekhout 2011). Unlike S. cerevisiae, K. phaffii can grow on glycerol in minimal media without requiring any additional supplements ($V_{\text{max}}$ of 0.26/h, $q_{\text{glycerol}}$ = 0.37 gs/gp/h; Jahic et al. 2002). Possession of four H$^+/$/glycerol symporters in addition to a Fps1-type glycerol facilitator demonstrates the superiority of the glycerol uptake metabolism compared to many other yeasts (Lages, Silva-Graca and Lucas 1999; Mattanovich et al. 2009). Of these four H$^+/$/glycerol transporters, GT1 (encoded by STL1-1, PP7435_Chr1-0321) was found to be one of the factors affecting the crosstalk between the glycerol and methanol metabolism in K. phaffii (Zhan et al. 2016). While the deletion of GT1 (STL1-1) did not cause a significant growth impairment in glycerol-based medium, it relieved glycerol repression on P\text{AOX1}. Additionally, overexpression of GT1 repressed MXR1 and AOX1 expression, whereas MXR1 overexpression repressed GT1 (Zhan et al. 2016, 2017; Li et al. 2018).

In addition to regulating the MUT pathway, glycerol metabolism in K. phaffii seems to have a global effect on NADPH balance. Metabolic flux analysis with $^{13}$C-labeled glycerol showed that the major cytosolic NADPH source might be the glycerol catabolic pathways (Tomás-Gamisans et al. 2019). In contrast to the previous assumption that the oxidative branch of the PPP is the main source for cytosolic NADPH, it was demonstrated that the flux through the PPP is almost negligible. Furthermore, it was hypothesized that, among the different glycerol catabolic pathways (Klein et al. 2017), the NADP-dependent glycerol oxidation pathway is the major cytosolic NADPH source in glycerol grown cultures. However, PPP seems to have a more profound effect on NADPH generation when the cells are grown on glucose: The split ratio of PPP flux is around 40–55% in K. phaffii (Baumann et al. 2010; Nocon et al. 2016; Ata et al. 2018) as opposed to S. cerevisiae which has a split ratio of 4–17% (Gombert et al. 2001; Maasheimo et al. 2001; Velagapudi et al. 2007).

L-rhamnose metabolism occurs in fungi via an oxidative (non-phosphorylated) pathway (Koivistoinen et al. 2012). A comparative genomics study showed that the genes of L-rhamnose
metabolism are clustered among the yeasts analysed in the study (Riley et al. 2016). It appears that K. phaffii has five genes (LRA1, LRA2, LRA3, LRA4 and TFC1) associated with L-rhamnose metabolism (Liu, Styles and Fink 2016; Valli et al. 2016). However, PP7435_Chr1-0845 (LRA3) does not cluster with the other genes, contrary to the gene arrangement in S. stipitis. Komagataella phaffii can grow on L-rhamnose as the sole carbon source with a slightly lower growth rate than on glucose and the expression of the related genes are induced by rhamnose and repressed in the presence of glucose (Liu, Styles and Fink 2016).

**GENE REGULATION AND TFs**

Methylo trophic yeasts offer a repertoire of regulated, strong promoters that are naturally regulating MUT pathway genes (Ergün et al. 2019b). Typically, the promoters of MUT pathway genes are tightly repressed on repressing carbon sources such as glucose and strongly induced when shifted to methanol. However, they demonstrate different modes of derepression among methylo trophic yeasts. The promoter of the K. phaffii AOX1 gene P_AOX1 is strongly induced by methanol while repressed by ethanol, glucose and glycerol (Ergün et al. 2020). Derepressed K. phaffii cells (repressing carbon source is depleted or non-repressing carbon source is present) display approximately 2% AOX1 transcriptional activity of the methanol-induced level, whereas methanol-induced cells display more than 1000-fold higher activity than fully repressed (glucose-grown) cells (Lin-Cereghino et al. 2006). In contrast, promoters of orthologous genes AOD (alcohol oxidase) in Candida boidinii, MOX (methanol oxidase) in H. polymorpha and MOD1 (methanol oxidase 1) in Pichia methanolica show respectively 3–30%, 60–70% and 60–70% derepression in glycerol compared to their methanol-induced levels (Hartner and Glieder 2006). Substantial amounts of heterologous protein were expressed by P_AOX1 in H. polymorpha on glycerol, and P_AOX1 was regulated in the same manner as P_AOX1 (Raschke et al. 1996). These findings suggest that rather than promoter cis-acting DNA elements, components of the cellular transcriptional machinery determine the expression mode of P_AOX1 on glycerol. Understanding the transcriptional regulation of P_AOX1 and other MUT pathway genes has paramount importance to enhance control over natural promoters and design new expression mechanisms in K. phaffii.

An overview of the characterized transcription factors (TFs) involved in regulation of carbon source utilization and their respective function in K. phaffii is given in Table 1 and Fig. 2.

