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A dual approach for improving homogeneity of a human-type N-glycan structure in *Saccharomyces cerevisiae*

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Abstract

N-glycosylation is an important feature of therapeutic and other industrially relevant proteins, and engineering of the N-glycosylation pathway provides opportunities for developing alternative, non-mammalian glycoprotein expression systems. Among yeasts, *Saccharomyces cerevisiae* is the most established host organism used in therapeutic protein production and therefore an interesting host for glycoengineering. In this work, we present further improvements in the humanization of the N-glycans in a recently developed *S. cerevisiae* strain. In this strain, a tailored trimannosyl lipid-linked oligosaccharide is formed and transferred to the protein, followed by complex-type glycan formation by Golgi apparatus-targeted human N-acetylglucosamine transferases. We improved the glycan pattern of the glycoengineered strain both in terms of glycoform homogeneity and the efficiency of complex-type glycosylation. Most of the interfering structures present in the glycoengineered strain were eliminated by deletion of the *MNN1* gene. The relative abundance of the complex-type target glycan was increased by the expression of a UDP-N-acetylglucosamine transporter from *Kluyveromyces lactis*, indicating that the import of UDP-N-acetylglucosamine into the Golgi apparatus is a limiting factor for efficient complex-type N-glycosylation in *S. cerevisiae*. By a combination of the *MNN1* deletion and the expression of a UDP-N-acetylglucosamine transporter, a strain forming complex-type glycans with a significantly improved homogeneity was obtained. Our results represent a further step towards obtaining humanized glycoproteins with a high homogeneity in *S. cerevisiae*.

Keywords: N-glycosylation, glycoengineering, *MNN1*, UDP-GlcNAc transporter, yeast, glycosylation efficiency

Introduction

Protein N-glycosylation is one of the most prevalent and complex posttranslational modifications of eukaryotic proteins, having an impact on various protein properties such as stability, biological activity, protein half-life in blood, and immunogenicity. A significant part of clinically approved therapeutic proteins is glycosylated, making glycosylation a key aspect in the function and safety of therapeutic proteins [1]. In addition, the efficacy of therapeutic proteins, such as antibodies or erythropoietin, can be improved through modification of the natural N-glycan structures or through introduction of additional N-glycosylation sites [2, 3]. Moreover, the impact of N-glycosylation on other industrially important proteins, such as hydrolytic enzymes, has recently been recognized [4].

Two main characteristics of N-glycans are their structural species-specificity and vast heterogeneity both in terms of structural variation as well as varying glycosylation site occupancy. Although the initial steps of the lipid-linked glycan donor biosynthesis and the N-glycan transfer step are conserved among eukaryotes, a high N-glycan diversity is created by trimming and further modification of the N-glycan structure in the Golgi apparatus. This complexity and diversity is founded on an enormous repertoire of enzymes; it has been estimated that 1 to 2% of the human genome encodes proteins that contribute to glycosylation, including N-linked glycosylation [5].

Due to the requirement for complex-type N-glycosylation, a majority of therapeutic glycoproteins is currently produced in mammalian expression systems despite their several limitations. These expression hosts are mainly of non-human origin, including Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells as well as murine cell lines such as NS0 or Sp2/0. In these hosts, the wide range of glycan-modifying enzymes can affect the N-glycosylation process and give rise to the incorporation of non-human sugars in the N-glycans. In addition, pharmaceutical glycoproteins produced by mammalian cells are often a mixture of a varying number of different glycoforms, and factors such as growth conditions can affect their glycan patterns [2]. Changes in the glycan patterns have indeed been observed between production lots in some commercially available therapeutic glycoproteins [6, 7].

In recent years, research has been conducted to develop alternative, non-mammalian expression systems for therapeutic glycoproteins, and engineering of the N-glycosylation pathway in the alternative host organisms is a central challenge in this development. *Saccharomyces cerevisiae* is a well-established production organism with a GRAS (generally recognized as safe) status, and it has been used for the production of a wide range of products, including various therapeutic proteins approved by FDA [3]. Therefore, *S. cerevisiae* is among the most attractive alternative hosts for glycoengineering and therapeutic glycoprotein production. In contrast to many other potential expression systems such as plant or insect cells, yeast also completely lacks the N-glycan processing machinery for creating hybrid and complex-type N-glycans. Therefore, yeast is the optimal host for rebuilding the N-glycan tailoring pathway as only the required activities need to be inserted and no interference by reactions from unwanted glycosyltransferases is to be expected. This provides an opportunity to develop a glycoprotein expression system

with well-defined, consistent and homogenous glycan profiles. This type of expression system offers major benefits in the context of therapeutic proteins, enabling consistent product properties combined with cost-effective production. In addition, a technology for producing single glycoforms would enable more detailed research on the relationship between glycan structures and protein properties, a topic which is currently poorly understood [1].

The glycan modifications in the Golgi apparatus of *S. cerevisiae* consist of mannosylation, and unlike in mammalian Golgi apparatus, no trimming of mannose residues occurs in yeast [8]. The mannosylation gives rise to two different glycan types: hypermannosylated glycans and smaller, core-type N-glycans. Processing of both glycan types in the yeast Golgi apparatus is initiated with the addition of an α-1,6-mannose to the α-1,3 mannose of the trimannosyl core, catalyzed by Och1p. In hypermannosylated glycans, the outer chain is further elongated with α-1,6-mannoses by two mannan polymerase complexes, M-Pol I and M-Pol II. This α-1,6 chain is then branched by the addition of α-1,2 mannoses by Mnn2p and Mnn5p, and the branches are terminated by the addition of α-1,3 linked mannose residues by Mnn1p [9]. These terminal α-1,3 mannoses have been reported to be immunogenic [10]. In core-type glycans, the α-1,6 backbone of the outer branch is not formed. Instead, an α-1,2 mannose is added to the first mannose of the outer branch by an unknown enzyme. Some studies suggest that M-Pol I may have a role in this reaction [11, 12]. Finally, a terminal α-1,3 mannose is added by Mnn1p to all terminal α-1,2 mannose [9].

