Characterization of the Pichia pastoris Protein-O-mannosyltransferase Gene Family

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Abstract

The methylotrophic yeast, Pichia pastoris, is an important organism used for the production of therapeutic proteins. However, the presence of fungal-like glycans, either N-linked or O-linked, can elicit an immune response or enable the expressed protein to bind to mannose receptors, thus reducing their efficacy. Previously we have reported the elimination of β-linked glycans in this organism. In the current report we have focused on reducing the O-linked mannose content of proteins produced in P. pastoris, thereby reducing the potential to bind to mannose receptors. The initial step in the synthesis of O-linked glycans in P. pastoris is the transfer of mannose from dolichol-phosphomannose to a target protein in the yeast secretory pathway by members of the protein-O-mannosyltransferase (PMT) family. In this report we identify and characterize the members of the P. pastoris PMT family. Like Candida albicans, P. pastoris has five PMT genes. Based on sequence homology, these PMTs can be grouped into three sub-families, with both PMT1 and PMT2 sub-families possessing two members each (PMT1 and PMT5, and PMT2 and PMT6, respectively). The remaining sub-family, PMT4, has only one member (PMT4). Through gene knockouts we show that PMT1 and PMT2 each play a significant role in O-glycosylation. Both, by gene knockouts and the use of Pmt inhibitors we were able to significantly reduce not only the degree of O-mannosylation, but also the chain-length of these glycans. Taken together, this reduction of O-glycosylation represents an important step forward in developing the P. pastoris platform as a suitable system for the production of therapeutic glycoproteins.

Introduction

Recent advances in genomics and proteomics have fuelled the increasing demand for large quantities of therapeutic proteins. Because the majority of these therapeutically relevant proteins require certain posttranslational modifications, such as N-glycosylation or O-glycosylation for proper function [1], most glycoproteins of commercial importance are currently expressed in mammalian cell culture. Fungal protein-expression systems are viewed as a potential alternative because of high volumetric productivity [2,3], low media cost, lack of retroviral contamination and ease of generation of stable cell lines. However, the presence of high mannose-type N- and O-glycans renders glycoproteins derived from fungal expression systems less desirable for human applications [4]. Recently the methylotrophic yeast Pichia pastoris has become a model system for protein expression, and our lab has described the humanization of the N-glycosylation pathway of this organism [5]. However, currently little has been reported on the engineering of O-linked glycans in this organism.

Protein O-mannosylation in fungi is initiated at the endoplasmic reticulum (ER) by a family of protein-O-mannosyltransferases (PMTs) that transfer mannose from dolichyl phosphate-activated mannose (Dol-P-Man) to serine or threonine residues of proteins entering the ER. In P. pastoris, additional mannosyltransferases then extend the mannose chain by up to four more residues [6] with GDP mannose serving as the carbohydrate donor (see Figure 1). The yeast Saccharomyces cerevisiae, where PMTs have been studied extensively for years, contains a highly redundant PMT gene...
family with up to seven members. They can be phylogenetically grouped into the three subgroups, PMT1 (containing PMT1, PMT5 and PMT7), PMT2 (containing PMT2, PMT3 and PMT6) and PMT4 (as the sole member of this group), encoding proteins with different protein substrate specificities [7]. In S. cerevisiae, the major transferase activity is performed by heterodimeric Pmt1p and Pmt2p subfamily member complexes and Pmt4p homodimeric complexes [7]. Orchard and colleagues described rhodanine-3-acetic acid (RAA) derivatives as potent and specific inhibitors of Candida albicans Pmt1p [8,9]. In S. cerevisiae, however, it was shown that these inhibitors affect the members of all three PMT subfamilies [10]. Here we describe the five members of the PMT family of P. pastoris. We provide data on the phenotypes of individual gene knockouts and the effect previously described inhibitors have on them. We also show that these inhibitors not only reduce O-glycan site occupancy but also O-glycan chain length via an unknown mechanism.