S. cerevisiae Adr1 (alcohol dehydrogenase synthesis regulator) has a pivotal role in the activation of glucose repressible genes, peroxisomal protein genes and ethanol, glycerol and fatty acid utilization pathway genes (Young et al. 2003). Alcohol dehydrogenase Adh2 is the first enzyme of the ethanol utilization pathway that catalyzes oxidation of ethanol to acetaldehyde. Adr1 and Cat8 synergistically activate S. cerevisiae ADH2 transcription and many other ethanol utilization pathway genes (Young et al. 2003). The K. phaffii Adr1-homologue Mxr1 (methanol expression regulator 1) has gained new functions and lost others through evolution as a result of changes in the environmental conditions, cell physiology and spectrum of genes that it controls. Deletion of MXR1 caused total transcriptional shut down of K. phaffii P_AOX1 and cells cannot grow on methanol, while it had less detrimental effect on K. phaffii P_ADX2 activation and growth on ethanol compared to S. cerevisiae transcriptional regulation (Ergün 2018). MXR1 is constitutively expressed at low levels and activates MUT pathway and peroxisome biogenesis (PEX) genes (Lin-Cereghino et al. 2006). Mxr1 is cytoplasmic in glucose-grown cells but localized to the nucleus in cells cultured on gluconogenic substances (Lin-Cereghino et al. 2006). Similar to S. cerevisiae Adr1, the 14–3–3 protein directly interacts with Mxr1 by phosphorylation and inhibits its activity (Parua et al. 2012). The ethanol-repressible nature of P_AOX1 has been investigated through Mxr1, however, the answer came from a different side. Promoter engineering of P_AOX1 by introducing a Cat8 cis-acting DNA motif converted ethanol-repressible P_AOX1 to the ethanol inducible P_AOX1 suc1-1,2 Variant (Ergün et al. 2020). This demonstrates that the ethanol repressible nature of P_AOX1 is due to the absence of an ethanol responsive cis-acting element. Addition of further Cat8 cis-acting motifs enhanced K. phaffii P_ADH2 expression 4.8-fold on ethanol (Ergün et al. 2019a), while the K. phaffii Δcat8-1Δcat8-2 mutant lost its ability to grow on ethanol (Ergün 2018; Barbay et al. 2021), emphasizing the importance of the K. phaffii Cat8-1 and Cat8-2 TFs for ethanol regulation.

In the methylo trophic yeast C. boidinii Trm1 and Trm2 (transcriptional regulation of methanol induction) are essential TFs for the expression of MUT pathway and PEX genes (Sasano et al. 2008, 2010). Trm2 is the homologue of K. phaffii Mxr1 and responsible for the activation of methanol-inducible genes by relieving glucose repression, and also essential for Trm1-dependent gene activation (Sasano et al. 2010). Komagataella phaffii Trm1, the homologue of C. boidinii Trm1, is another positively acting TF of MUT pathway and PEX genes. The respective Δtrm1 mutant showed impaired P_AOX1 activity and growth on methanol (Sahu, Krishna Rao and Rangarajan 2014), which could be rescued by MTR1 overexpression (Wang et al. 2016b).

Mit1 (methanol-induced TF1) positively regulates MUT pathway genes, but not peroxisomal genes and activates P_AOX1 in response to methanol while it represses P_AOX1 transcription on glycerol (Wang et al. 2016b). Komagataella phaffii Δmit1 cannot grow on methanol and P_AOX1 is not active. Complementation of the Δmit1 mutant with H. polymorpha Mpp1 (methylotrophic peroxisomal protein) restored growth and P_AOX1 activity on methanol, and furthermore, lead to remarkable AOX1 expression levels on glycerol (Wang et al. 2016b). Structural differences between Mit1 and Mpp1 likely contribute to the differential expression mode of P_AOX1 and P_MOX.

Mxr1, Mit1 and Trm1 are binding to P_AOX1 at different sites and cooperatively activate P_AOX1, but overexpression of Mit1 or Trm1 does not restore P_AOX1 activity in the Δmxr1 mutant. Firstly, derepression of P_AOX1 is mediated by the master TF Mxr1, then Trm1 and Mit1 contribute to promoter activation (Wang et al. 2016b). TRM1 is expressed constitutively in glucose, glycerol and methanol, while MIT1 expression is strongly induced on methanol. Both Mit1 and Trm1 are localized to the nucleus in glucose, glycerol and methanol conditions. Mit1 binds to P_AOX1 in the presence of all three carbon sources, however, Trm1 only binds when cells are grown on methanol or glycerol but not on glucose (Wang et al. 2016b).

Rop1 (repressor of phosphoenolpyruvate carboxykinase; Kumar and Rangarajan 2012) and Nrg1 (Wang et al. 2016a) are negative regulators of K. phaffii MUT pathway and PEX genes. Rop1 and Mxr1 function antagonistically but exhibit the same DNA binding specificity whereby Rop1 binds to DNA with higher affinity than Mxr1 (Kumar and Rangarajan 2012). S. cerevisiae Nrg1 and Nrg2 (negative regulator of glucose-repressed genes) mediate glucose repression (Zhou and Winston 2001). K. phaffii Nrg1 represses MUT pathway and PEX genes in glucose and glycerol conditions. It directly binds to five positions on P_AOX1, two of which overlap with MXR1s (Wang et al. 2016a). The Δnrg1 mutant showed a growth defect when cultivated on glucose,
### Table 1. List of TFs that are experimentally confirmed to regulate carbon source utilization in *K. phaffii*.

