A Combined System for Engineering Glycosylation Efficiency and Glycan Structure in *Saccharomyces cerevisiae*

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We describe a novel synthetic N-glycosylation pathway to produce recombinant proteins carrying human-like N-glycans in *Saccharomyces cerevisiae*, at the same time addressing glycoform and glycosylation efficiency. The $\Delta alg3 \Delta alg11$ double mutant strain, in which the N-glycans are not matured to their native high-mannose structure, was used. In this mutant strain, lipid-linked $Man_3GlcNAc_2$ is built up on the cytoplasmic side of the endoplasmic reticulum, flipped by an artificial flippase into the ER lumen, and then transferred with high efficiency to the nascent polypeptide by a protozoan oligosaccharyltransferase. Protein-bound $Man_3GlcNAc_2$ serves directly as a substrate for Golgi apparatus-targeted human N-acetylglucosaminyltransferases I and II. Our results confirmed the presence of the complex human-like N-glycan structure $GlcNAc_2Man_3GlcNAc_2$ on the secreted monoclonal antibody HyHEL-10. However, due to the interference of Golgi apparatus-localized mannosyltransferases, heterogeneity of N-linked glycans was observed.

More than 70% of the approved biopharmaceuticals are glycosylated proteins (e.g., antibodies and growth factors). The glycan structures influence biophysical and biochemical properties of a protein, like solubility, stability, serum half-life, bioactivity, receptor binding, and immunogenicity (1, 2). A common feature of protein glycosylation is its natural heterogeneity with respect to single glycan structures and of N-glycosylation site usage (3).

Glycosylation patterns are species specific, but most therapeutic glycoproteins are currently produced in mammalian cells of nonhuman origin, leading to small but significant alterations in glycosylation patterns relative to those of human serum. Cell lines currently used by industry include CHO cells, mouse myeloma cells (NSO and SP2/O), and hybridomas; human-derived cells, such as HEK293 or Per.C6, are not widely distributed (4). Bacteria, yeasts, and fungal cells are commonly used when posttranslational modifications are considered unimportant (5). The growing demand for production capacity for therapeutic glycoproteins is driving extensive efforts to generate alternative expression systems for glycoproteins, including plants (6), yeast, fungi (7), and bacteria (8). The most challenging issues of these alternative expression systems are to prevent the introduction of non-human-like, potentially immunogenic sugar moieties and to reproduce the human N-glycosylation patterns or even tailor them for production of highly active therapeutic glycoproteins (9).

N-linked glycosylation is initiated at the cytosolic side of the endoplasmic reticulum (ER) membrane, where in a stepwise manner $Man_5GlcNAc_2$ is assembled on dolichol pyrophosphate (PP-Dol) (Fig. 1). The $Man_5GlcNAc_2$ -PP-Dol is flipped into the ER lumen and is further glycosylated. The $Glc_3Man_9GlcNAc_2$ glycan structure assembled on PP-Dol is transferred to asparagine in certain glycosylation sequons (N-X-S/T, where X is any amino acid except P) of substrate proteins by oligosaccharyltransferase (OST) complex in the ER lumen. The N-linked $Glc_3Man_9GlcNAc_2$ glycan structure is subsequently trimmed to $Man_8GlcNAc_2$, after which the glycoprotein is transported to the Golgi apparatus (10).

N-glycan diversity is mainly generated by trimming and mod-

ification of the N-glycan structure in the Golgi apparatus, creating a plethora of species-specific structures. Despite species-specific differences, the trimannosyl core N-glycan structure ($Man_3GlcNAc_2$) is common to all biosynthetic N-glycans (11). In yeast, the highly conserved precursor $Man_8GlcNAc_2$, which is leaving the ER compartment, is further mannosylated (12, 13). Golgi modifications in yeast start with the addition of an α -1,6-mannose by Och1p to the α -1,3-mannose of the trimannosyl core. To form the high-mannose glycan structure, the outer chain is elongated by sequential action of two protein complexes, mannan polymerase I and II. α -1,6-Mannose is further elongated by addition of more α -1,2-mannoses by Mnn2 and Mnn5 proteins, mannosylphosphate by Mnn4 and Mnn6 proteins, and finally capping α -1,3-mannoses by Mnn1p. Core-type N-glycans lack the outer chain and have only an α -1,2-mannose (mannosylphosphate) and α -1,3-capping mannoses (12).

In mammals, the $Man_8GlcNAc_2$ glycan is trimmed to $Man_5GlcNAc_2$ by an α -1,2-mannosidase (ManI) enzyme. The first $GlcNAc$ then is added by N-acetylglucosaminyltransferase I (GnTI). The $GlcNAcMan_5GlcNAc_2$ glycan is further trimmed by a second α -mannosidase activity (ManII), and the second $GlcNAc$ is added by N-acetylglucosaminyltransferase II (GnTII). Galactose, fucose, and/or additional $GlcNAc$ residues then can be transferred by various glycosyltransferases. The final step is the eventual terminal capping of the N-glycans with sialic acid (11).

The pivotal step in the humanization of N-glycosylation in yeast is the generation of the substrates for GnTI and GnTII by the

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FIG 1 Cartoon of the N-glycan biosynthesis pathways in mammals and yeast, of the engineered N-glycosylation in *Pichia pastoris*, and of the artificial N-glycan biosynthesis pathway in *S. cerevisiae*. (A) The lipid-linked oligosaccharide (LLO) synthesis in the ER (dark gray) is a highly conserved process. Dolichol-phosphate serves as the acceptor for the first phosphate-activated GlcNAc residue. Lipid-linked $\text{Man}_3\text{GlcNAc}_2$ is flipped from the cytoplasmic face of the ER membrane into the ER lumen and is further elaborated by Alg3 and other luminal mannosyl- and glucosyltransferases. The final structure, $\text{Glc}_3\text{Man}_3\text{GlcNAc}_2$, is the preferred substrate for the oligosaccharyltransferase (OST) complex and is transferred to the nascent polypeptide. After trimming of three glucose and one mannose residues, N-glycosylation pathways of mammals and yeast diverge in the Golgi apparatus (light gray). In mammals, the Golgi resident mannosidase I (ManI) trims three mannose residues, leaving a $\text{Man}_3\text{GlcNAc}_2$ structure, which is the substrate for GnTI. Mannosidase II (ManII) trims two additional mannose residues, generating the substrate for GnTII. Numerous additional enzymes catalyze the transfer of galactose, fucose, and sialic acid and of additional branching GlcNAc residues. In contrast, N-glycan processing in the Golgi apparatus of *S. cerevisiae* comprises only attachment of mannose residues. These additions are catalyzed by a number of mannosyltransferases, which differ in their specificities for the substrate and in the type of linkage. The final structures are highly mannosylated N-glycans with various numbers of added Man residues (indicated by “n”). (B) Alternative N-linked glycosylation pathway in *P. pastoris*. A $\text{Man}_3\text{GlcNAc}_2$ structure is generated in the yeast by deleting the *alg3* gene. The $\text{Man}_3\text{GlcNAc}_2$ structure is a poor substrate for glucosyltransferases and the endogenous OST complex. The substrate for GnTI is generated by expression of ManI activity, either as an ER-retained fusion or targeted into the Golgi apparatus. The $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ structure is generated by overexpression of GnTII. (C) In glycoengineered *S. cerevisiae*, the trimannosyl core structure serves as a basis to generate complex N-glycans. The lipid-linked $\text{Man}_3\text{GlcNAc}_2$ structure is generated by the deletion of the two mannosyltransferases (Δalg3 and Δalg11). The deletion of these two genes abrogates LLO biosynthesis, giving rise to the lipid-linked $\text{Man}_3\text{GlcNAc}_2$ structure. An artificial flippase (Flc2*P) and a protozoan OST (POT) are employed to increase glycosylation efficiency in this system. The N-linked $\text{Man}_3\text{GlcNAc}_2$ structure serves as a substrate for Golgi apparatus-targeted GnTI and GnTII, giving rise to the complex N-glycan

introduction of heterologous mannosidase activities. Furthermore, early experiments indicated the importance of proper localization of the heterologous enzymes in the secretory pathway for complete activity (reviewed in reference 7).