Materials and Methods

Strains and DNA manipulations

Escherichia coli strain DH5α was used for recombinant DNA work. P. pastoris strains NRRL-Y11430 (wild type), PBP33 (a Δoch1 derivative of NRRL-Y11430) or YGLY11 (a Δpro1, Δbmt2, Δmnn4L1 derivative of NRRL-Y11430) were used for construction of yeast strains. PCR reactions were performed according to suppliers’ recommendations, using ExTan™ (Takara Bio, Mountain View, CA), Taq Poly (Promega, Madison, WI) or Pfu Turbo® (Stratagene, La Jolla, CA). Restriction and modification enzymes were from New England Biolabs (Ipswich, MA). PCR analysis of the PMT knockout strains was performed as described in [11]. The program used to determine the P. pastoris PMT homologues of the corresponding S. cerevisiae genes was BLAST [12]. The partial genomic sequence of P. pastoris was provided by Integrated Genomics Inc., with a public version of the Pichia genome now available at http://www.Pichiagenome.org/.

Transformation of yeast strains

Yeast transformations were performed as described in [11]. In short, yeast cultures in logarithmic growth phase were washed twice in distilled water and once in 1 M sorbitol. 5–50 µg linearized DNA in 10 µl (TE: 10 mM Tris, 1 mM EDTA, pH 8.0) were mixed with 100 µl yeast cells and electroporated, using α BTX electroproporation system (BTX, San Diego, CA). After addition of 1 ml recovery medium (1% yeast extract, 2% peptone, 2% dextrose, 4 × 10^{-5}% biotin, 1 M sorbitol, 0.4 mg/ml ampicillin, 0.136 mg/ml chloramphenicol), the cells were incubated without agitation for 4 h at room temperature and then spread onto appropriate media plates.