| TF     | Uniprot ID* | Homologs in other yeasts | Function                                                                 | References                        |
|--------|-------------|--------------------------|--------------------------------------------------------------------------|-----------------------------------|
| Aft1   | F2QPE8.KOMPC|                          | Regulates genes of carbohydrate metabolism and recombinant protein secretion | Ruth et al. (2014)                 |
| Cat8-1 | F2QS26.KOMPC| *S. cerevisiae Cat8, K. lactis Cat8* | Activates glyoxylate cycle and EUT pathway in ethanol grown *K. phaffii*, required for growth on acetate | Barbay et al. (2021)              |
| Cat8-2 | F2QYX3.KOMPC| *S. cerevisiae Cat8/Sip4, K. lactis Cat8/Sip4* | Activates carnitine shuttle and EUT pathway in ethanol grown *K. phaffii* | Barbay et al. (2021)              |
| Cra1   | F2QF5.KOMPC | *S. cerevisiae Gal4*    | Controls glycolysis and fermentation metabolism                           | Ata et al. (2018)                 |
| Flo8   | F2QYE9.KOMPC| *S. cerevisiae Flo8*    | Master regulator of filamentous growth and surface adherence, also involved in glucose repression | Rebnegger et al. (2016)           |
| Mig1-1 | F2QZJ1.KOMPC| *S. cerevisiae Mig1, H. polymorpha Mig1* | Repressor of MUT pathway and PEX genes                                   | Wang et al. (2017); Shi et al. (2018) |
| Mig1-2 | F2QPW6.KOMPC| *S. cerevisiae Mig1, H. polymorpha Mig2* | Repressor of MUT pathway and PEX genes                                   | Wang et al. (2017); Shi et al. (2018) |
| Mit1   | F2QV89.KOMPC| *H. polymorpha Mpp1*   | Activator of MUT pathway but not PEX genes on methanol, represses *P*<sub>AOX1</sub> in response to glycerol | Wang et al. (2016b)               |
| Mxr1   | F2QZ27.KOMPC| *S. cerevisiae Adr1, C. boidinii Trm2* | Activator of MUT pathway and PEX genes                                   | Lin-Cereghino et al. (2006)       |
| Trm1   | F2QZY1.KOMPC| *C. boidinii Trm1*     | Activator of MUT pathway and PEX genes                                   | Sahu et al. (2014)                |
| Trc1   | F2QZI4.PICP7| *S. stipitis Trc1*     | TF suggested to be involved in the regulation of LRA genes of the rhamnose metabolism | Liu, Styles and Fink (2016)       |
| Nrg1   | F2QUX2.KOMPC| *S. cerevisiae Nrg1/2* | Repressor of MUT pathway and PEX genes                                   | Wang et al. (2016a)               |
| Rop1   | F2QW29.KOMPC|                          | Repressor of MUT pathway, PEX genes and phosphoenolpyruvate carboxykinase | Kumar and Rangarajan (2012)        |

*For some TF genes different annotations are used in literature. In order to avoid ambiguity, their UniProt IDs are provided.*
S. cerevisiae Mig1 and Mig2 are involved in glucose repression of different carbon metabolism genes (Schüller 2003). Komagataella phaffii Mig1-1 and Mig1-2 are mainly localized in the nucleus in glucose or glycerol conditions, while they translocate to the cytosol when cells are grown on methanol (Wang et al. 2017). Glycerol-induced suppression of P\textsubscript{AOX1} is partially removed in the Δmig1-1 mutant, while no effect is observed in the Δmig1-2 mutant. The double knock-out Δmig1-1Δmig1-2 mutant showed increased transcriptional activation (Wang et al. 2017), which seems to be mediated through activation of Mit1 (Shi et al. 2021). On the other hand, neither deletion of MIG1-1 nor MIG1-2 deregulated P\textsubscript{AOX1} on glucose. The double deletion of MIG1-1 and MIG1-2 led to a growth defect on glycerol and glucose (Shi et al. 2018).

**MATING AND MATING-TYPE SWITCHING**

*K. phaffii* is a preferentially haploid yeast usually propagating by mitotic cell division. Sexual reproduction (mating and spore formation) is possible but can only be observed under nitrogen starvation conditions (Feng et al. 2020). In contrast to *S. cerevisiae*, where haploid cells mate spontaneously to form stable diploids, the mating-type (MAT) genes and most other mating-relevant genes of *K. phaffii* are not expressed in rich medium (Heistinger, Gasser and Mattanovich 2018). Once mating has occurred, diploid *K. phaffii* cells rapidly undergo meiosis and sporulation if no selective pressure is applied. This coupling of mating and sporulation has also been observed in other yeasts like Candida lusitaniae or *K. lactis* but is absent in Saccharomyces species, where the lack of nitrogen and presence of a non-fermentable carbon source act as a trigger for sporulation of diploid cells (Booth, Tuch and Johnson 2010; Merlino, Dudin and Martin 2013; Sherwood et al. 2014; Hanson and Wolfe 2017).

Although the mating-type and mating behavior of yeasts is generally regulated by the MAT genes, there are significant differences in the mechanism of mating-type switching and the control of cell type regulations between different species. *K. phaffii* has a two-locus mating-type system, where both loci, containing either MATa1 and MATa2 or MATα1 and MATα2, are located at the beginning of chromosome 4 (Fig. 3). The two loci are flanked by inverted repeat (IR) sequences containing one (DIC1) and three (SLA2, SUI1 and CWC25) genes and are separated by around 135 kb of DNA sequence also containing the centromere (Hanson, Byrne and Wolfe 2014). Under mating conditions, the MAT locus next to the telomeric region remains silenced, while the genes in the second MAT locus are transcribed and thereby determine the mating-type of the cell (Heistinger, Gasser and Mattanovich 2018).

*K. phaffii* is a secondary homothallic yeast and mating-type switching takes place by homologous recombination at the ‘outer’ inverted repeat region containing the DIC1 genes. This leads to an inversion of the whole chromosomal region between the two MAT loci and a swap of the MAT allele in the active mating-type locus (Hanson, Byrne and Wolfe 2014). A similar, well studied mating-type system is found in the methylotrophic yeast *O. polymorpha*, where the mating loci are flanked by one inverted repeat region and silencing of the silent MAT locus is mediated by its proximity to centromeric heterochromatin (Hanson, Byrne and Wolfe 2014; Maekawa and Kaneko 2014). A recent study analysing the MAT loci of more than 300 budding yeast species found that such two-locus flip-flop switching mechanisms have evolved independently at least ten times, while the three-locus mating-type system as it is found in *S. cerevisiae* and closely related species seems to have evolved only once within the budding yeasts (Krasowski et al. 2019). In *S. cerevisiae*, two silent MAT loci (HMLα and HMRα) serve as template for mating-type switching via a synthesis-dependent strand annealing mechanism. Switching is initiated by the HO endonuclease, which introduces a double strand break at the active MAT locus (Strathern et al. 1982; Ira, Satory and Haber 2006; Haber 2012; Hanson and Wolfe 2017). The genomes of methylotrophic yeasts do not contain orthologs of HO endonuclease and although it has been shown that nitrogen starvation induces switching via an Rme1 and Ste12 dependent pathway in *O. polymorpha*, it remains unclear by which molecular mechanism mating-type switching is initiated (Hanson, Byrne and Wolfe 2017; Yamamoto et al. 2017).