Glycoengineering in yeasts has mainly focused on *Pichia pastoris* and *S. cerevisiae*, and various approaches have been used to create hybrid and complex-type glycans in yeasts. Many glycoengineering approaches are based on the elimination of the yeast-specific hypermannosylation by the deletion of a Golgi-resident mannosyltransferase *OCH1* either alone or in combination with other mannosyltransferase deletions, and on the creation of a substrate for mammalian glycosyltransferases by the expression of mannosidases [13]. In *S. cerevisiae*, an alternative approach was recently used, based on the direct formation of the lipid-linked oligosaccharide (LLO) $Man_3GlcNAc_2$ in the endoplasmic reticulum (ER) and its transfer onto the protein [14]. In this approach, the $Man_3GlcNAc_2$ structure, present in all eukaryotic N-glycans, directly functioned as a substrate for mammalian GlcNAc transferases I and II expressed in yeast Golgi apparatus, obviating the need for *OCH1* deletion and mannosidase expression. In order to maintain a high glycosylation efficiency, the introduction of an artificial flippase (Flc2*p) and an oligosaccharyltransferase from *Leishmania brasiliensis* (POT) was necessary to compensate for reduced flipping and transfer of the LLO to the nascent protein. Although this yeast strain produced complex type N-glycans, a substantial amount of the $Man_3GlcNAc_2$ was modified with additional mannose residues.

In this work, we followed a dual strategy to improve the glycan homogeneity in the production of complex-type N-glycans in glycoengineered *S. cerevisiae*. By deleting the mannosyltransferase *MNN1*, we have been able to improve the glycan homogeneity by eliminating most of the interfering mannosyltransferase activities. We also obtained an increased relative abundance of a complex-type $GlcNAc_2Man_3GlcNAc_2$ glycan by enhancing UDP-GlcNAc transport into the Golgi apparatus through expression of a UDP-GlcNAc transporter. By a combination of these two approaches, a glycan pattern containing a complex-type N-glycan with an improved homogeneity was obtained.

Materials and methods

Strains, reagents and growth conditions

The chemicals and reagents used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The reagents for DNA work were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All *S. cerevisiae* strains and plasmids used in the experiments are presented in Table 1.

Cloning

Escherichia coli strain TOP10 (Invitrogen, Carlsbad, CA, USA) was used as a host organism for recombinant DNA work. Oligonucleotides were purchased from Eurofins Genomics (Ebersberg, Germany) and are listed in Supplementary table S1. The gene encoding a UDP-GlcNAc transporter in *Kluyveromyces lactis* (*KlMNN2-2*) was isolated from the genomic DNA of *K. lactis* strain ATCC 8585 (VTT Technical Research Centre of Finland, Espoo, Finland) by colony PCR using oligonucleotides OJR47 and OJR48 and Phusion High Fidelity polymerase. The SpeI XhoI digested PCR product was ligated into the corresponding restriction sites of pRS415-derived plasmid series [15], placing the gene under the control of *TEF*, *GPD* and *GAL1* promoters and creating plasmids pMP001, pMP002 and pMP003, respectively. Plasmid pAX428 was used for the expression of the Golgi-localized human GlcNAc transferases I and II, and its construction has been described earlier [14]. Plasmids were transformed into yeast cells using the lithium acetate method [16].

Generation of yeast deletion strains

For homologous recombination-based gene deletions, the complete open reading frames of target genes were replaced with a PCR product containing the *kanMX4* or *natNT2* cassette and 50 bp long extensions flanking the target gene coding sequence. For the *MNN1* and *MNN2* deletions in strain YG1429, the *kanMX4* cassette was amplified from plasmid pRS305K [17] using oligonucleotides OJR43 and OJR44 for *MNN1* deletion and OJR41 and OJR42 for *MNN2* deletion. For the *MNN1* deletion in strain YAF39, the *natNT2* cassette was amplified from plasmid pUG74 [18] using oligonucleotides OMP01 and OMP02. The *LEU2* locus in YG1429 was replaced with the PvuII linearized plasmid pRS305N [17]. Linear fragments were transformed into yeast cells using the lithium acetate method [16]. Transformants containing the *kanMX4* or *natNT2* selection markers were selected in the presence of 200 µg/ml G418 or 100 µg/ml nourseothricin (Jena Biosciences, Jena, Germany), respectively. The *MNN1* and *MNN2* deletions were confirmed by colony PCR, using oligonucleotide pairs OJR45 and OCS4 or OJR45 and OJR61 for *MNN1* deletion with *kanMX4* or *natNT2* cassette, and OJR46 and OCS4 for *MNN2* deletion with *kanMX4* cassette, respectively. The *LEU2* deletion was confirmed by absence of growth in leucine-deficient growth medium.