Using a combinatorial library composed of fragments encoding yeast-derived ER- and Golgi membrane-localizing leader peptides and catalytic domains of vertebrate glycosidases and glycosyltransferases, human-like hybrid and complex N-glycans were generated in *Pichia pastoris* (14–16). The substrate for GnTI is generated by the activity of an α -1,2-mannosidase activity targeted to the ER-*cis* Golgi compartment. After transfer of the GlcNAc residue, the substrate for GnTII is created by the activity of Golgi-targeted ManII activity. A simplification of the system was achieved by using a *P. pastoris* strain devoid of the *ALG3* gene in the lipid-linked oligosaccharide (LLO) biosynthesis pathway, leading to the generation of a truncated N-glycan structure and necessitating the insertion of a ManI activity only (17). At the moment, terminally galactosylated or sialylated N-glycans are being produced in *P. pastoris* (18, 19).

Although the initial steps in the expression of heterologous glycosyltransferases have been performed in *Saccharomyces cerevisiae* (20), baker’s yeast has not been the focus of glycoengineering. However, in contrast to *P. pastoris*, *S. cerevisiae* has a long history of approved therapeutic proteins which are used, including insulin, glucagon, hirudin, human growth hormone, and vaccines (21).

In this work, we describe a synthetic biology approach to generate humanized N-glycans in *S. cerevisiae*. We have used a strain with deletions in *ALG11* and *ALG3* genes involved in LLO synthesis to generate genetically tailored lipid-linked $\text{Man}_3\text{GlcNAc}_2$ structures. The $\text{Man}_3\text{GlcNAc}_2$ structure then serves as a substrate for Golgi-localized GnTI and GnTII. Our data confirmed the presence of the complex-type human-like glycans on a model antibody. The strategy disclosed here obviates the need for the introduction of heterologous mannosidase activities. Furthermore, besides the N-glycan structure we also address N-glycosylation efficiency (Fig. 1).

MATERIALS AND METHODS

Strains. All strains are described in Table 1. Strains YG1429 and YG1430 were derived from YG1363 after UV mutagenesis and were selected for better growth.

Cloning. All recombinant DNA work has been done according to standard protocols using *Escherichia coli* TOP10 (Invitrogen) as the expression host. Genes encoding STT3 proteins were isolated from *Leishmania brasiliensis* and *L. infantum* using PCR and were inserted into pCR2.1-TOPO (Invitrogen) for sequencing. Genomic DNAs were obtained from the Swiss Tropical and Public Health Institute, Basel, Switzerland. Isolation and cloning of *L. major* LmSTT3D and *Trypanosoma brucei* STT3 (TbSTT3B and TbSTT3C) genes has been described before (22, 23). For expression, all genes have been inserted into SpeI and XhoI restriction sites of the yeast shuttle vectors pRS426-GPD and pRS416-GPD. All transferases except for TbSTT3B and TbSTT3C were C-terminally tagged with a single hemagglutinin (HA) tag (YPYDVPDYA). TbSTT3B and TbSTT3C contained a combined C-terminal Myc and hexahistidine tag (EQKLISEEDLNSAVDHHHHHH). Cloning and isolation of a truncated version of yeast FLC2, termed FLC2* (NC_001133.9; bp 1

GlcNAc $_2$ Man $_3$ GlcNAc $_2$ in yeast cells. No trimming of mannose residues is required in this artificial N-glycan biosynthesis pathway. Gray circle, mannose; black circle, glucose; black square, N-acetylglucosamine.

TABLE 1 Strains used in this study

Name	Genotype	Reference or source
YG1363	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg11::HIS3</i>	18
YG1365	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg11::HIS3</i>	18
YG1429/YG1430	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg11::HIS3</i> UV mutagenized and selected for better growth	This work
YAF39	YG1429, <i>MATα ade2-101 his3Δ200 lys2-801 ura3-52 Δalg3::His3 Δalg11::His3 Δleu2::KanMX4::LbSTT3_3::Flc2*</i>	This work
YAF49	YAF39 with pAX428 (Ura3) and pAX550 (Leu2)	This work
YFP149	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg11::HIS3 Δmnn1::HIS3MX</i>	This work
YFP157	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg11::HIS3 Δmnn2::KanMX4</i>	This work
YFP159	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg11::HIS3 Δktr1::KanMX4</i>	This work
YFP160	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg11::HIS3 Δmnn5::KanMX4</i>	This work
YFP163	<i>MATα ade2-101 ura3-52 his3Δ200 Δalg3::HIS3 Δalg11::HIS3 Δktr3::KanMX4</i>	This work
YFP168	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg11::HIS3 Δkre2::KanMX4</i>	This work

to 1323), has been described (C. Neupert, F. M. Pujol, H. Savilahti, A. J. Kastaniotis, K. Hiltunen, and M. Aebi, unpublished data). FLC2*, under the control of its native promoter, is present as an SmaI-NheI fragment on YEplc2* but is lacking a terminator sequence. A HindIII site was inserted 3' of the last transmembrane domain of FLC2* by PCR to allow in-frame insertion of an HA tag followed by the CYC1 terminator. The CYC1 terminator was amplified by PCR, extending its 5' end by the coding sequence of the HA epitope followed by a stop codon, and inserted into HindIII NheI restriction sites of YEplc2*. An additional BglII restriction site was inserted between the terminator and NheI restriction site. Expression of FLC2* under the control of the *PGK1* promoter has been achieved by exchanging the SbfI-EcoNI fragment comprising the native promoter and the 5' end of FLC2* with the *PGK1* promoter isolated from genomic DNA using PCR. To enable promoter exchange, its 3' end then was elongated with 27 bp of the 5' end of FLC2* sequence.

The composite system was constructed by inserting the Ecl136II-Acc65I fragments, encompassing promoter, multiple cloning site, and terminator from pRS426-GPD and pRS426-TEF, into SmaI-Acc65I restriction sites of the FLC2* expression plasmids. Finally, the transferases were inserted into SpeI-XhoI restriction sites. Four versions of the composite system were generated, comprising LmSTT3D and LbSTT3_3, which were under the control of either the *TEF* or *GPD* promoter, respectively. To allow coexpression with GnTI and GnTII, the BglII-SacI DNA fragments comprising the composite system were transferred into BamHI-SacI sites of YEplac181 carrying a *LEU2* selection marker, generating the plasmids pAX447, containing LmSTT3D, and pAX448, containing LbSTT3_3. For integration into the *LEU2* locus of the genome of *S. cerevisiae*, the BglII-Ecl136II DNA fragments comprising the composite system were placed into BglII-SmaI sites of pRS305K, generating plasmids pAX451 (containing LmSTT3D) and pAX453 (containing LbSTT3_3).