Construction of expression plasmids

The plasmid expressing the human plasminogen domains Kringle 1-3 was constructed the following way: Primers K1-3/UP (5’- CCGAATTCTCAGAGCTGGAATGAAATGAA-3’) and K1-3/LP1 (5’- ATGATGATGACCACCCGTCCTGAGAACAGGTTAG-3’) were used to amplify the K1-3 domain from a plasmid encoding human plasminogen (a gift from Nick Menhart, Illinois Institute of Technology, Chicago) and add an EcoRI site at the 5’-end and a GGGHHH tag at the 3’-end (marked by italics). The resulting PCR product was then re-amplified using K1-3/UP and primer K1-3/LP2 (5’- TTAATGATGATGATGATGATGATGATGATGACCACCACCGTCCTGAGAACAGGTTAG-3’), extending the His-tag to 9 units and adding a stop codon. The resulting product was then cloned into the expression plasmid pBK118. In order to remove the N-glycosylation site in the K3 domain (position 308), the asparagine at this position was mutated to serine by inverse PCR, using pBK105 as a template and primers K3f (5’- ACCCCTCACACACATACACCACCCGTCCTGAGAACAACTTCTACACCACACGACGACGAC-3’). The resulting PCR product was then ligated to produce plasmid pBK118. Subsequently, the K1-3 insert was sequenced to confirm the mutation. Plasmid pBK118 was then digested with EcoRI, and the insert was cloned into the EcoRI site of pPICZalphaA (Invitrogen (now Life Technologies), Carlsbad, CA) to yield pBK105. In order to remove the N-glycosylation site in the K3 domain (position 308), the asparagine at this position was mutated to serine by inverse PCR, using pBK105 as a template and primers K3f (5’- ACCCCTCACACACATACACCACCCGTCCTGAGAACAACTTCTACACCACACGACGACGAC-3’). The resulting PCR product was then ligated to produce plasmid pBK118. Subsequently, the K1-3 insert was sequenced to confirm the mutation. Plasmid pBK118 was then digested with EcoRI, and the insert was cloned into the EcoRI site of pPICZalphaA (Invitrogen (now Life Technologies), Carlsbad, CA) to yield pBK119 (the K1-3 expression plasmid). Before transformation into yeast strains, pBK119 was linearized with Pmel. This facilitates integration of the vector into the AOX1 promoter locus. Two plasmids expressing an IgG1 were constructed the following way: Construction of pDX580, which directs expression of the heavy chain (human IgG1) as well as the light chain (human Kappa) were codon optimized for expression in P. pastoris and synthesized by Life
Variable regions were made in-house by overlap PCR using oligonucleotides purchased from IDT Inc. (Coralville, IA). Full length heavy and light chains with respective signal sequences (Aspergillus niger α-amylase for heavy chain, and chicken lysozyme for light chain) and restriction sites (BstBI on the 5'-end and NotI on the 3'-end) were assembled by overlap PCR, and the resulting heavy and light chains were cloned into pCR2.1 TOPO to generate pDX344 and pDX349 respectively. pDX344 was then digested with BstBI and NotI and the IgG1 heavy chain was cloned into pPICZA creating pDX518. Similarly, the light chain was released by BstBI and NotI digest and also cloned into pPICZA, creating pDX490. To allow for integration into the P. pastoris genome, a plasmid was created by cloning a 1.5 kb fragment of the PpAOX2 promoter into the BglII / EcoRI sites of pPICZA, effectively replacing the AOX1 promoter region with that of AOX2. This plasmid was named pBK85. The heavy chain cassette, encompassing the AOX1 promoter, the antibody heavy chain and the AOX1 terminator, was then released from pDX518 by BglII / BamHI digest and cloned into the BamHI site of pBK85 creating pDX564. The light chain cassette, encompassing the AOX1 promoter, the antibody light chain and the AOX1 terminator, was then released from pDX490 by BglII / BamHI digest and cloned into the BamHI site of pDX564 creating the final IgG1 expression plasmid pDX580. This plasmid can be integrated into the AOX2 promoter genomic region by linearization at the unique PmI site in the plasmid and transformation into P. pastoris. Construction of pGLY2988, which directs integration of the antibody genes into the TRP2 locus of P. pastoris: Expression/ integration plasmid vector pGLY2988 contains expression cassettes under control of the methanol-inducible P. pastoris AOX1 promoter that encode the heavy (Hc) and light (Lc) chains of an IgG1. The IgG1 Hc and Lc fused at the N-terminus to the S. cerevisiae α-MAT pre signal peptide were synthesized by Life Technologies/ GeneArt AG (Regensburg, Germany). Each was synthesized with unique 5' EcoRI and 3' FseI sites. Both nucleic acid fragments encoding the Hc and Lc proteins fused to the α-MAT pre signal peptide were separately subcloned using 5' EcoRI and 3' FseI unique sites into an expression plasmid vector pGLY2198, which contains the P. pastoris TRP2 gene as targeting region and the Zeoence-resistant marker and generates expression cassettes under the control of the AOX1 promoter and S. cerevisiae CYC1 terminator, to form plasmid vectors pGLY2987 and pGLY2338, respectively. The Lc expression cassette was then removed from plasmid vector pGLY2338 by digesting with BamHI and NotI, and subcloned into plasmid vector pGLY2987 digested with BamHI and NotI, thus generating the final expression plasmid vector pGLY2988.

Construction of PMT knockout strains

The PpPMT deletion alleles were generated either using the URA5-blaster system described in [13], or by the PCR overlap method [14] using primers designed based on the genomic sequence. In the first PCR reaction, DNA comprising about 1kb of nucleotide sequences for 5'- and 3'-regions flanking the PMT ORFs and the NAT, HYG or KAN resistance markers [15,16] were individually PCR amplified. P. pastoris genomic DNA (strain NRRL-Y11430) was used as a template for the PpPMT flanking regions, whereas NAT, HYG and KAN resistance marker fragments were amplified using plasmids as described in [15,16]. Then, in a second PCR reaction, the three first round PCR products per knockout were used as templates to generate an overlap product that contained all three fragments in a single linear allele. The final PCR product was then directly employed for transformation. Transformants were selected on YPD medium containing 200 µg/ml of hygromycin, 100 µg/ml of Nourseothricin, or 100 µg/ml of kanamycin. In each case the proper integration of the mutant allele was confirmed by PCR.