Cultivation of yeast strains

S. cerevisiae strains were grown in YPD medium (1% yeast extract (Lab M, Heywood, UK), 2% peptone (BD, Franklin Lakes, NJ, USA), 2% glucose), minimal medium (0.67% yeast nitrogen base without amino acids (BD, Franklin Lakes, NJ, USA) supplemented with adenine and lysine) or SD medium (0.67 % yeast nitrogen base without amino acids supplemented with a dropout mix lacking leucine and uracil), using 2 % glucose or 1 %

raffinose as the carbon source. Growth media for YAF39 and strains derived from YAF39 was supplemented with sorbitol. *S. cerevisiae* cultures were grown in shake flasks at 30 °C except for strains YMP05, YMP06, YMP07 and YMP09, which were grown at 28 °C. Cell densities of the liquid cultivations were monitored by measuring their optical density at 600 nm (OD_{600}).

For cell wall protein N-glycan analysis, empty yeast strains were grown in YPD medium and collected in the mid-log phase. Yeast strains containing plasmids were cultivated in SD medium containing 1 % raffinose, except for strains YMP15 and YMP16, which were cultivated in minimal medium containing 1 % raffinose. Expression of the glycosyltransferases GnTI and GnTII and the UDP-GlcNAc transporter *KlMNN2-2* was induced at an OD_{600} of 1.0 using 2 % galactose, after which the cells were grown for 24h.

Isolation of N-glycans

Cell wall proteins from a cell amount corresponding to 50 OD_{600} units were prepared for N-glycan isolation using a method described earlier [19] with the following modifications. The cells were lysed using 0.5 mm acid washed glass beads in 10 mM Tris-HCl buffer, pH 7.4 containing protease inhibitor cocktail (cOmplete EDTA-free, Roche, Basel, Switzerland) and 1 mM phenylmethanesulfonyl fluoride (PMSF). The reduction and alkylation of cysteines was performed at room temperature, and after washing the pellet five times with 2 M thiourea, 7 M urea, 2 % SDS, 50 mM Tris-HCl, pH 8, additional five washes were performed with H_2O.

N-glycans were released from cell wall glycoproteins using PNGase F (500 U/µl, New England Biolabs). Samples were resuspended in 200 µl of deglycosylation mix (Glycoprotein Denaturation Buffer, G7 buffer, 1 % NP-40 and 1 µl of PNGase F), and the deglycosylation reaction was performed at 37 °C for 16 hours.

The released glycans were purified using C18 and graphitized carbon columns (Supelclean ENVI-18 and ENVI-Carb, Sigma-Aldrich, St. Louis, MO, USA). Both columns were washed two times with 500 µl of 95% acetonitrile and equilibrated two times with 500 µl of 2 % acetonitrile. The sample and the effluent from washing the sample tube were applied to the C18 column in 2 % acetonitrile, and the column was washed two times with 200 µl of 2 % acetonitrile. The flow-through from the C18 column was applied to a graphitized carbon column, which was subsequently washed two times with 500 µl of 2 % acetonitrile. The glycans were eluted using two times 200 µl of 70 % acetonitrile and dried by evaporation at 65 °C. The purified glycans were labeled with 2-aminobenzamide (2-AB) as described earlier [20]. Briefly, the glycans were dissolved in labeling solution (0.35 M 2-AB, 1 M $NaCNBH_3$ in 70 % DMSO, 30 % acetic acid) and incubated for two hours at 65 °C. Excess label was removed as described earlier [21], using two discs of Whatman 3 mm chromatography paper (GE Healthcare, Little Chalfont, UK) inserted in a 2-ml syringe. The labeled glycans were desalted in a graphitized carbon column as described above.

MALDI-TOF

The 2-AB labeled glycans were analyzed by MALDI-TOF MS operated in the positive ion mode (Autoflex II, Bruker Daltonics, UK). The glycan samples were dissolved in 0.1 % trifluoroacetic acid, mixed 1:1 with a 2,5-dihydroxybenzoic acid (DHB) matrix (10 g/l DHB in a 1:1 mixture of acetonitrile and 0.1% trifluoroacetic acid), and 1 µl of the mixture was spotted onto the target plate and dried. The MALDI-TOF data were processed and

analyzed using mMass 5.5 software [22], and the peaks corresponding to the m/z values of the 2-AB labeled glycan structures were identified using GlycoMod Tool [23]. The relative abundances of the glycan structures were defined as the peak intensity corresponding to each glycan structure normalized to the peak intensity sum of all glycan structures in the sample.

Results

Increasing glycan homogeneity by removing interfering mannosyltransferase activity

According to earlier results, the tailored trimannosyl glycan formed in the ER of the Δ*alg3* Δ*alg11* strain can serve as a substrate not only for GlcNAc transferase I [24], but unexpectedly also for yeast mannosyltransferases present in the Golgi apparatus [14]. These mannosyltransferase activities competed with the GlcNAc transferase activities and resulted in the formation of $Man_{4-6}GlcNAc_2$ glycans which could not be converted to complex-type N-glycans by the glycoengineered yeast. Based on enzymatic digests and glycan analysis, the fourth mannose was identified as an α-1,2 mannose most likely linked to the α-1,6 arm of the core glycan, which in turn was capped with one or two additional mannose residues. The deletion of *MNN1*, encoding an α-1,3 mannosyltransferase located in the Golgi apparatus [25], resulted in a less heterogeneous glycan pattern in a Δ*alg3* Δ*alg11* strain, consisting of mostly the $Man_4GlcNAc_2$ glycan [14].