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**Figure 2.** TFs involved in the regulation of *K. phaffii*’s central carbon metabolism. Blue rectangles represent the transporters. Activators and repressors are shown in green and red, respectively. See Table 1 for detailed information on the TFs. XuMP: Xylulose monophosphate pathway. TCA: Tricarboxylic acid cycle.
As described above, the Mat TFs encoded in the active MAT locus determine the mating-type of a cell. In K. phaffii, Mata2 and Matα1 in a- and α-type cells, respectively, activate the expression of mating-type specific genes like the pheromone and pheromone surface receptor genes and are thereby essential for mating. In diploid cells, MATα1 and MATa2 are required for sporulation and the repression of mating (Heistinger, Gasser and Mattanovich 2018). In S. cerevisiae and other post-WGD yeasts, a rewiring of this regulatory network resulted in the loss of a-specific gene activation by Mata2 and the requirement for a-specific gene repression by Matα2 in α-type cells (Tsong et al. 2006; Sorrells et al. 2015).

Due to the requirements of prolonged nitrogen starvation and solid media, mating of K. phaffii is unlikely to occur under industrial production conditions, even though there are always cells of both mating-types present in the culture when working with homothallic strains. However, mating represents a useful tool for strain development as it can be applied to combine interesting traits by methods like quantitative trait loci (QTL) mapping or other post-WGD yeasts, a rewiring of this regulatory network resulted in the loss of a-specific gene activation by Mata2 and the requirement for a-specific gene repression by Matα2 in α-type cells (Tsong et al. 2006; Sorrells et al. 2015).

Due to the requirements of prolonged nitrogen starvation and solid media, mating of K. phaffii is unlikely to occur under industrial production conditions, even though there are always cells of both mating-types present in the culture when working with homothallic strains. However, mating represents a useful tool for strain development as it can be applied to combine interesting traits, investigate genetic determinants of relevant traits by methods like quantitative trait loci (QTL) mapping or the generation of combinatorial libraries (Chen et al. 2012; Liti and Louis 2012; Swinnen, Thevelein and Nevoigt 2012). The use of heterothallic strains, such as the K. phaffii CBS2612 Δαic1-2 strains generated by the deletion of the ‘outer’ homologous region required for mating-type switching, further enables the use of K. phaffii for classical genetic studies (Heistinger, Gasser and Mattanovich 2018).

**CENTROMERES**

The centromeres of K. phaffii consist of a 2–2.7 kb inverted repeat region with a central core sequence of around 1 kb. Each of the four chromosomes has one centromere which is unique in sequence. The centromere of chromosomes 3 and 4 are found close to one chromosome end, with the centromere on chromosome 4 being located within the invertible region flanked by the MAT loci (Figure 3). The centromeres were identified as large non-transcribed regions and replicate early during cell division (Coughlan et al. 2016; Sturmberger et al. 2016). A ChIP-seq experiment showed that the centromere-specific histone variant Cse4 is most abundant in the core sequence but its signal can be detected all along the non-transcribed centromeric region. The orientation of the centromeres is variable in different K. phaffii isolates, indicating that recombination at the inverted repeats can occur (Coughlan et al. 2016). The inverted repeat centromeres of K. phaffii are highly different from the small point centromeres found in S. cerevisiae and closely related species, which are only around 125 bp long and defined by a clear consensus sequence (Hegemann and Fleig 1993). Although inverted repeat centromeres are also found in other yeasts like Candida tropicalis, the small genome and efficient tool for manipulation make K. phaffii an interesting system to study centromere function (Chatterjee et al. 2016). So far, two studies have reported the characterization of K. phaffii plasmid vectors carrying whole centromeric sequences. Those plasmids were found to increase mitotic stability while maintaining a low copy number when compared to classical ARS plasmids (Nakamura et al. 2018; Piva et al. 2020).

**MORPHOLOGY SWITCHES**

Upon experiencing adverse environmental conditions, budding yeasts can switch from unicellular to multicellular lifestyle, leading to flocculation, pseudohyphae formation or invasive growth (Brückner and Mösch 2012). These morphogenetic events give rise to subpopulations of cells exhibiting different phenotypes, thus providing advantage for adaptation to environmental changes and increasing chances of survival. In S. cerevisiae these morphology switches are associated with the flocculin (FLO) gene family, which has five dominant members encoding GPI-anchored cell-wall proteins (Verstrepen and Klis 2006; Willaert 2018). Out of these FLO1, FLO5, FLO9 and FLO10 are involved in flocculation, while FLO11 is responsible for filamentous growth (Guo et al. 2000; Van Mulders et al. 2009; Goossens and Willaert 2010).

**Figure 3.** Mating-type systems of K. phaffii and S. cerevisiae. (A) Homologous recombination at the outer inverted repeat (IR) region results in mating-type switching by inversion of the genomic region between the two MAT loci, including the centromere of chromosome 4 in K. phaffii. (B) In S. cerevisiae, HO endonuclease initiates mating-type switching. The two silent MAT loci (HMLα and HMαα) serve as template for mating-type switching via a synthesis dependent strand annealing mechanism.