Synthesis of Pmt inhibitors

Preparation of PMT-1, (5-[(3,4-bis(phenylmethoxy)phenyl)methylene]-4-oxo-2-thioxo-3-thiazolidine-4-carboxylic acid), was as follows: The procedure was adapted from Orchard et al. in U.S. Pat. No. 7,105,554 and the compound is referred to in [8] as 1c. A solution of rhodamine-3-acetic acid (1 g, 5.20 mmol, 1 eq), 3,4-dibenzyloxybenzaldehyde (2.04 g, 6.25 mmol, 1.2 eq), and sodium acetate (1.3 g, 15.6 mmol, 3 eq) in acetic acid (30 mL) was heated to reflux, and stirred overnight. As the reaction mixture was cooled to room temperature, the product was precipitated and filtered and washed with acetic acid, then petroleum ether. The residue was dissolved in hot DMSO, filtered, and precipitated by addition of water. Upon cooling, the precipitate was filtered and recrystallized from ethyl acetate and petroleum ether to give a product which was suspended in water and freeze-dried overnight in vacuo to give the final product as a fluffy yellow powder.

Preparation of PMT-3, (5-[3-(1-Phenyl-2-hydroxy)ethoxy]-4-(2-phenylethoxy)phenyl)methylene-[4-oxo-2-thioxo-3-thiazolidine-4-carboxylic acid], (Orchard et al. in U.S. Pat. No. 7,105,554) was synthesized in three steps as follows: Step 1: Production of (+)-(S)-2-Acetoxy-1-bromo-1-phenylethane. Cold HBr-acetic acid (12.4 g, 52.2 mmol) was added drop-wise to (-)-(R)-1-phenylethanol-1,2-diol (2.4 g, 17.4 mmol) during about five minutes and the mixture was stirred at room temperature for 40 minutes. Water (25 mL) was added and the solution was neutralized with sodium carbonate and extracted with ether (3x30 mL). The combined extracts were dried and evaporated to give (+)-(S)-2-acetoxy-1-bromo-1-phenylethane. Cold acetate and water (brine was added to help break up the emulsion that formed). The organic layer was washed twice more with water, brine, and then dried over sodium sulfate and evaporated to dry a dark oil. The residue was purified by chromatography on silica gel, and elution with diethyl ether and petroleum ether to give a product which was suspended in water and freeze-dried overnight in vacuo to give the final product as a fluffy yellow powder.

Preparation of PMT-4, (5-[(3,4-bis(phenylmethoxy)phenyl)methylene]-4-oxo-2-thioxo-3-thiazolidine-4-carboxylic acid), (Orchard et al. in U.S. Pat. No. 7,105,554) was synthesized in three steps as follows: Step 1: Production of (+)-(S)-2-Acetoxy-1-bromo-1-phenylethane. Cold HBr-acetic acid (12.4 g, 52.2 mmol) was added drop-wise to (-)-(R)-1-phenylethanol-1,2-diol (2.4 g, 17.4 mmol) during about five minutes and the mixture was stirred at room temperature for 40 minutes. Water (25 mL) was added and the solution was neutralized with sodium carbonate and extracted with ether (3x30 mL). The combined extracts were dried and evaporated to give (+)-(S)-2-acetoxy-1-bromo-1-phenylethane. Cold acetate and water (brine was added to help break up the emulsion that formed). The organic layer was washed twice more with water, brine, and then dried over sodium sulfate and evaporated to give a dark oil. The residue was purified by chromatography on silica gel, and elution with diethyl ether yielded an orange oil. This oil was dissolved in methanol (100 ml) and to the solution was added an aqueous solution of sodium hydroxide (7 mL, 1M). After 30 minutes, the mixture was evaporated to remove the methanol and the residue...
partitioned between dichloromethane and water. The organic layer was dried over sodium sulfate and evaporated. The residue was purified by chromatographed on silica gel, and elution with petroleum ether: diethyl ether (1:2) yielded the product as a cream colored powder. Step 3: Production of Pmt inhibitor PTM1-3. A solution of rhodanine-3-acetic acid (158 mg, 0.828 mmol, 1 eq), 3-(1-phenyl-2-hydroxy)ethoxy)-4-(2-phenylethoxy) benzaldehyde (300 mg, 0.828 mmol, 1 eq) (the product of Step 2), and ammonium acetate (191 mg, 3 eq) in toluene (10 mL) was heated to reflux for 3.5 hours, cooled to room temperature, and diluted with ethyl acetate (50 mL). The organic solution was washed with 1M HCl (2x200 mL) and brine (200 mL) then dried over sodium sulfate and evaporated. After work-up, the residue was purified by chromatography on silica gel. Elution with ethyl acetate yielded a yellow gum, which was recrystallized from diethyl ether and petroleum ether to give the product as a yellow powder.