Human GnTI and GnTII genes were isolated from genomic DNA clones (genomic clones 737 and 753; Image). Fusion of yeast leader sequences and catalytic domains of GnTI and GnTII have been constructed as described previously (24). They are composed of amino acids 1 to 100 of *S. cerevisiae* Kre2p and amino acids 103 to 445 of human GnTI. The fusion of *S. cerevisiae* Mnn2-GnTII comprises amino acids 1 to 36 of Mnn2p and 88 to 443 of human GnTII. Both constructs have been Flag tagged (DYKDDDDK). Kre2-GnTI and Mnn2-GnTII fusions were inserted into SpeI-HindIII and SpeI-EcoRI restriction sites, respectively, of pRS426-GPD. A PCR-generated and XhoI-Acc65I-digested PGK1 terminator fragment was used to replace the CYC1 terminator of the plasmid carrying the GnTI fusion. For expression of the GnTI and GnTII fusions,

the glycosyltransferases were inserted into pRS426, carrying the bidirectional yeast native *GAL1-10* promoter, placing Kre2-GnTI under the control of *GAL1* and Mnn2-GnTII under the control of the *GAL10* promoter. First, the fragment comprising Mnn2-GnTII and the CYC1 terminator was isolated using SpeI digestion, followed by filling in of the overhang using the Klenow fragment and NdeI digestion, and the resulting fragment was inserted into the EcoRI restriction site and made blunt using the Klenow fragment and NdeI restriction site of pRS426-GAL1-10. Kre2-GnTI followed by PGK1 terminator sequences was isolated using Acc65I digestion, followed by filling in of the overhang using the Klenow fragment and SpeI digestion, and the resulting fragment was inserted into Ecl136II-SpeI restriction sites, generating plasmid pAX428.

A hybrid human monoclonal antibody directed against hen egg lysozyme (HyHEL IgG) was created. The HyHEL-10 IgG heavy and light chains were constructed based on protein sequences derived from 1CO8_A (V_L) and 1CO8_B (V_H), the crystal structure of a hen egg lysozyme binding HyHEL-10 Fab molecule. The V_L and V_H sequences of HyHEL-10 molecules were fused to the corresponding human IgG1 C_L or C_{H1}, C_{H2}, and C_{H3} sequences, respectively, generating light and heavy chains. Based on the literature, an evolved yeast *MATα* pre- and propeptide sequence was selected to secrete IgG into the culture medium (25). All DNA sequences were codon optimized for expression in *S. cerevisiae* and assembled by DNA synthesis (Geneart, Germany). Expression of light and heavy chains was driven by the galactose-inducible *GAL1* promoter, and both expression cassettes were combined on the low-copy-number plasmid pRS415-*GAL1* using a *LEU2* selection marker, generating plasmid pAX550.

Transformation of yeast strains. The lithium acetate method was used for transformation of yeast cells. For integration of a composite system into the *LEU2* locus of YG1363 and YG1429, plasmid DNA was linearized and yeast strains were transformed. After transformation, cells were grown overnight in nonselective medium and then transferred to medium supplemented with 200 μg/liter G418.

Generation of mannosyltransferase deletion strains. The *MNN1* locus was deleted in yeast wild-type cells by replacement with a PCR product containing the *HIS3MX* cassette and flanking *MNN1* coding sequences. The *Δmnn1* null deletion strain was combined with the *Δalg3* deletion strain (YG247) by mating the two mutant strains. The resulting diploid strain was sporulated, and tetrads were dissected. A haploid strain carrying both *mnn1* and *alg3* deletions was selected, and the deletions were further confirmed by genomic DNA (gDNA) PCR analysis. To generate the *Δalg3 Δalg11 Δmnn1* triple mutant strain, the constructed *Δalg3*

$\Delta mnn1$ haploid double mutant strain was mated with strain YG1363 ($\Delta alg3 \Delta alg11$). The resulting diploid strain was sporulated, and tetrads were dissected. A haploid triple mutant strain containing all three deletions, $\Delta alg11$, $\Delta alg3$, and $\Delta mnn1$, was selected, and the deletions were further confirmed by gDNA PCR analysis.

Deletion of α -1,2-mannosyltransferase genes was done in the $\Delta alg3 \Delta alg11$ strain carrying the *ALG11* gene on a *URA3* plasmid. The target gene loci were deleted by replacement with a PCR product containing the *KanMX4* cassette and flanking target gene-coding sequences. Deletions were verified by gDNA PCR analysis. Counterselection on 5-fluoroorotic acid was used to select for cells that had lost the *ALG11* plasmid.

Growth of yeast strains. All medium components were obtained from Difco (yeast nitrogen base [YNB], raffinose, and galactose), Sigma (amino acids), and Applichem (sorbitol). Growth of the temperature-sensitive strain YG1363 was done at 25°C. The UV-mutagenized strains YG1429 and YG1430 were grown at 30°C. All experiments were done in batch mode using shake flasks.

For optimizing N-glycosylation efficiency, cells were grown in synthetic dropout medium lacking uracil and were supplemented with 2% glucose and 1 M sorbitol.

For initial N-glycan analysis, cells were grown in synthetic dropout medium lacking uracil supplemented with 1% raffinose and 1 M sorbitol. Induction of Kre2-GnII and Mnn2-GnTII expression was done at an A_{600} of 0.5, and cells were grown for the indicated times. For expression in glycoengineered yeast, we used a chemically defined medium containing 6.7 g/liter YNB, 30 mg/liter lysine, 20 mg/liter adenine, 1% raffinose, and 0.25 M sorbitol. Kre2-GnTI- and Mnn2-GnTII-expressing cells were induced at an A_{600} of 0.5 with 2% galactose. Cells were grown at 25°C with shaking at 180 rpm.

For expression of IgG in glycoengineered yeast, a synthetic dropout medium lacking uracil and leucine was used. The medium was supplemented with 0.25 M sorbitol. Precultures were grown in 1% raffinose. Induction of IgG and of Kre2-GnTI and Mnn2-GnTII expression was done by addition of 2% galactose. Expression was done at 20°C with shaking at 180 rpm for 36 h. To stabilize secreted IgG, bovine serum albumin (to a final concentration of 0.05 mg/ml; Sigma) and protease inhibitor cocktail without EDTA (0.5 times the recommended concentration; Roche) were added to the cultures.

Purification of protein. Secreted IgG was purified using affinity chromatography. Culture supernatant was cleared by centrifugation at $15,000 \times g$ at 4°C for 20 min. Cleared culture broth was adjusted to 300 mM NaCl, 20 mM Tris, pH 7.5, and passed through a 0.22- μ m-filter unit. The filtrate was loaded on a protein G Sepharose column (GE Healthcare) with a 1-ml bed volume at a flow rate of 1 ml/min. The column was washed with 10 column volumes of 300 mM NaCl, 20 mM Tris, pH 7.5, and the bound IgG was eluted with 300 mM NaCl, 100 mM glycine, pH 2.7. One-ml fractions were collected and the pH was neutralized by the addition of 50 μ l 2.5 M Tris, pH 8.5.