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Most S. cerevisiae laboratory strains are devoid of flocculation and filamentous growth, as there is a defect in the FLO8 gene encoding the master transcriptional activator (Liu, Styles and Fink 1996). Morphological differentiations have also been observed in Komagataella species, however, until recently not much was known of the genetic and biochemical basis underlying these phenotypes. K. phaffii possesses an expanded FLO gene family consisting of 12 members containing different Flo-domains, and a Flo8-type TF (De et al. 2020). Pseudohyphae formation and surface adherence are absent when Flo8 is deleted, indicating that Flo8 is also the major regulator of filamentous growth in K. phaffii (Rebnegger et al. 2016; De et al. 2020). In both, S. cerevisiae and C. albicans, Flo8 was demonstrated to form a heterodimer with Mas11 via their N-terminal LisH domains that cooperatively regulates filamentous growth (Su et al. 2009; Kim et al. 2014). However, while also in K. phaffii Flo8 contains an N-terminal LisH domain no Mas11 homolog was identified (De et al. 2020), suggesting that Flo8 acts as a homodimer in this organism. While there is a clear ortholog of FLO11, for the other structural FLO genes no distinct homologs can be identified (Kock et al. 2018; Brückner et al. 2020; De et al. 2020). Kock et al. (2018) studied the nine predicted adhesins with a lectin-like PA14 domain present in Komagataella spp, and identified that the Komagataella adhesins form a unique clade in the fungal kingdom, thus stressing that it is hard to infer FLO gene function from one yeast species to another. Among them KpFlo1 (also named Cea1 or Flo5-2) was discovered as the first fungal adhesin showing high specificity for terminal β-GlcNAc capped glycans including chitinous polymers. The authors speculate that the Komagataella PA14 domain containing proteins evolved to adapt to the cells to their specific habitat to govern cell-substrate interactions different from flocculation.

Only very little is known about the mechanisms leading to floc formation in K. phaffii. Floculation is caused by homotypic cell-cell adhesion, whereby yeast cells aggregate into multicellular masses (flocs) that sediment out of the medium (Soares 2011). In S. cerevisiae, flocculation is triggered by carbon source limitation, pH variations, external stressors or the presence of ethanol or ions e.g. Ca²⁺ (Soares 2011). Cell–cell adhesion is depending on the N-terminal PA14 lectin domains present in S. cerevisiae Flo1, Flo5, Flo9 and Flo10 and their reversible binding of cell wall mannanns (Goossens et al. 2015). The respective genes are located adjacent to telomeres, and are silenced through their subtelomeric localization during normal growth conditions (Soares 2011; Cullen and Sprague 2012). It is not known so far which members of the FLO gene family are responsible for floculation in K. phaffii. Unlike in S. cerevisiae, no silencing of the subtelomeric FLO genes was observed in exponential growth conditions at pH 5.0 (De et al. 2020).

Mbawala et al. (1990) showed that cells exhibiting higher flocculation show elongated mannose chains containing alpha-1,2 and beta-1,2 linkages, indicating that also in K. phaffii lectin-like mechanisms are involved in this cell–cell adhesion process. Addition of 2 mM EDTA which captures Ca²⁺ involved in glycan cross-linking was reported to reduce floc formation observed during growth of K. phaffii in unbuffered YPD media (Tanneberger et al. 2007). In our experience, floc formation can be observed macroscopically at a pH around 4.0, and is reversible upon shifting the pH (De 2019).

Importantly, flocculation and sedimentation can be advantageous in industrial bioprocesses, as a rapid and efficient means of separation of the biomass from the product containing supernatant (e.g. in industrial ethanol fermentation processes of S. cerevisiae, (Soares 2011)). In this respect, K. phaffii cells engineered for increased rhamnose metabolic flux were shown to exhibit strong flocculation and sedimentation in rhamnose-containing media (Yan et al. 2018).

During pseudohyphal growth, filament-like structures are formed as cells divide but remain attached to each other (Cullen and Sprague 2012). If the filaments extend into a solid substrate, the phenomenon is termed invasive growth. In S. cerevisiae, pseudohyphal growth is dependent on Flo11 and more prevalent in diploid cells while invasive growth is more prevalent in haploid cells (Soares 2011; Cullen and Sprague 2012; Song and Kumar 2012). Despite being haploid, invasive growth has so far not been observed in K. phaffii. Also the environmental triggers leading to pseudohyphal growth are different between the two species: In contrast to S. cerevisiae, K. phaffii morphology is not affected by fusel alcohols or nitrogen starvation (De et al. 2020). So far, an elongated phenotype representing pseudohyphae was only observed when K. phaffii was cultivated at slow growth rates below μ = 0.075/h in glucose-limited chemostats (Rebnegger et al. 2014; De et al. 2020). The transition of K. phaffii from yeast to pseudohyphal form is associated with transcriptional changes of at least three FLO genes (FLO11, FLO400 and FLO5-1, all under control of Flo8) as well as chromatin remodeling. In contrast to S. cerevisiae, deletion of FLO11 reduced but did not abolish pseudohyphae formation in K. phaffii. On the other hand, deletion of either FLO400 or FLO5-1 prevented the morphological changes. This was associated with a lack of FLO11 induction upon switching to slow growth rates in glucose-limited chemostats, suggesting that K. phaffii Flo400 and/or Flo5-1 act as upstream signals for the activation of FLO11. However, it is not known which signaling cascades are responsible (De et al. 2020). Surprisingly, some strategies preventing morphological differentiations also resulted in higher productivity of secreted recombinant proteins (Gasser, Mattanovich and Buchetics 2014). Representative microscopic images of different morphological states of K. phaffii as described in this and preceding chapters are shown in Fig. 4.