Therefore, we deleted the *MNN1* gene from YG1429, a UV-mutagenized Δ*alg3* Δ*alg11* strain with improved growth properties and therefore more suitable as a production strain. In the resulting glycan profile, no relevant amounts of $Man_5GlcNAc_2$ and larger glycan structures (M5 and M6) were observed but a structure containing four mannoses (M4) was still present (Fig. 1a, b), which was in accordance with the earlier findings. Also when the *MNN1* deletion was performed in YAF39, a UV-mutagenized Δ*alg3* Δ*alg11* strain containing Flc2*p and POT for compensated glycosylation efficiency, a similarly improved glycan pattern was obtained (Fig. 1c). Since defects in glycosylation often result in growth phenotypes, we compared growth of the parental strain with the *MNN1* deletion strain. Deletion of the *MMN1* gene had only a minor effect on the viability of the strain (Fig. 2).

After a successful improvement in the glycan homogeneity by *MNN1* deletion, we then focused on the remaining single interfering structure that was still present in the glycans of cell wall glycoproteins. In this $Man_4GlcNAc_2$ glycan, one additional mannose is added to the tailored trimannosyl glycan via an α-1,2 linkage. We tried to identify the responsible enzyme for this interfering mannosyltransferase activity based on literature and information available in *Saccharomyces* genome database on all known yeast mannosyltransferases [26]. The Golgi-localized enzymes with reported or putative α-1,2 mannosyltransferase activity include Mnn2p, Mnn5p, Kre2p/Mnt1p, Ktr1p and Ktr3p (Table 2). Based on its putative specificity and acceptor site, we considered Mnn2p as the most likely enzyme to transfer the α-1,2 mannose to the α-1,6 arm of the core glycan. We deleted the *MNN2* gene from the strain YG1429, but no changes in the glycan profile were observed (data not shown).

Availability of UDP-GlcNAc in the yeast Golgi apparatus is limiting GlcNAc transfer

In order to obtain complex-type glycosylation in the glycoengineered yeast, we expressed Kre2p-GnTI and Mnn2p-GnTII fusion proteins, containing the catalytic domains of human GlcNAc transferases I and II fused to the targeting domains of yeast Kre2p and Mnn2p, respectively [14]. In our experiments, the expression of GnTI and GnTII resulted in approximately 18-19 % relative abundance of the complex-type $GlcNAc_2Man_3GlcNAc_2$ target glycan (G2M3; strains YMP08 and YMP09 in Table 3). Therefore, UDP-GlcNAc seemed to be available for the GlcNAc transfer reactions in the Golgi apparatus of *S. cerevisiae*, even though the native N-glycan modification processes in *S. cerevisiae* Golgi apparatus do not require UDP-GlcNAc and no endogenous transporters specific for UDP-GlcNAc have thus far been identified in the Golgi apparatus of *S. cerevisiae*. UDP-GlcNAc is synthesized in the cytoplasm and used in addition to the LLO biosynthesis in the cell wall chitin and GPI anchor biosynthetic processes [27]. As complex-type glycans were formed in the glycoengineered strain this suggests that yeast has an endogenous ability to import UDP-GlcNAc in the Golgi apparatus. A similar observation was also reported earlier, when GlcNAc residues were observed in the glycans of a modified hen egg lysozyme produced in *S. cerevisiae* [28]. Thus, it is safe to postulate that UDP-GlcNAc can enter the Golgi apparatus via an unspecific transporter protein or an unidentified UDP-GlcNAc transporter. However, we were hypothesizing that an increased availability of UDP-GlcNAc could improve the formation of the complex-type N-glycan.

In order to enhance the import of UDP-GlcNAc to the Golgi apparatus, we expressed the *KlMNN2-2* gene from *K. lactis* in *S. cerevisiae*. *KlMNN2-2* encodes a UDP-GlcNAc transporter, a transmembrane protein that imports UDP-GlcNAc from the cytoplasm to the Golgi apparatus, thereby providing substrate molecules for the GlcNAc transferases [29]. We co-expressed the GnTI and GnTII fusion proteins, regulated by the bidirectional *GAL1-10* promoter, with the *KlMNN2-2* gene under the control of the constitutive *GPD1* and *TEF1* promoters or the galactose-inducible *GAL1* promoter in strain YAF39. In order to keep the growth media composition constant and to eliminate possible biases in growth and other cellular processes that can be caused by differences in auxotrophy and nutritional supplementation [30], the glycan patterns were compared to a strain in which the *KlMNN2-2* expression plasmid was replaced by the empty pRS415-GPD plasmid. A clear increase in the relative abundance of the target glycan was obtained in all three strains expressing *KlMNN2-2* under the control of different promoters. The most significant improvement in the relative abundance of the target glycan was achieved in the strain expressing *KlMNN2-2* under the control of constitutive *GPD1* promoter, in which the obtained relative abundance of the target glycan (G2M3) was 27 % (Fig. 3 and Table 3). The relative abundances of the interfering $Man_4GlcNAc_2$ (M4) and $Man_5GlcNAc_2$ (M5) glycans were correspondingly decreased by the *KlMNN2-2* expression. However, a slight increase in the relative abundance of the $Man_3GlcNAc_2$ glycan (M3) was unexpectedly observed in the strains expressing *KlMNN2-2*.