Expression of reporter proteins

Protein expression for the transformed yeast strains was carried out either in shake flasks or 96 well deep well plates at 24°C with buffered glycerol-complex medium (BMGY) consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, 4x10^{-5} M biotin, and 1% glycerol. The induction medium for protein expression was buffered methanol-complex medium (BMMMY) consisting of 1% methanol instead of glycerol in BMGY. Pmt inhibitor Pmt-1 or Pmt-3 in methanol was added to the induction medium to the appropriate concentrations. Cells were harvested and centrifuged at 2000 rpm in a tabletop centrifuge for five minutes. For Western analysis, seven µl of the supernatant from the expression cultures was separated by polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [17] and then electroblotted onto nitrocellulose membranes (Schleicher & Schuell (now Whatman, Inc., Florham Park, N.J.)). Kringle 1-3 protein was detected on the Western blots using an anti-His antibody (H-15 at 1:300 dilution) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and developed using the ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce Biotechnology, Rockford, IL.). The IgG1 was detected using an HRP-conjugated anti-human IgG (H&L at 1:1000 dilution) to detect heavy and light chains. O-glycan site occupancy and length was determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

To measure O-glycan content, the protein of interest was purified from the growth medium using either nickel chelation chromatography (for K1-3) [18], or protein A (for the IgG1). Subsequently the O-glycans were released from the protein of interest by alkaline elimination (beta-elimination). This process also reduces the newly formed reducing terminus of the released O-glycan (either oligomannose or mannose) to mannitol. The mannitol group thus serves as a unique indicator of each O-glycan. 0.5 nmol or more of protein, contained within a volume of 100 μl PBS buffer, was required for beta elimination. The sample was treated with 25 μl alkaline borohydride reagent and incubated at 50°C for 16 hours. About 20 μl arabitol internal standard was added, followed by 10 μl glacial acetic acid. The sample was then centrifuged through a Millipore filter containing both SEPABEADS and AG 50W-X8 resin and washed with water. The samples, including wash, were transferred to plastic autosampler vials and evaporated to dryness in a centrifugal evaporator. 150 μl 1% AcOH/Methanol was added to the samples and the samples were evaporated to dryness in a centrifugal evaporator. This last step was repeated five more times. 200 μl of water was added and 100 μl of the sample was analyzed by high pH anion-exchange chromatography coupled with pulsed electrochemical detection-Dionex HPLC (HPAEC-PAD). Average O-glycan occupany was determined based upon the amount of mannitol recovered. For a more detailed protocol please refer to [19].

Microarray analysis

The genome of Pichia pastoris strain NRRL-Y11430 was sequenced by Agencourt (now Beckman-Coulter) and annotated by Biomax and will be reported in greater detail in an accompanying manuscript [Mickus et al, in preparation]. The annotated genome was used to generate Agilent microarray probes for all 5424 genes for 3’ biased hybridization protocol to a density of 2-3 probes per gene (4207 genes with 3 probes/transcript and 1217 genes with 2 probes/transcript). This custom-designed Agilent P. pastoris 15 k 3.0 array (8x15K) gene microarray was used for all whole genome gene-chip RNA expression analyses. To generate the transcript analysis discussed herein of the PMT genes, the wild type strain NRRL-Y11430 was cultivated in quadruplicate in 0.5 L fermenters (Sixfors multifermentation system, ATR Biotech, Laurel, MD) with a standard glycerol-to-methanol batch/fed-batch process as described previously [20] with pH 6.5, 24°C, 300 ml airflow/min, and initial stirrer speed of 550 rpm, which increased to 1200 rpm linearly between hours 1 to 10 using IRIS multifermenter software (ATR Biotech, Laurel, MD). The batch phase was initiated in BMGY medium (Invitrogen, a subsidiary of Life Technologies, Carlsbad, CA) with an initial charge of 4% glycerol and cultivated until glycerol was exhausted, followed by a fedbatch methanol induction phase where a methanol solution comprised of 100% methanol, 5 mg/L biotin and 12.5 ml PTM1 salts was fed at 0.6 g/h. Samples were taken in quadruplicate by removing enough cell broth to obtain ~1x10^6 cells, and each aliquot was centrifuged for 30 seconds at 10,000 x g and flash frozen. The frozen samples were shipped to Cogenics (now Beckman-Coulter Genomics, Morrisville, NC) for RNA extraction and microarray hybridization. Raw intensity values were normalized using standard statistical methods [21]. Normalized intensity values from four independent samples were averaged to generate the expression values reported.