**NITROGEN METABOLISM**

Although nitrogen is an essential macronutrient for yeasts, its metabolism has been poorly investigated. Moreover, as most of available data concern S. cerevisiae, highlighting the non-conventional traits of K. phaffii is less obvious as compared to carbon metabolism for instance. In both K. phaffii and S. cerevisiae, ammonia and organic nitrogen are the main N-sources, and N-metabolism is mainly based on deamination and transamination reactions with ammonia, glutamate and glutamine as key compounds. From BLASTp searches (E value cut-off 10⁻⁶), all transporters for ammonia (three genes; Marini et al. 1997) and amino acids (21 genes; Bianchi et al. 2019) characterized so far in S. cerevisiae seem to have a counterpart in K. phaffii (data not shown). However, despite this similarity, it turned out that one needs to be careful by drawing conclusions from S. cerevisiae. For example, branched-chain amino acid (BCAA) biosynthesis is differentially compartmentalized in K. phaffii, with solely cytosolic ɑ-isopropylmalate synthase Leu4 and BCAA aminotransferase Bat1 (Förster et al. 2014). Furthermore, enzymes involved in alanine (Alt1), aspartate (Aat2), glutamate (Gdh2, Gdh3 and Glt1) and lysine (Lys20 and Lys21) synthesis are found primarily in the cytosol of K. phaffii, whereas they localize also to the mitochondria in S. cerevisiae (Valli et al. 2020). In S. cerevisiae, two NADPH-glutamate dehydrogenases encoded by GDH1 and GDH3 catalyze the transamination of ɑ-ketoglutarate with the formation of glutamate. K. phaffii possesses only one Gdh enzyme,
termed Gdh3 (CP014584, with about 70% of identity in amino acid sequence to *S. cerevisiae* Gdh1 and Gdh3), similarly to *Yarrowia lipolytica* (Trotter et al. 2020) and *S. stipitis* (Freese et al. 2011). Glutamate can be further transaminated by glutamine synthase (gene GLN1) to yield glutamine. Inversely, ammonia can be released by deamination of glutamine or glutamate by glutamate synthase (gene GLT1) and NAD-glutamate dehydrogenase (gene GDH2), respectively (Magasanik 2003). These three key metabolites are the major precursors of amino acid biosynthesis while α-ketoglutarate is the hinge between C- and N-metabolisms.

In some methylotrophic yeasts such as *H. polymorpha*, nitrate assimilation occurs by its reduction into ammonium through the consecutive action of nitrate and nitrite reductases encoded by the genes YNR1 and YNL1, respectively (Siverio 2002). This is not the case for *K. phaffii* that does not assimilate nitrate or possess nitrate reductase activity (Unkles et al. 2004). Even though the majority of media contain ammonium as nitrogen source, *K. phaffii* can also efficiently grow on urea at least in complex medium (Guo et al. 2012), and the addition of urea to the fermentation medium was discussed to be beneficial for protein production purposes (Adivitiya, Mohanty and Khasa 2021). By contrast to *S. cerevisiae*, *K. phaffii* and other non-conventional yeasts are able to grow in minimal medium containing aspartate or glutamate as sole N- and C-sources (Sahu and Rangarajan 2016). This ability has been found to be correlated to the activity of Gdh2. A Δgdh2 mutant of *S. stipitis* cannot utilize glutamate as C-source while in *Y. lipolytica* the activity of Gdh2 increases up to 18-fold as compared to Gdh1 when glutamate is both, C- and N-source (Trotter et al. 2020).

In *K. phaffii*, TFs that regulate methanol metabolism have also been found involved in this process. For instance, Mrx1 but not Trm1 or Rop1, regulates the activity of GDH2 expression at post-transcriptional level. Mxr1 also regulates at the transcriptional level the genes AAT1 and AAT2 encoding mitochondrial and cytosolic aspartate aminotransferase, respectively, and the gene GLN1. Mxr1 Response Elements (MXREs) have been found in the promoter sequence of AAT2 and GLN1. Therefore, methanol metabolism, a peculiar feature of *K. phaffii*, also controls N-metabolism. Beside this, nitrogen sources such as casamino acid have also been reported to regulate methanol metabolism (Velastegui et al. 2019). Indeed, expression of the genes AOX1, DAS1 and FLD1 is reduced in the presence of 0.1% of casamino acid.

Recently, a genomic survey of nitrogen assimilation pathways in budding yeast has been published (Linder 2019). By contrast to *S. cerevisiae*, the genome of *K. phaffii* contains the gene AMO1 encoding amine oxidase that catalyzes the deamination degradation in *S. cerevisiae* is composed by a single copy ASP1 gene and four repeated copies of ASP2 encoding cytosolic and cell-wall asparaginases (League, Slot and Rokas 2012). These enzymes catalyze the deamination of asparagine into aspartate with the release of ammonium. From BLASTp analysis, no clear Asp2 counterpart could be identified in the *K. phaffii* genome, while sequence NC_012965.1 from strain GS115 shows 57% identity with Asp1 (data not shown).