N-glycan isolation from cell wall proteins and purified protein. N-glycans from purified proteins were released using 1 μ l of 500 U/ μ l PNGase F (New England Biolabs) according to the manufacturers' instructions. The N-glycans were purified using C_{18} (Sep-Pak C_{18} Cartridges; Waters) and a graphitized carbon column (Supelco; Supelco ENVI-Carb).

N-glycans from cell wall protein were isolated as described previously (26) but using 1 μ l of 500 U/ μ l PNGase F instead of EndoH. As starting material, cell pellets corresponding to 50 optical density (OD) units of cells measured at 600 nm were used. Released glycans were purified using C_{18} and a graphitized carbon column.

For matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) analysis, N-glycans were permethylated or 2-aminobenzamide (2-AB) labeled. 2-AB labeling was performed as described previously (27). For permethylation, purified and dried N-glycans were dissolved in a slurry of finely ground NaOH pellets in dimethyl sulfoxide (~0.2 ml), followed by addition of 0.1 ml of methyl iodide. The

reaction mixture was vortexed for 20 min at room temperature and quenched with 0.2 ml of water, followed by chloroform–water extraction, and the permethylated N-glycans were dried under an air stream.

Glycosidase treatment. 2-AB-labeled glycans were resuspended in 50 mM sodium citrate (pH 5) for α -mannosidase (20 U/mg; Jack bean; Sigma) treatment and in 50 mM sodium acetate (pH 5.2) for α -1,2 mannosidase treatment (*Trichoderma reesei*; a gift from R. Contreras, University of Ghent). 2-AB-labeled glycans were resuspended in 20 mM sodium citrate-phosphate (pH 6.0) and treated with β -galactosidase (Jack bean; PROzyme). Reaction mixtures were incubated overnight at 37°C. Glycans were desalted and purified from enzymes with a small-scale carbon column (27).

Mass spectrometry. MALDI-TOF MS and tandem MS (MS/MS) analysis of permethylated and 2-AB-labeled glycans were performed on a 4800 Proteomics Analyzer (Applied Biosystems) operated in the positive reflectron mode. For MS acquisition, the samples were dissolved in acetonitrile and mixed 1:1 with 2,5-dihydroxybenzoic acid (DHB) matrix (10 mg/ml in 50% acetonitrile) for spotting onto the target plate. For MS/MS analysis, the potential difference between the source acceleration voltage and the collision cell was set to 2 kV to obtain the high-energy collision-induced dissociation (CID) fragmentation pattern. Air was used as the collision gas, and the structural assignment was performed as described in detail previously (28).

Quantification of [3 H]mannose-labeled LLO using HPLC. To analyze lipid-linked oligosaccharides (LLOs), 2×10^9 cells in the exponential growth phase were labeled for 20 min at room temperature in 450 μ l YPD containing 0.1% dextrose, 1 M sorbitol, and 200 μ Ci [3 H]mannose (21.6 Ci/mmol) (Hartmann Analytic GmbH, Glattbrugg, Switzerland). LLO extraction and high-performance liquid chromatography (HPLC) analysis of the radiolabeled oligosaccharides were performed as described by Zufferey et al. (29).

SDS-PAGE and immunoblotting. Cell extracts were prepared in SDS-PAGE sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol) supplemented with protease inhibitor cocktail (Roche). Equal amounts of cell extracts were resolved on SDS-polyacrylamide gels. Separated proteins were electrophoretically transferred onto Immobilon-nitrocellulose membranes at 25 mA and room temperature. All incubation and washing steps were performed in 1% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20. The primary antibodies were directed against carboxypeptidase Y (CPY) (30), Flag tag (Sigma), and HA tag (Santa Cruz). A horseradish peroxidase (HRP)-conjugated antibody directed against the F_c portion of human IgG was obtained from Sigma. Secondary antibodies were anti-rabbit IgG horseradish peroxidase conjugate and anti-mouse IgG horseradish peroxidase conjugate (Santa Cruz). Chemiluminescent detection was performed with ECL Western blotting detection reagent (Pierce, Rockford, IL) according to the suppliers' recommendations.

RESULTS

Generation of lipid-linked trimannosyl core structure Man₃GlcNAc₂-PP-Dol. A *S. cerevisiae* strain producing exclusively the trimannosyl core structure Man₃GlcNAc₂ has been described before, and it was observed that overexpression of Rft1p improved growth of the $\Delta alg3 \Delta alg11$ strain (31). In this strain, deletion of *alg3* and *alg11* genes abrogates LLO synthesis before and after flipping of the LLO into the ER lumen, generating Man₃GlcNAc₂-PP-Dol. HPLC analysis of the glycan moiety of [3 H]mannose-labeled and hydrolyzed LLO isolated from the $\Delta alg11$ strain revealed the presence of three distinct species, including Man₃GlcNAc₂, Man₆GlcNAc₂, and Man₇GlcNAc₂. Upon the additional deletion of the *alg3* gene, Man₆GlcNAc₂ and Man₇GlcNAc₂ disappeared and only Man₃GlcNAc₂ was present in the HPLC profile (Fig. 2), revealing that a single defined species is generated. LLOs, which are incompletely assembled on the cyto-

FIG 2 $\Delta alg3 \Delta alg11$ yeast strain produces $Man_3GlcNAc_2$. Shown are HPLC profiles of [3H]mannose-labeled LLO isolated from $\Delta alg11$ (YG1365) (A) and $\Delta alg3 \Delta alg11$ (YG1363) (B) yeast strains. Deletion of the *alg11* gene yields three structures, $Man_3GlcNAc_2$ (M3), $Man_6GlcNAc_2$ (M6), and $Man_7GlcNAc_2$ (M7). The intermediate structure, $Man_3GlcNAc_2$, is accumulating, since flipping of this structure is limited, but it is further elongated after flipping by Alg3p and other luminal mannosyltransferases. Deletion of the *alg11* gene in combination with the *alg3* gene yields the target structure $Man_3GlcNAc_2$ (M3). The further elaboration of this structure is blocked by the deletion of the *alg3* gene.

plasmic side of the ER membrane, can still be flipped into the ER lumen and, from there, transferred to proteins. However, the strain displays severe hypoglycosylation and a growth retardation phenotype (Fig. 3; see Fig. S2 in the supplemental material).

Improving N-glycosylation efficiency. Efficient N-linked protein glycosylation depends on two essential processes, the flipping of the LLO across the ER membrane into the ER lumen and on the subsequent transfer of the oligosaccharide from the lipid donor to the nascent peptide chain.

However, genetically tailored LLOs, such as $Man_3GlcNAc_2$ -PP-Dol, are only poor substrates for the endogenous flippase and OST complex. Analysis of N-glycosylation efficiency of the endogenous model glycoprotein carboxypeptidase Y (CPY) in the parental strain revealed a strong hypoglycosylation pattern (Fig. 3, lane 1). Therefore, to compensate for the reduced N-glycosylation efficiency in the $\Delta alg3 \Delta alg11$ strain, the flux through the LLO biosynthesis pathway and the transfer of the glycans to the nascent polypeptide must be improved in order to restore the full N-glycosylation of proteins.