A dual approach for improving complex-type N-glycosylation

In order to combine the increased glycan pattern homogeneity obtained by the *MNN1* deletion with the improved target glycan formation described above, we expressed GnTI and II both with and without *KlMNN2-2* in the Δ*alg3* Δ*alg11* Δ*mnn1* strain YMP10, which was derived from the UV-mutagenized strain YG1429. As expected, the

glycan pattern homogeneity was improved; in addition to the target glycan, only one interfering structure, $Man_4GlcNAc_2$ (M4), was present in the cell wall glycans in significant amounts (Fig. 4a, b). Moreover, a significantly increased extent of complex-type glycosylation was obtained by the expression of *KlMNN2-2*. Also in this strain background, the highest relative abundance of the complex-type target glycan (G2M3, 32 %) was obtained upon *KlMNN2-2* expression under the control of *GPD1* promoter (Table 3).

After these successful glycan profile improvements were achieved, we tried to obtain a similarly improved glycan pattern in a corresponding strain with compensated glycosylation efficiency. However, in the Δ*alg3* Δ*alg11* Δ*mnn1* strains with compensated glycosylation efficiency and expressing GnTI/II (YMP15 and YMP16), the increase in the relative abundance of the G2M3 target glycan caused by *KlMNN2-2* expression was lower than was obtained in the strains without glycosylation efficiency compensation. After optimization of the growth medium for these strains, a slight increase in the relative abundance of the target glycan was observed upon *KlMNN2-2* expression under the control of *GPD1* promoter, resulting in a target glycan relative abundance of 25 % (Fig. 4c, d and Table 3).

Discussion

In this work we followed a dual approach to increase glycan homogeneity and to improve complex-type glycosylation in a glycoengineered Δ*alg3* Δ*alg11* yeast strain. Our earlier studies have indicated that the $Man_3GlcNAc_2$ structure presented on a protein to the Golgi localized GnTI and GnTII was unexpectedly also a substrate for the endogenous mannosyltransferases, leading to the generation of a mixture containing up to eight interfering glycan structures in addition to the complex-type target glycan [14]. These additional glycan structures were formed as a result of both interfering endogenous mannosyltransferase activity and incomplete processing of the tailored trimannosyl glycan to complex-type glycans by the GlcNAc transferases. Thus, we sought to reduce the amount of the interfering mannosyltransferase activity and to increase the activity of GnTI and GnTII by providing more nucleotide sugar substrate in the Golgi apparatus.

We improved the glycan pattern homogeneity by the deletion of the *MNN1* gene. Mnn1p is an α-1,3 mannosyltransferase that adds terminal mannose residues to the α-1,2 mannose branches of the outer chain in wild-type yeast glycans [10]. Based on the disappearance of $Man_{5-6}GlcNAc_2$ structures, *MNN1* also seems to be responsible for the addition of the fifth, α-1,3 linked mannose in the glycans of the Δ*alg3* Δ*alg11* strain. As the terminal α-1,3 linked mannoses have shown to be very immunogenic [10], the elimination of this mannose residue is an important feature considering the potential therapeutic applications of the proteins produced in glycoengineered yeast. However, $Man_4GlcNAc_2$ glycans were still present in the cell wall proteins of the glycoengineered yeast after *MNN1* deletion.

Considering the substrate specificities of Mnn1p and the glycan analysis of mannosidase digests conducted earlier on the glycoengineered strains [14], this enzyme seems to add the α-1,3 mannose residue to a tailored glycan that has already received an additional α-1,2 mannose. Therefore, the α-1,2 mannosyltransferase activity which adds the first additional mannose residue to the tailored glycan is the interfering reaction which competes for the glycan

donor with the GlcNAc transferases. As an attempt to eliminate this fourth mannose, we deleted *MNN2*, a gene encoding an α-1,2 mannosyltransferase which adds mannose to the α-1,6 linked mannoses of the outer chain [31]. However, no effect on the glycan pattern was observed.

Individual deletions of all other known Golgi-resident α-1,2 mannosyltransferases have been conducted earlier, but no single deletion has resulted in the elimination or decrease of the $Man_4GlcNAc_2$ structure [14]. Therefore, it is possible that the intefering α-1,2 mannose could be added by either an unidentified or uncharacterized mannosyltransferase or that multiple mannosyltransferases with redundant α-1,2 mannosyltransferase activities are responsible for this reaction.

A similar addition of an α-1,2 linked mannose to an α-1,6 mannose residue of a glycan also takes place in yeast when core-type glycans are formed. It is possible that the unknown enzyme responsible for the addition of the α-1,2 mannose to core-type glycans could be the same enzyme which adds the fourth mannose to our tailored glycan. Some results have indicated that components of M-PolI and M-PolII complexes, in particular Mnn9p, have both α-1,6 and α-1,2 mannosyltransferase activities *in vitro* [11, 12, 32]. It has therefore been speculated that Mnn9p, a member of both M-PolI and M-PolII complexes, could have a role in the formation of core-type glycans and their α-1,2 mannosylation [11, 33]. Therefore, subunits of the two mannan polymerase complexes, especially *MNN9*, would be interesting candidates for the search of the interfering mannosyltransferase. However, some studies using recombinant *ScMNN9* do not support the role of *MNN9* in α-1,2 mannosylation and suggest that the α-1,2 mannosylation found in the earlier studies may be a result of a contaminant [34, 35]. Other possibilities for the interfering mannosylation could be found in the *KTR* gene family, consisting of *KRE2, YUR1, KTR1, KTR2, KTR3, KTR4, KTR5, KTR6* and *KTR7* [36]. This family includes several mannosyltransferases the activities of which have not been characterized in detail, including Ktr2p, Ktr4p, Ktr5p and Ktr7p (Table 2). It has been reported that some enzymes in the *KTR* family have redundant activities and that Ktr1p, Ktr2p, Ktr3p, Yur1p and Kre2p would be partially responsible for α-1,2 mannosylation of N-glycans [36].