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Some specificities of *K. phaffii* could be also highlighted from amino acid catabolic pathways. For instance, the asparagine degradation in *S. cerevisiae* is composed by a single copy ASP1 gene and four repeated copies of ASP2 encoding cytosolic and cell-wall asparaginases (League, Slot and Rokas 2012). These enzymes catalyze the deamination of asparagine into aspartate with the release of ammonium. From BLASTp analysis, no clear Asp2 counterpart could be identified in the *K. phaffii* genome, while sequence NC_012965.1 from strain GS115 shows 57% identity with Asp1 (data not shown).
of aliphatic primary amine (R-NH₂) with the release of ammonia. It also contains the gene AOC1 encoding lysyl oxidase. The corresponding enzyme has been biochemically characterized in detail (Kucha and Dooley 2001). Regarding uracil catabolism, K. phaffii genome contains genes URC1 and URC4 encoding putative cyclohydrolase and ribosyl-urea degrading enzymes which are missing in S. cerevisiae. In budding yeast, purines, uric acid and allantoin are all catabolized in a common pathway with ammonia missing in cyclohydrolase and ribosyl-urea degrading enzymes which are missing in S. cerevisiae. In budding yeast, purines, uric acid and allantoin are all catabolized in a common pathway with ammonia as the final product. A total of 10 genes are involved in this pathway namely, XAN1, XAN2, URO1, URO2, URO3, DAL1, DAL2, DAL3 and DUR1,2, although some of them are not present in the S. cerevisiae genome. These are the two XAN genes and the three UBO genes that are putatively involved in the conversion of xanthine into uric acid and of uric acid into allantoin. Xanthine oxidoreductase (XAN genes) are known to require a molybdenum cofactor (MoCo) to be active (Mendel 2013). At least six genes, MOC1–MOC6 are involved in molybdenum cofactor biosynthesis in eukaryotic cells. The homologs of these six genes were identified in K. phaffii but not in S. cerevisiae (Linder 2019).

PROTEIN SECRETION

Based on the status of K. phaffii as a popular recombinant protein production platform, a large body of dedicated research aimed at elucidating (or manipulating) the molecular mechanics governing protein synthesis, secretion and post-translational modifications (PTMs) such as disulfide bond formation, proteolytic processing as well as N- and O-glycosylation. According to published literature, secretion yields of recombinant products processing as well as N- and O-glycosylation. According to published literature, secretion yields of recombinant products are often exceed those of mammalian cells, although some of them are not present in the S. cerevisiae genome. These are the two XAN genes and the three UBO genes that are putatively involved in the conversion of xanthine into uric acid and of uric acid into allantoin. Xanthine oxidoreductase (XAN genes) are known to require a molybdenum cofactor (MoCo) to be active (Mendel 2013). At least six genes, MOC1–MOC6 are involved in molybdenum cofactor biosynthesis in eukaryotic cells. The homologs of these six genes were identified in K. phaffii but not in S. cerevisiae (Linder 2019).

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Protein N-glycosylation in eukaryotes is initiated in the ER by the linking of the precursor Glc3Man9GlcNAc2 to an asparagine residue in the consensus sequence asparagine-X-serine/threonine (Asn-X-Ser/Thr, where X is any amino acid except for proline). Subsequently, the terminal α-1,2 and α-1,3 glucose residues are removed by respective glucosidases and one α-1,2-mannose is removed by an ER-residing α-1,2-mannosidase, resulting in a Man9GlcNAc2 glycan. Further N-glycan modifications of properly folded proteins take place in the Golgi apparatus, where yeasts add mannose and mannose-phosphate sugars to the Man9GlcNAc2 glycan core, generating N-glycans of the high-mannose type (Hamilton and Gerngross 2007; De Wachter, Van Landuyt and Callewaert 2018). N-glycan structure and side chain composition can differ substantially between yeast species (Thak et al. 2018) but can also be very heterogeneous in regard to a specific N-glycosylation site. S. cerevisiae N-glycans typically carry longer mannose outer chains, containing up to 150–200 mannose residues in total, while in K. phaffii outer mannose chain length is shorter with a total of 8–18 mannose residues (Herscovics and Orlean 1993; Kang et al. 1998; Krainer et al. 2013; Thak et al. 2018). Different to S. cerevisiae, K. phaffii glycan do not have immunogenic terminal α-1,3-linked mannose residues, due to the lack of the corresponding Mnn1 enzyme family (Delic et al. 2013; Thak et al. 2018). Instead, K. phaffii possesses Bmt enzymes which catalyse the addition of beta-1,2 mannoses.

Mannose outer chain elongation is initiated by the introduction of an α-1,6-mannose residue by the mannosyltransferase Och1. Disruption of this gene in K. phaffii leads to a reduction from 10 to 8 mannose residues in the dominant glycan (Krainer et al. 2013) and co-overexpression of a recombinant α-1,2-mannosidase in the ER mainly yields Man8GlcNAc2 structures (De Wachter, Van Landuyt and Callewaert 2018). In contrast, in S. cerevisiae two more enzymes acting on Man8GlcNAc2 were required to be deleted, which are not present in the K. phaffii genome. While it was initially believed that K. phaffii lacking Och1 shows only a minor phenotype in contrast to S. cerevisiae Δoch1, it was later elucidated that the true K. phaffii Δoch1 knock-out has a wrinkly morphology and a growth deficit (Krainer et al. 2013; De Wachter, Van Landuyt and Callewaert 2018), which was not observed in the initial insertional mutant (Choi et al. 2003; Vervecken et al. 2004). Nevertheless, in combination with the fact that less knockouts are required, this is probably one explanation why glycoengineering proceeded mainly with K. phaffii rather than S. cerevisiae. De Wachter, Van Landuyt and Callewaert (2018) comprehensively reviewed recent advances in N- and O-glycoengineering of yeasts. So far, most progress regarding the humanization of N-glycans has been made in K. phaffii, allowing even for the production of proteins containing complex-type sialylated N-glycans or mucin-type O-glycans. Nevertheless, to date no biopharmaceuticals produced in glycoengineered yeasts have reached the market.