The protein catalyzing the flipping of the LLO into the ER lumen has been shown to be encoded by the *RFT1* gene (31). Recently, a C-terminally truncated version of the endogenous ER-localized yeast protein Flc2p (named Flc2*p) was shown to functionally replace Rft1p, restoring growth and N-glycosylation defects. Furthermore, Flc2*p, in contrast to Rft1p, displayed a more relaxed substrate specificity toward truncated LLO (C. Neupert et al., unpublished). Therefore, we evaluated whether overexpres-

FIG 3 Coexpression of an artificial flippase (Flc2*p) and the protozoan oligosaccharyltransferase LmSTT3D increases N-glycosylation efficiency in the $\Delta alg3 \Delta alg11$ strain (YG1363). Protein extracts were separated on SDS-polyacrylamide gels. After transfer of proteins, the membrane was analyzed using an anti-CPY antibody. CPY has four N-glycosylation sites, and hypoglycosylation is manifested by the appearance of a ladder pattern indicating missing N-glycans. Complete glycosylation yields a single band on the immunoblot. The position of un (-4)-, mono (-3)-, di (-2)-, and triglycosylated (-1) CPY and mature CPY (mCPY) are indicated. Expression of either flippase or transferase improves glycosylation efficiency and reveals that in the parental strain both processes, i.e., flipping and transfer, are limiting. However, the glycosylation efficiency can be improved by engineering of the LLO biosynthesis pathway. v.c., vector control.

sion of Flc2*p reduced hypoglycosylation in the $\Delta alg3 \Delta alg11$ strain. N-glycosylation of CPY in the $\Delta alg3 \Delta alg11$ (YG1363) strain overexpressing Flc2*p was improved relative to that of the control strain (Fig. 3, lanes 1 and 2). In order to verify that the flipping of $Man_3GlcNAc_2$ -PP-Dol across the ER membrane is improved by the expression of Flc2*p, we analyzed the relative distribution of cytoplasmic and luminal LLO precursors. For this experiment, we employed a $\Delta alg11$ (YG1365) strain, since in this strain the cytoplasmic LLO biosynthesis steps are indistinguishable from the $\Delta alg3 \Delta alg11$ (YG1363) strain, but after flipping the LLOs are further modified, allowing differentiation between cytoplasmic and luminal LLOs. Comparison of the LLO profiles of the strain expressing Flc2*p and the control strain revealed that less $Man_3GlcNAc_2$ -PP-Dol accumulated on the cytoplasmic side of the ER membrane in the Flc2*p-expressing strain (31.0%) than in the control strain (43.5%). Therefore, expression of Flc2* shifted the relative distribution of LLOs in favor of the luminal LLO species, indicating an improved flipping (data not shown).

We next addressed the transfer of the oligosaccharide onto the nascent polypeptide. The oligosaccharyltransferase of higher eukaryotes is integrated into a multisubunit protein complex (OST complex), which, besides the catalytic subunit (Stt3), contains several other partially essential subunits (32). In contrast, kinetoplastids such as *Leishmania* spp. and *Trypanosoma* spp. possess single-subunit OST complexes (33, 34).

We and others previously characterized protozoan oligosaccharyltransferases (POT) in yeast (23, 35, 36). *L. major* encodes four Stt3 proteins, three of which complemented a $\Delta stt3$ deletion in yeast. In particular, one of the four paralogous proteins, LmSTT3D, was tested. LmSTT3D did not integrate into the yeast OST complex, and its catalytic activity was independent of the presence of the other subunits of the yeast OST complex. Additionally, overexpression of LmSTT3D improved N-glycosylation of CPY in yeast strains lacking genes involved in LLO biosynthesis, indicating that this type of POT has a relaxed specificity toward

FIG 4 Expression of the POT improves glycosylation of CPY in the $\Delta alg3 \Delta alg11$ yeast strain. YG1363 was transformed with low-copy-number plasmids for the expression of POTs. Protein extracts were prepared and separated on SDS-PAGE gels. After transfer of proteins, the membrane was analyzed using an anti-CPY antibody. Expression of LbSTT3_1 and LbSTT3_3 strongly improved N-glycosylation of CPY. For details about N-glycosylation levels, see the legend to Fig. 3. v.c., vector control.

LLO structure (23, 36). Indeed, overexpression of LmSTT3D in the $\Delta alg3 \Delta alg11$ (YG1363) strain increased glycosylation of CPY (Fig. 3, lane 1 and 3). Finally, by coexpressing Flc2* and LmSTT3D, further improvements were achieved (Fig. 3).

We therefore performed a screen to identify POTs which would cure hypoglycosylation in the $\Delta alg3 \Delta alg11$ strain. We expressed POTs from *L. major*, *L. brasiliensis*, *L. infantum*, and *T. brucei*. With the exception of *L. major*, all other genomes encode three STT3 paralogues. These genes were expressed from either low-copy- or high-copy-number plasmids in various yeast strains (wild-type, $\Delta alg11$, and $\Delta alg3 \Delta alg11$ strains), and their ability to improve growth and N-glycosylation was analyzed. In particular, LbSTT3_3, isolated from *L. brasiliensis*, improved glycosylation of CPY in a $\Delta alg3 \Delta alg11$ strain most strongly (Fig. 4) and also displayed relaxed substrate specificity, as evidenced by its activity profile in the different yeast strains. Phylogenetic analysis showed that LbSTT3_3 is most closely related to LmSTT3D. In addition, a second paralogue from *L. brasiliensis* improved N-glycosylation activity to a similar extent. Interestingly, LbSTT3_1 is phylogenetically more distant from LbSTT3_3 than from some of the less active homologues. Furthermore, the experiment revealed that optimized expression levels are crucial, as overexpression of these integral membrane proteins was unfavorable for growth despite improving N-glycosylation efficiency.

Previous experiments showed that both insufficient flipping and transfer reactions contributed to the hypoglycosylation observed in the $\Delta alg3 \Delta alg11$ strain. However, we were able to improve both processes individually by selecting a POT and introducing an artificial flippase activity. Therefore, by combining both elements in a composite system, we envisioned that further improvements could be accomplished.

Two versions of the composite system were generated, one on a replicative plasmid and one designed for integration into the host strains' genome. Several adjustments were made to improve the composite system. Increasing Flc2*p expression levels by using the strong constitutive *PGK1* promoter did not improve the effect of Flc2*p when present on a high-copy-number plasmid, but it proved to be essential when integrating the composite system as a single copy into the genome. Expression of LmSTT3D and LbSTT3_3 under the control of two different constitutive promoters (*GPD* and *TEF*) was evaluated in combination with Flc2*p. N-glycosylation of CPY and growth was improved to different extents by the various constructs. In contrast to the expression of LmSTT3D, expression of LbSTT3_3 from the very strong *GPD*

promoter relative to the weaker *TEF* promoter did not further elevate glycosylation levels of CPY. The final constructs were composed of LbSTT3_3 under the control of the *TEF* promoter and *FLC2** under its native promoter when expressed from a high-copy-number plasmid or driven by the strong *PGK1* promoter when integrated into the genome. Expression of the composite system enabled complete N-glycosylation of CPY, both when present on plasmid and when integrated into the genome of the $\Delta alg3 \Delta alg11$ strains (Fig. 5).