In our experiments, the expression of Kre2p-GnTI and Mnn2p-GnTII fusion proteins in the glycoengineered yeast resulted in the formation of a complex-type $GlcNAc_2Man_3GlcNAc_2$ glycan, indicating that endogenous import activity of UDP-GlcNAc to the Golgi apparatus takes place in *S. cerevisiae* to some extent. However, the expression of *KlMNN2-2* increased the relative abundance of the complex-type glycan, suggesting that the endogenous import rate of UDP-GlcNAc might be limiting efficient GlcNAc transferase activities in *S. cerevisiae*. In glycoengineering studies with *P. pastoris*, controversial results have been published regarding the necessity to express the UDP-GlcNAc transporter in order to obtain efficient hybrid or complex-type glycosylation [37, 38]. These differences in results may depend on strains and localization of the GlcNAc transferases [38].

In order to enhance UDP-GlcNAc transport, we expressed *KlMNN2-2* under the control of three different promoters. The increase in the relative abundance of the target glycan was the highest when *KlMNN2-2* was expressed under the control of the strongest promoter, *GPD1*. This was observed both in strains with and without glycosylation efficiency compensation, although the differences between promoters were not high. Since a higher expression level

of *KlMNN2-2* resulted in a higher increase in the extent of complex-type glycosylation, it can be postulated that a further increase of the expression of this gene, such as expression in a high copy-number plasmid, might lead to further increase in complex-type glycosylation efficiency.

In addition to optimization of single steps contributing to complex-type glycosylation such as UDP-GlcNAc transport, identification of the limiting steps for complex-type glycosylation as well as balancing of expression among all relevant genes and assessing all factors affecting complex-type glycosylation will help to further improve the glycan pattern and also to minimize potential excess metabolic burden caused by heterologous gene overexpression. This includes optimization of both expression levels, activities and localization of the relevant genes, but also investigating other genes and factors such as culture conditions, the effect of which on complex-type glycosylation are not yet well known in yeast. Our results imply that the import of UDP-GlcNAc limits complex-type glycosylation to some extent, and this limitation can be relieved by expression of a UDP-GlcNAc transporter. Our data also suggest that on coexpression of a UDP-GlcNAc transporter and GnTI/II, the GlcNAc transfer step may become a limiting step for complex-type glycosylation if expressed in a low copy number plasmid, as indicated by a somewhat lowered relative abundance of complex-type glycosylation (results not shown).

An aspect that should be taken into account in further optimization of efficient complex-type glycosylation and in identification of potential limiting reactions is that the energy required for the nucleotide sugar import into Golgi apparatus is obtained from the equimolar export of the corresponding nucleoside monophosphate, UMP in the case of UDP-GlcNAc [39]. UMP is formed when the UDP released from UDP-GlcNAc in the GlcNAc transfer reaction is cleaved by a uridine diphosphatase (UDPase). UDP has also been reported to act as an inhibitor of GlcNAc transferases [40]. Two enzymes with reported UDPase activity, Gda1p and Ynd1p, have been identified in *S. cerevisiae* [41, 42]. However, both of these enzymes have higher activity towards GDP than UDP. Since native glycan processing steps in the yeast Golgi apparatus do not require UDP-linked sugars, it is possible that the endogenous UDPase activities of the yeast Golgi apparatus are not high enough to cleave UDP at a rate required to reach efficient complex-type glycosylation, especially when overexpressing glycoproteins and when the glycosylation efficiency is not impaired, making UDPase activity a potential limiting step for complex-type glycosylation.

As a conclusion, the data in this work represent a further advance towards the production of a homogeneous and tailored glycan profile in *S. cerevisiae*, reflecting the potential of yeast for glycoprotein production for therapeutic purposes. Obtaining a homogeneous glycan profile will also enable research on single glycoforms and their impact on protein properties. In the future, rebuilding the glycosylation pathway can also allow possibilities beyond humanization of the N-glycan structures, enabling the formation of modified or new-to-nature glycoforms which may offer improved properties or novel functions for therapeutic as well as other industrially relevant proteins.

Competing interests

The authors declare that they have no competing interest.