In yeasts, O-glycosylation is also initiated in the ER by a family of protein O-mannosyltransferases (PMTs) that transfer mannos from dolichol phosphate β-D-mannose (Dol-P-Man) to Ser/Thr residues in the nascent proteins. Linear elongation of the O-glycans takes place in the Golgi, where mannosyltransferases catalyze the transfer of mannos from GDP mannos (Neubert et al. 2016). As true for many other genes, the number of PMT genes is reduced to five in K. phaffii compared to seven in S. cerevisiae. Similar to other yeasts, K. phaffii PMT1 and PMT2 appear both to have a predominant role in protein O-glycosylation, with PMT2 showing the highest expression levels and the Δpmt2 mutant strain the most severe growth defect (Govindappa et al. 2013; Nett et al. 2013; Radoman et al. 2021) comprehensively analysed the occurrence and composition of
O-glycans of secreted proteins that were produced in K. phaffii, and found that the degree of O-mannosylation of a recombinant protein proved to be higher when methanol was used as a carbon source. The majority of O-glycans was composed of one mannose residue, while a maximum of five mannose residues was observed. It is not known, however, which enzymes are responsible for chain elongation, as K. phaffii possesses six homologs of the Golgi-resident Ktr/Kre-family mannosyltransferases compared to nine family members in S. cerevisiae, making their correct functional assignment as well as their disruption more difficult in K. phaffii.

Major differences between S. cerevisiae and non-conventional yeasts are seen in the structural organization of the Golgi apparatus, which consists of disk-shaped membranes called cisterna. Golgi resident proteins, mainly involved in N- and O-glycan as well as lipid processing, are spatially organized, depending on their respective function. Freshly synthesized proteins that pass the ER-quality control, exit the ER via COPII-coated transport vesicles at so-called transitional ER (tER) sites and enter the Golgi at the cis site, move through medial cisternae and eventually arrive at the trans compartment (Papanikou and Glick 2009; Suda and Nakano 2011). In most eukaryotes, Golgi cisternae form stacks. In vertebrates, the structural organization of the Golgi is even more complex, appearing as a twisted, ribbon-like network (Wei and Seemann 2010). S. cerevisiae is one of only a few known eukaryotic organisms where the respective Golgi compartments are not organized in stacks but scattered across the cytoplasm (Mowbrey and Dacks 2009). K. phaffii on the other hand, contains 2–5 stacks of ca. 4 cisternae each per cell and shares several other Golgi characteristics with mammalian cells such as the presence of a cisternae-surrounding matrix as well as fenestration and tubular extension of the cisternae (Papanikou and Glick 2009). It, therefore, serves as a model organism for Golgi-related research. Disruption of Golgi stacking in mammalian cells has been demonstrated to reduce total N-linked protein glycosylation and decrease N-glycan complexity. Reversible unstacking of the Golgi in K. phaffii was observed in a SEC16 temperature sensitive mutant (Connerly et al. 2005) as well as the KO mutants of PlMLH1, encoding a GRIP domain Golgin (Jain, Dahara and Bhattacharyya 2019). Permanent unstacking under physiological conditions was achieved by the disruption of the genes RSN1, CSC1-2 or TVP18, which are thought to be involved in calcium transport or signaling. However, unstacking of the Golgi by disruption of these genes did not lead to significant changes in the cellular N-glycome nor in N-glycan abundance or composition of glycoGFP (Aw et al. 2021).

One of the major advantages attributed to K. phaffii is that it secretes comparatively few host cell proteins, facilitating down-stream processing, and thereby reducing production costs. According to Lum and Min (Lum and Min 2011) the predicted secretome size of S. cerevisiae is 50%, and that of Y. lipolytica, another popular host for recombinant protein production, is even 300% larger than that of K. phaffii. However, these numbers do not account for growth conditions or for proteins regularly observed extracellularly such as metabolic enzymes, chaperones or proteins involved in translation that are not actively secreted but reach the extracellular space either by passive transport, unconventional secretion or cell lysis (Nombela, Gil and Chaffin 2006; Miura and Ueda 2018). Several studies have been conducted in order to characterize the secretome of K. phaffii under industrially relevant conditions (Dragosits et al. 2009; Huang et al. 2011; Burgard et al. 2020). The most recent study carried out by Burgard et al. (2020) investigated how the secretome of K. phaffii changes throughout a typical recombinant protein production process and how the choice of carbon source (glucose or glycerol/methanol) affects native protein secretion. In total 51 proteins were identified, concordant with previous observations. A similar study conducted in K. lactis, whose predicted secretome size varies between 113 and 178 proteins (Swaim et al. 2008; Lum and Min 2011), found up to 120 proteins when cells were grown on galactose and a total of 151 proteins across all growth conditions. In both, K. lactis and K. phaffii, a majority of identified proteins possessed a predicted signal peptide. While the core secretome (proteins identified in every condition) of K. phaffii mainly consisted of cell wall proteins, in K. lactis also many proteins with a function in glycosylation, carbohydrate metabolism and proteolysis were identified. To the authors knowledge no studies investigating the full secretome of other important yeast recombinant protein production hosts like S. cerevisiae, H. polymorpha or Y. lipolytica under industrially relevant conditions have been published so far, hindering relevant comparisons to these organisms.

CONCLUSIONS

Genetic diversity is large among budding yeasts. Their morphological similarity should not make us believe that they function very similarly. Partly, differences between S. cerevisiae and non-conventional yeasts can be explained by the WGD event and subsequent functional specialization that S. cerevisiae and its close relatives went through. Non-conventional species such as K. phaffii have rather adapted their proteome to the ecological niches they inhabit. But even where gene sets are similar, different transcriptional control creates multitude. Such regulatory differentiations have recently been identified in carbon metabolism and contribute to the different physiology of K. phaffii in comparison to S. cerevisiae. Also, differential localization of proteins was observed. It remains to be identified in future if such regulatory differences also contribute to the higher secretion efficiency and different glycan pattern that make K. phaffii an excellent protein production host. Overall, we strongly recommend being careful when drawing analogies solely based on sequence analysis.

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AUTHOR CONTRIBUTIONS

All authors contributed equally to the manuscript. OA and LH prepared the final figures. All authors have read and agreed to the final version of the manuscript.

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