Humanization of the N-glycans by expression of GnTI and GnTII. As a next step, we aimed to explore the feasibility of using Man₃GlcNAc₂ as the substrate for Golgi apparatus-localized GnTI and GnTII. We followed recently published strategies for the expression of mammalian N-glycosylation enzymes in the yeast Golgi apparatus (19, 24), creating fusions between localization domains from endogenous Golgi apparatus-localized yeast enzymes (Kre2p and Mnn2p) and the catalytic domains from glycosyltransferases, creating Kre2-GnTI and Mnn2-GnTII fusions, respectively. The bidirectional galactose-inducible *GAL1-10* promoter was employed to coexpress both protein activities in *S. cerevisiae*.

The coexpression of Kre2-GnTI and Mnn2-GnTII in YG1363 was confirmed by immunoblot assay using an anti-FLAG antibody (Fig. 6A). The N-linked oligosaccharides were isolated from the cell wall proteins of YG1363 cells expressing Kre2-GnTI and Mnn2-GnTII, and the 2-AB-labeled sample was analyzed by MALDI-TOF MS. The presence of a peak at m/z 1,459.7 in the MS profile confirmed the transfer of two GlcNAc residues onto the Man₃GlcNAc₂ structure, yielding GlcNAc₂Man₃GlcNAc₂, albeit at a low efficiency (data not shown).

Permethyated oligosaccharides of YG1363 cells expressing Kre2-GnTI and Mnn2-GnTII were further analyzed by tandem mass spectrometry analysis. The fragmentation pattern of the permethylated GlcNAc₂Man₃GlcNAc₂ glycan confirmed the composition and linkages of the expected sugar structure (28). High-energy CID MS/MS of permethylated N-glycan generated the D ion at m/z 676, which is characteristic for complex-type biantennary N-glycan, and also the B/Y ion pair at m/z 260/1,402, which is characteristic for a terminal HexNAc residue (see Fig. S1 in the supplemental material).

In order to improve efficiency and viability of YG1363, two UV-mutagenized, more robust, and better-growing strains were isolated (YG1429 and YG1430) (see Fig. S2 in the supplemental material). The N-glycan analysis confirmed that the N-glycan profile is the same as that in the parental strain YG1363 (see Fig. S3 in the supplemental material). Improved strain viability in combination with medium optimization strongly increased GlcNAc₂Man₃GlcNAc₂ abundance.

MALDI-TOF MS analysis of the 2-AB-labeled glycans isolated from YG1430 expressing Kre2-GnTI and Mnn2-GnTII showed a significant increase in GlcNAc transferase activity, in which the peak at m/z 1,459.7, representing GlcNAc₂Man₃GlcNAc₂, was the major glycan peak. (Fig. 6B; also see Fig. S4 in the supplemental material). HPLC analysis of 2-AB-labeled glycans showed that the target structure GlcNAc₂Man₃GlcNAc₂ accounted for $35.48\% \pm 3.23\%$ of the total N-glycans (Table 2). Other peaks detected in the MS spectra corresponded to the hybrid N-glycans (GlcNAc₁Man₃GlcNAc₂, GlcNAc₁Man₄GlcNAc₂, and GlcNAc₁Man₅GlcNAc₂ at m/z 1,256, 1,418, and 1,580, respectively). However, the MS profile also revealed the presence of N-glycans containing additional mannose residues (Man₄GlcNAc₂, Man₅GlcNAc₂, and Man₆GlcNAc₂), and the man-

FIG 5 N-glycosylation efficiency can be improved in the $\Delta alg3 \Delta alg11$ strain by coexpressing the artificial flippase Flc2*p in combination with LbSTT3_3. (A) The parental strain YG1363 and a strain expressing a model protein (YAF19) are included. YAF19 was transformed with the plasmid for expression of Kre2-GnTI/Mnn2-GnTII (pAX428) and with plasmids expressing Flc2*p in combination with LmSTT3D (pAX447) or Flc2*p with LbSTT3_3 (pAX448). Expression of Kre2-GnTI/Mnn2-GnTII was induced with 2% galactose (Gal). Protein extracts were separated on SDS-PAGE gels. After the transfer of protein, membranes were analyzed using an anti-CPY antibody. Full glycosylation of CPY was obtained by coexpression of Flc2*p and LbSTT3_3. Expression of *FLC2** was under the control of its native promoter, and LbSTT3_3 was expressed under the control of the *TEF* promoter. Expression of Kre2-GnTI/Mnn2-GnTII had no negative effect on glycosylation efficiency. (B) The strain YAF39 has been generated by integration of the composite system (Flc2*p and LbSTT3_3; pAX453) into the genome of the UV-mutagenized $\Delta alg3 \Delta alg11$ strain YG1429. YAF39 was transformed with the plasmid for expression of Kre2-GnTI/Mnn2-GnTII (pAX428). Expression of Kre2-GnTI/Mnn2-GnTII was induced by the addition of 2% galactose. Protein extracts were separated on SDS-PAGE gels. After transfer of protein, membranes were analyzed using an antibody directed against the HA tag of LbSTT3_3, against the Flag tag of Kre2-GnTI/Mnn2-GnTII, and against CPY. Full glycosylation of CPY was obtained by coexpression of Flc2*p and LbSTT3_3. Expression of *FLC2** is under the control of the *PGK1* promoter, and LbSTT3_3 is expressed under the control of the *TEF* promoter. Expression of Kre2-GnTI/Mnn2-GnTII has no negative effect on glycosylation efficiency. For details about N-glycosylation levels, see the legend to Fig. 3.

nosylation of the $\text{Man}_3\text{GlcNAc}_2$ structure also occurred in the non-induced control strain (see Fig. S4). This finding indicated the presence of competing mannosyltransferase activities.

Expression of antibody in the glycoengineered strain. With the generation of the composite system yielding complete N-glycosylation of CPY, its integration into the more robust yeast strain, and the establishment of tools to humanize yeast N-glycosylation, we aimed at the production of a model IgG directed against hen egg lysozyme (HyHEL) decorated with complex N-glycans.

The expression of the IgG was verified by immunoblot analysis using antibodies specific for the F_c portion of the heavy chain (Fig. 7). The presence of the light chain was verified with an antibody specific for the constant region of the light chain (data not shown). The mobility difference after PNGase F treatment indicated that the heavy chain was glycosylated. In comparison, the mobility shift of the heavy chain from the IgG expressed in the wild-type yeast strain was much bigger, which is characteristic for a high-mannose-type structure (Fig. 7).

Expression of proteins was performed in batch mode at 20°C for 36 h. Secreted IgG was purified via affinity chromatography using Protein G. Permethylated N-glycans released from IgG were analyzed using MALDI-TOF MS. The spectra revealed the pres-

ence of the targeted species $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ at m/z 1,661.9; however, all interfering mannose-type structures ($\text{Man}_4\text{GlcNAc}_2$, $\text{Man}_5\text{GlcNAc}_2$, and $\text{Man}_6\text{GlcNAc}_2$) were also detected in the spectra (Fig. 7). In contrast to the N-glycans isolated from cell wall proteins, a substantial fraction of $\text{Man}_3\text{GlcNAc}_2$ was still present, indicating that not all $\text{Man}_3\text{GlcNAc}_2$ is currently modified in the Golgi apparatus, neither by Kre2-GnTI and Mnn2-GnTII nor by Golgi apparatus-localized mannosyltransferases.