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Tables

Table 1 *S. cerevisiae* strains and plasmids used in this study

Name	Genotype/description	Reference
YAF39	MATα *ade2-101 his3Δ200 lys2-801 ura3-52* Δ*alg3*::*HIS3* Δ*alg11*::*HIS3* Δ*leu2*::*KanMX4*::*LbSTT3_3*::*Flc2**	[14]
YG1429	MATα *ade2-101 his3Δ200 lys2-801 ura3-52* Δ*alg3*::*HIS3* Δa*lg11*::*HIS3* UV mutagenized and selected for better growth	[14]
YMP01	YG1429 Δ*mnn1*::*kanMX4*	This work
YMP05	YAF39 with pAX428 (*URA3*) and pMP001 (*LEU2*)	This work
YMP06	YAF39 with pAX428 (*URA3*) and pMP002 (*LEU2*)	This work
YMP07	YAF39 with pAX428 (*URA3*) and pMP003 (*LEU2*)	This work
YMP08	YG1429 Δ*mnn1*::*kanMX4* with pAX428 (*URA3*)	This work
YMP09	YAF39 with pAX428 (*URA3*) and pRS415-GPD1 (*LEU2*)	This work
YMP10	YG1429 Δ*leu2*::*natNT2* Δ*mnn1*::*kanMX4*	This work
YMP11	YG1429 Δ*leu2*::*natNT2* Δ*mnn1*::*kanMX4* with pAX428 (*URA3*) and pMP001 (*LEU2*)	This work
YMP12	YG1429 Δ*leu2*::*natNT2* Δ*mnn1*::*kanMX4* with pAX428 (*URA3*) and pMP002 (*LEU2*)	This work
YMP14	YAF39 Δ*mnn1*::*natNT2*	This work
YMP15	YAF39 Δ*mnn1*::*natNT2* with pAX428 (*URA3*) and pMP002 (*LEU2*)	This work
YMP16	YAF39 Δ*mnn1*::*natNT2* with pAX428 (*URA3*) and pRS415-GPD1 (*LEU2*)	This work
pAX428	Kre2-GnTI and Mnn2-GnTII fusion under control of *GAL1-10* promoter, *URA3* selection marker	[14]
pRS415-GPD	*LEU2* selection marker, *GPD1* promoter for gene expression	[15]
pMP001	Native *MNN2-2* from *K. lactis* in SpeI, XhoI of pRS415-*TEF1* promoter, *LEU2* selection marker	This work
pMP002	Native *MNN2-2* from *K. lactis* into SpeI, XhoI of pRS415-*GPD1* promoter, *LEU2* selection marker	This work
pMP003	Native *MNN2-2* from *K. lactis* into SpeI, XhoI of pRS415-*GAL1* promoter, *LEU2* selection marker	This work

Table 2 Golgi-localized mannosyltransferases of *S. cerevisiae* involved in N- and O-linked glycosylation

Gene Name	Description	Glycan acceptor substrate	Systematic Name	Glycosylation pathway
ANP1 = MNN8	Subunit of a Golgi mannosyltransferase complex[a]	α-1,6 mannose	YEL036C	N- and O-linked glycosylation
HOC1	Subunit of a Golgi mannosyltransferase complex[a]	α-1,6 mannose	YJR075W	N-linked glycosylation
KRE2 = MNTI	α-1,2-mannosyltransferase	α-1,2 mannose, non-terminal α-1,6 mannose, Man-O-Ser/Thr	YDR483W	N- and O-linked glycosylation
KTR1	α-1,2-mannosyltransferase	α-1,2 mannose, non-terminal α-1,6 mannose, Man-O-Ser/Thr	YOR099W	N- and O-linked glycosylation
KTR2	Mannosyltransferase	Unknown	YKR061W	N-linked glycosylation
KTR3	Putative α-1,2-mannosyltransferase	α-1,2 mannose, Man-O-Ser/Thr	YBR205W	N- and O-linked glycosylation
KTR4	Mannosyltransferase	Unknown	YBR199W	unknown
KTR5	Putative mannosyltransferase	Unknown	YNL029C	unknown
KTR7	Putative mannosyltransferase	Unknown	YIL085C	unknown
MNN1	α-1,3-mannosyltransferase	α-1,2 mannose	YER001W	N- and O-linked glycosylation
MNN2	α-1,2-mannosyltransferase	non-terminal α-1,6 mannose	YBR015C	N-linked glycosylation
MNN5	α-1,2-mannosyltransferase	α-1,2 mannose	YJL186W	N-linked glycosylation
MNN9	Subunit of a Golgi mannosyltransferase complex[a,b]	α-1,6 mannose	YPL050C	N-linked glycosylation
MNN10	Subunit of a Golgi mannosyltransferase complex[a]	α-1,6 mannose	YDR245W	N-linked glycosylation
MNN11	Subunit of a Golgi mannosyltransferase complex[a]	α-1,6 mannose	YJL183W	N-linked glycosylation
MNT2	α-1,3 mannosyltransferase	α-1,3 mannose (α-1,2 mannose)	YGL257C	O-linked glycosylation
MNT3	α-1,3-mannosyltransferase	α-1,3 mannose (α-1,2 mannose)	YIL014W	O-linked glycosylation
MNT4	Putative α-1,3-mannosyltransferase	Unknown	YNR059W	unknown
OCH1	α-1,6 mannosyltransferase	non-terminal α-1,3 mannose	YGL038C	N-linked glycosylation
VAN1	Subunit of a Golgi mannosyltransferase complex[b]	α-1,6 mannose	YML115C	N-linked glycosylation
YUR1	Mannosyltransferase	Unknown	YJL139C	N-linked glycosylation

[a] Mnn9p, Anp1p (= Mnn8p), Mnn10p, Mnn11p and Hoc1p form a complex

[b] Van1p and Mnn9p form a complex

Table 3 Relative abundances of N-glycans isolated from cell wall glycoprotein samples, presented as percentages of total glycans based on the relative peak intensities of the 2-AB labeled glycans in MALDI-TOF spectra. Average values (N=2) for peaks having a relative abundance of at least 1 % are shown