Identification of the interfering mannosyltransferase activity. When we analyzed glycan profiles of humanized yeast strains, peaks representing $\text{Man}_4\text{GlcNAc}_2$, $\text{Man}_5\text{GlcNAc}_2$, and $\text{Man}_6\text{GlcNAc}_2$ were identified. However, only N-linked $\text{Man}_3\text{GlcNAc}_2$ can serve as the substrate for Kre2-GnTI and Mnn2-GnTII. In order to identify the interfering mannosyltransferases, we analyzed the N-glycans from $\Delta alg3 \Delta alg11$ strains by combining enzymatic mannosidase treatment with glycan profiling using MALDI-TOF MS. Treatment of N-glycans with an α -1,2-mannosidase revealed that the fourth mannose is linked α -1,2 to the trimannosyl core, as judged from the disappearance of the $\text{Man}_4\text{GlcNAc}_2$ peak (see Fig. S6 in the supplemental material). The recalcitrance of $\text{Man}_5\text{GlcNAc}_2$ and $\text{Man}_6\text{GlcNAc}_2$ glycan species to α -1,2-mannosidase treatment indicated that the terminal mannose residues are linked differently. Upon treatment of the N-glycans with the linkage-unspecific Jack bean

FIG 6 Expression of Kre2-GnTI and Mnn2-GnTII leads to the generation of complex-type N-glycan in *S. cerevisiae*. (A) Immunoblot analysis of $\Delta alg3 \Delta alg11$ (YG1363) strain cell extracts expressing Kre2-GnTI and Mnn2-GnTII (pAX428) under the control of the galactose-inducible promoter *GAL1-10*. Cells were induced with 2% galactose. Whole-cell extracts were separated on SDS-PAGE, and the transferred proteins were probed with anti-Flag antibody. Kre2-GnTI and Mnn2-GnTII were detected already after 2 h of induction. (B) MALDI-TOF MS spectra of the 2-AB-labeled glycans isolated from cell wall proteins of the UV-mutagenized $\Delta alg3 \Delta alg11$ (YG1430) strain carrying the plasmid pAX428, which encodes Kre2-GnTI and Mnn2-GnTII under the control of the galactose-inducible *GAL1-10* promoter. Cells were induced with 2% galactose. The peak at m/z 1,459 represents the target structure $GlcNAc_2Man_3GlcNAc_2$. The peaks at m/z 1,256, 1,418, and 1,580 represent the hybrid structures $GlcNAc_1Man_3GlcNAc_2$, $GlcNAc_1Man_4GlcNAc_2$, and $GlcNAc_1Man_5GlcNAc_2$. Mannosylation of $Man_3GlcNAc_2$ (m/z of 1053; M3) yielded peaks at m/z 1,215 ($Man_4GlcNAc_2$; M4), 1,377 ($Man_5GlcNAc_2$; M5), and 1,539 ($Man_6GlcNAc_2$; M6). Grey circle, mannose; black square, N-acetylglucosamine.

α -mannosidase, all glycan structures collapsed into $Man_1GlcNAc_2$ and $Man_2GlcNAc_2$, proving that all modifications were α -linked mannoses (see Fig. S6). Based on these experiments and on the literature (37), a list of the yeast Golgi mannosyltransferases was compiled to evaluate whether these enzymes were involved in transferring the extra mannoses on the $Man_3GlcNAc_2$ glycan structure in the Golgi apparatus (Table 3).

Mnn1p is an α -1,3-mannosyltransferase in the yeast Golgi apparatus, capping the terminal α -1,2-mannose in each branch of the high-mannose N-linked glycans (38). We deleted this locus and combined it with the $\Delta alg3 \Delta alg11$ strain (YG1363). We isolated N-linked glycans from the triple mutant strain, and the N-glycan analysis showed the reduction by one mannose unit in the N-glycan profile compared to the $\Delta alg3 \Delta alg11$ strain (YG1363). The $Man_5GlcNAc_2$ peak was eliminated and the major peak was $Man_4GlcNAc_2$ (Fig. 8A). However, the fourth α -1,2-mannose on the trimannosyl core structure remained unaffected, as shown by enzymatic treatment (Fig. 8B).

Therefore, we individually deleted all known Golgi apparatus-localized α -1,2-mannosyltransferase activities in the $\Delta alg3 \Delta alg11$

(YG1363) strain, in which Mnn2p and Mnn5p were involved in N-linked glycosylation (39) and Kre2p, Ktr1p, and Ktr3p were involved in N- and O-linked glycosylation in the Golgi apparatus (12, 40). The deletion of either of these α -1,2-mannosyltransferase-expressing genes in the $\Delta alg3 \Delta alg11$ strain did not affect the abundance of the $Man_4GlcNAc_2$ peak or the linkage type (see Fig. S7 in the supplemental material).

DISCUSSION

In this work, we describe an alternative strategy to produce complex-type N-glycans in yeast. Our system is based on the reengineering of the LLO biosynthesis pathway in order to create the substrate for GnTI and GnTII, $Man_3GlcNAc_2$. By presenting the protein-linked trimannosyl core to GnTI and GnTII, no heterologous mannosidase activity is required, simplifying the mammalian N-glycosylation pathway (Fig. 1). As proposed by *in vitro* studies, the $Man_3GlcNAc_2$ structure can serve *in vivo* as the substrate for GnTI and GnTII (41), although the preferred substrate for GnTI is $Man_5GlcNAc_2$ (11). Our process differs from previously described systems for humanization of the N-glycosylation pathway in *P. pastoris* and other fungi, which are based on either the overexpression of two mannosidase activities or on the deletion of the *alg3* gene in combination with the overexpression of

mannosidase activity (15–17). A drawback inherent to the manipulation of LLO biosynthesis is the resulting reduction of glycosylation efficiency. Hypoglycosylation results from the fact that the tailored $Man_3GlcNAc_2$ structure is not the natural substrate for the endogenous flippase and OST (10, 31). Both of these steps, the flipping and the transfer of the oligosaccharide, depend in our novel system on heterologously expressed proteins: an artificial flippase, Flc2*_p, and an OST from *L. brasiliensis*. Optimization of both steps finally led to complete glycosylation of model proteins.

As deletion of *alg3* leads to a hypoglycosylation phenotype in *S. cerevisiae* (42), it can be speculated that the deletion of *P. pastoris alg3* has similar deleterious effects on protein glycosylation. Although deletion of the homologous enzyme in *P. pastoris* has been described, the effects of its deletion on glycosylation efficiency were not studied (43). However, a glycoengineering approach based on the deletion of the *alg3* gene and without compensatory measures as shown here would likely result in reduced glycosylation efficiency and growth defects. Notably, very recently the effect of overexpression of LmSTT3D on glycosylation efficiency on a recombinant antibody in glycoengineered *P. pastoris* was reported (44). Overexpression of LmSTT3D but of none of the other *Leishmania* STT3 paralogues improved site occupancy of a therapeutic glycoprotein to almost 100%. The glycoengineering strategy in this strain was based on the introduction of two mannosidase activities and not on the deletion of the *P. pastoris* *ALG3* gene. It

TABLE 2 Relative glycan abundances^a in humanized *S. cerevisiae*

Glycoform	Avg $\pm$ SD
$Man_3GlcNAc_2$	1.92 $\pm$ 0.57
$Man_4GlcNAc_2$ plus $GlcNAc_1Man_3GlcNAc_2$	27.27 $\pm$ 2.44
$Man_5GlcNAc_2$ plus $GlcNAc_1Man_4GlcNAc_2$	25.24 $\pm$ 0.52
$GlcNAc_2Man_3GlcNAc_2$	35.48 $\pm$ 3.23
$Man_6GlcNAc_2$	6.23 $\pm$ 4.65
$Man_7GlcNAc_2$	3.62 $\pm$ 0.23

^a Total N-glycan abundance was set to 100; $n = 3$.

FIG 7 Model antibody HyHEL-10 decorated with complex-type N-glycan. Expression of antibody was achieved by transforming YAF39 with plasmids encoding Kre2-GnTI and Mnn2-GnTII (pAX428) and a plasmid for expression of HyHEL-10 antibody (pAX550). Expression of Kre2-GnTI/Mnn2-GnTII and antibody under galactose-inducible promoters was induced at an A_{600} of 0.5 and was continued for 36 h. Secreted HyHEL-10 antibody was purified using protein G Sepharose affinity chromatography. The purified antibody was concentrated, and N-glycans were released with PNGase F. Samples before and after N-glycan release were separated on SDS-PAGE and the protein was transferred to membranes, and the mobility difference was detected using an antibody specific for the F_c portion of the antibody (α -IgG-HRP). For comparison, the identical HyHEL-10 antibody has been purified from a wild-type yeast strain (BJ3500). The antibody carries a single N-glycosylation site, and enzymatic removal of the N-glycans leads to a mobility shift, the extent of which depends on the mass of the N-glycan. MALDI-TOF spectra of permethylated N-glycans released from HyHEL-10 antibody is shown. The peak appearing at m/z 1,662 is the targeted $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ structure. The peaks at m/z 1,171, 1,375, 1,580, and 1,784 represent $\text{Man}_3\text{GlcNAc}_2$ (M3), $\text{Man}_4\text{GlcNAc}_2$ (M4), $\text{Man}_5\text{GlcNAc}_2$ (M5), and $\text{Man}_6\text{GlcNAc}_2$ (M6), respectively. An asterisk in the spectrum indicates permethylation artifacts. Grey circle, mannose; black square, N-acetylglucosamine; glyco., glycosylated form; deglyco., deglycosylated form.

therefore would be highly interesting to know what would be the effect of LmSTT3D on the glycosylation efficiency and growth of glycoengineered *P. pastoris* strains based on *alg3* gene deletion as used before (17, 18).

In-depth qualitative and quantitative analysis of glycan structures using sequencing of permethylated N-glycans by MS/MS analysis proved the generation of the target structure $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ but also revealed the presence of the trimannosyl core structure carrying additional mannoses. Enzymes in the Golgi apparatus, on one hand the endogenous mannosyltransferases and on the other hand GnTI/GnTII, are competing for the substrate, the trimannosyl core. The use of UV-mutagenized strains and medium optimization shifted the equilibrium toward GnTI and GnTII activities. However, further improvements will be needed to shift the balance between these processes in favor of the GlcNAc addition. Support for the hypothesis that interfering mannosyltransferase activities can be outcompeted arises from work in *P. pastoris*. Expression of mannosidase I and GnTI in the $\Delta\text{alg3 } \Delta\text{och1}$ deletion strain also resulted in mannosylation of the glycan. Upon coexpression of GnTII, no such modifications occurred anymore (17). Therefore, activity of GnTI/II could be improved either by modulating ex-

pression levels and availability of UDP-GlcNAc or by choosing alternative pairs of localization and catalytic domains.

Our results showed that Golgi mannosyltransferases utilize the $\text{Man}_3\text{GlcNAc}_2$ N-glycan as a substrate, yielding mainly $\text{Man}_4\text{GlcNAc}_2$ and $\text{Man}_5\text{GlcNAc}_2$ structures. The deletion of the *MNN1* locus, encoding a Golgi α -1,3 mannosyltransferase, resulted in the generation of a less heterogeneous N-glycan pattern, leaving mainly the fourth α -1,2-linked mannose. However, the individual inactivation of all known Golgi α -1,2 mannosyltransferases in the $\Delta\text{alg3 } \Delta\text{alg11}$ mutant strain did not abolish the generation of the $\text{Man}_4\text{GlcNAc}_2$ structure, suggesting that combined deletions of α -1,2 mannosyltransferases are required or an unidentified α -1,2 mannosyltransferase is active.

Interestingly, when analyzing the N-glycans of the secreted IgG, substantial amounts of $\text{Man}_3\text{GlcNAc}_2$ were still detected which were modified by neither mannosyltransferases nor GnTI/II, indicating that the activities of GnTI and GnTII are more rate limiting if overexpressing a glycoprotein than when analyzing cell wall proteins. The synthesis of the latter is limited to the necessary amounts to maintain cell wall integrity. A similar observation has been reported previously by Jacobs et al. (24), who compared N-glycan profiles of total secreted protein to N-glycan profiles of

TABLE 3 Golgi mannosyltransferases involved in N- and O-linked glycosylation pathways potentially competing for $\text{Man}_3\text{GlcNAc}_2$ substrate

Protein	Description	Gene name	Glycosylation pathway
Mnn1	α -1,3-Mannosyltransferase	YER001W	N-linked glycosylation
Mnn2	α -1,2-Mannosyltransferase	YBR015C	N-linked glycosylation
Mnn5	α -1,2-Mannosyltransferase	YJL186W	N-linked glycosylation
Kre2/Mnt1	α -1,2-Mannosyltransferase	YDR483W	N- and O-linked glycosylation
Ktr1	α -1,2-Mannosyltransferase	YOR099W	N- and O-linked glycosylation
Ktr3	α -1,2-Mannosyltransferase	YBR205W	N- and O-linked glycosylation

FIG 8 Deletion of *MNN1* reduces mannosylation of $\text{Man}_3\text{GlcNAc}_2$ structure. MALDI-TOF MS spectra of 2-AB-labeled N-glycans isolated from the cell wall proteins of the $\Delta\text{alg3 } \Delta\text{alg11 } \Delta\text{mnn1}$ triple mutant strain (YFP149). (A) Analysis of 2-AB-labeled N-glycans from the cell wall proteins of the $\Delta\text{alg3 } \Delta\text{alg11 } \Delta\text{mnn1}$ strain yielded mainly $\text{Man}_4\text{GlcNAc}_2$. Mannosylation of $\text{Man}_3\text{GlcNAc}_2$ (m/z 1,053; M3) yielded peaks at m/z 1,215 ($\text{Man}_4\text{GlcNAc}_2$; M4) and 1,377 ($\text{Man}_5\text{GlcNAc}_2$; M5). (B) Treatment of the sample with *T. reesei* α -1,2-mannosidase enzyme shifted the peak at m/z 1,215 to one at m/z 1,053, representing $\text{Man}_3\text{GlcNAc}_2$ (M3).

overexpressed murine interleukin-10 and observed a higher structural N-glycan heterogeneity on interleukin-10.

Our data show that the glycoengineering of *S. cerevisiae* is a promising alternative for the production of therapeutic proteins. Furthermore, when designing protein expression factories, not only the type of glycan but also glycosylation efficiency are crucial factors for success.

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