Strain	*ΔMNN1*	*KlMNN2-2* [a]	M3[b]	M4 [b]	GM3 [b]	M5 [b]	GM4 [b]	G2M3 [b]	M6 [b]
YMP09[c]	No	-	7 %	54 %	-	16 %	2 %	19 %	1 %
YMP05[c]	No	*TEF1*	11 %	47 %	-	13 %	1 %	26 %	2 %
YMP06[c]	No	*GPD1*	12 %	46 %	1 %	11 %	1 %	27 %	1 %
YMP07[c,d]	No	*GAL1*	10 %	52 %	-	12 %	-	25 %	1 %
YMP16[c,e]	Yes	-	6 %	65 %	-	2 %	4 %	22 %	-
YMP15[c,e]	Yes	*GPD1*	5 %	66 %	-	2 %	2 %	25 %	-
YMP08	Yes	-	4 %	74 %	1 %	-	2 %	18 %	-
YMP11	Yes	*TEF1*	8 %	62 %	-	-	-	28 %	-
YMP12	Yes	*GPD1*	7 %	60 %	-	-	-	32 %	-

[a] Promoter used for the expression of *KlMNN2-2*

[b] M3=$Man_3GlcNAc_2$, M4=$Man_4GlcNAc_2$, GM3=$GlcNAcMan_3GlcNAc_2$, M5=$Man_5GlcNAc_2$, GM4=$GlcNAcMan_4GlcNAc_2$, G2M3=$GlcNAc_2Man_3GlcNAc_2$, M6=$Man_6GlcNAc_2$

[c] Strains contain Flc2*p and POT for glycosylation efficiency compensation

[d] N=1

[e] Strain grown in minimal medium

Figure legends

Fig. 1 *MNN1* deletion improves glycan homogeneity. MALDI-TOF MS spectra of 2-AB labeled N-glycans isolated from the cell wall glycoproteins of Δ*alg3* Δ*alg11* strain YG1429 (a), Δ*alg3* Δ*alg11* Δ*mnn1* strain YMP01 (b) and YMP14, an Δ*alg3* Δ*alg11* Δ*mnn1* strain containing FLC2* and POT (c). Strains were grown in YPD medium at 30 °C and collected at mid-log phase. The peaks at m/z 1053, 1215, 1377, 1539 and 1701 correspond to the sodium adducts of glycan structures $Man_3GlcNAc_2$ (M3), $Man_4GlcNAc_2$ (M4), $Man_5GlcNAc_2$ (M5), $Man_6GlcNAc_2$ (M6) and $Man_7GlcNAc_2$ (M7), respectively

Fig. 2 Deletion of the *MNN1* gene is accompanied by a minor growth defect. Growth curves of YG1429 (Δ*alg3* Δ*alg11*) and YMP01 (Δ*alg3* Δ*alg11* Δ*mnn1*) duplicate cultures grown in YPD medium at 30 °C. ■ YG1429; ● YMP01

Fig. 3 Expression of a UDP-GlcNAc transporter increases the relative abundance of the complex-type $GlcNAc_2Man_3GlcNAc_2$ glycan. MALDI-TOF MS spectra of 2-AB labeled N-glycans isolated from the cell wall glycoproteins of FLC2* and POT containing Δ*alg3* Δ*alg11* strains YMP09 (a) and YMP06 (b). Both strains carry plasmid pAX428 for GnTI and GnTII expression, and YMP06 expresses *KlMNN2-2* under the control of *GPD1* promoter. Strains were grown in SD medium supplemented with 0.2 M sorbitol at 28 °C, induced at OD_{600} 1.0 with 2% galactose, and samples were collected 24h after induction. The peaks at m/z 1053, 1215, 1257, 1377, 1418, 1460 and 1540 correspond to the sodium adducts of glycan structures $Man_3GlcNAc_2$ (M3), $Man_4GlcNAc_2$ (M4), $GlcNAcMan_3GlcNAc_2$ (GM3), $Man_5GlcNAc_2$ (M5), $GlcNAcMan_4GlcNAc_2$ (GM4), $GlcNAc_2Man_3GlcNAc_2$ (G2M3) and $Man_6GlcNAc_2$ (M6), respectively. Squares, GlcNAc; circles, mannose

Fig. 4 Expression of a UDP-GlcNAc transferase combined with the *MNN1* deletion in the Δ*alg3* Δ*alg11* strain results in an improved glycan pattern containing fewer interfering structures and increased relative abundance of the target glycan. The improvement in the relative abundance of the target glycan through *KlMNN2-2* expression is more pronounced in a strain without compensated glycosylation efficiency. MALDI-TOF MS spectra of Δ*alg3* Δ*alg11* Δ*mnn1* strains YMP08 (a), YMP12 (b), YMP16 (c) and YMP15 (d). All strains carry plasmid pAX428 for expression of GnTI and GnTII, and strains YMP12 and YMP15 also express *KlMNN2-2* under the control of *GPD1* promoter. Strains YMP16 and YMP15 contain FLC2* and POT for compensated glycosylation efficiency. Strains were grown at 30 °C, induced at OD_{600} 1.0 with 2 % galactose, and samples were collected 24h after induction. YMP08 and YMP12 were grown in SD medium and strains YMP15 and YMP16 in minimal medium containing 0.5 M sorbitol. The peaks at m/z 1053, 1215, 1256, 1377, 1418 and 1460 correspond to sodium adducts of glycan structures $Man_3GlcNAc_2$ (M3), $Man_4GlcNAc_2$ (M4), $GlcNAcMan_3GlcNAc_2$ (GM3), $Man_5GlcNAc_2$ (M5), $GlcNAcMan_4GlcNAc_2$ (GM4) and $GlcNAc_2Man_3GlcNAc_2$ (G2M3), respectively. Squares, GlcNAc; circles, mannose