Results

1): The Pichia pastoris genome contains five protein mannosyltransferases of which PMT2 is most highly expressed

Querying a P. pastoris genomic sequence database with the open reading frames of all seven S. cerevisiae PMTs, we
identified the P. pastoris PMT1 (770 amino acids with 53% identity; Accession no. XP002491100), PMT2 (750 amino acids with 64% identity; Accession no. XP002491148), PMT4 (741 amino acids with 54% identity; Accession no. CCA36946), PMT5 (741 amino acids with 35% identity; Accession no. XP002489942), and PMT6 (752 amino acids with 51% identity; Accession no. XP002494221) homologues. No homologues for ScPMT3 or ScPMT7 were found. The importance of PMTs in fungal cell wall integrity have made them attractive targets for antifungal therapies, and their sequences have been elucidated in many biologically relevant species [10,22–25]. Phylogenetic analysis of the P. pastoris PMT genes compared with their homologues from S. cerevisiae, Schizosaccharomyces pombe, Candida albicans and Aspergillus nidulans showed that the P. pastoris PMT genes are most closely related to their C. albicans counterparts (see Figure 2). Similar to P. pastoris, the PMT gene family of C. albicans consists of five genes (PMT1, PMT2, PMT4, PMT5 and PMT6) [22], whereas A. nidulans as well as S. pombe each only contain three PMT genes, one for each subgroup. To determine the expression level of the five identified PMT homologs in P. pastoris, an Agilent RNA expression microarray was employed. The wild type NRRL-Y11430 strain was cultivated in quadruplicate in 0.5 L fermentation vessels in a standard glycerol-to-methanol fermentation process that is typically used for heterologous protein production [20]. Samples were taken when the cultures reached 50 mg/ml of wet cell weight (approximately halfway through glycerol batch phase), at the end of batch phase, and then 4 h and 24 h into methanol induction. These samples were then applied to an Agilent array containing probes directed against the NRRL-Y11430 annotated geneset as described in an accompanying manuscript (Mickus et al., in preparation). From these whole genome expression data, normalized intensity values for the five annotated PMT genes was compared to the Actin (ACT1) and Glyceraldehyde-3-Phosphate Dehydrogenase (GPD) control genes and data are plotted for the glycerol and 24 h methanol time points (see Figure 3). Comparative expression levels varied significantly among the PMTs; however, transcripts for all of the genes were detected under all conditions tested with even the most weakly expressed family member (PMT6) in the ~60th percentile of transcripts detected under both conditions. PMT2 was the most highly expressed gene among the P. pastoris PMTs with PMT1 and PMT4 showing intermediate expression and PMT5 and PMT6 the weakest. This is consistent with what has been reported in S. cerevisiae where the Pmt1p/Pmt2p heterodimer accounts for the majority of O-mannosyl transfer. This is also consistent with genetic manipulation where elimination of Pmt2p activity resulted in the greatest effects both on viability and in terms of reducing O-mannosyl transfer to proteins. Expression of control genes is consistent with previous reports [26]. Notably, all five of the PMTs were dramatically repressed on methanol compared to glycerol (Figure 3), which could simply be a function of reduced growth rate on methanol due, in turn, to a lower energy availability from methanol compared to glycerol, combined with a limiting feed rate [27].

2): Δpmt1 and Δpmt2 strains secrete protein with significantly reduced O-glycan site occupancy

In order to determine which of the PMT genes in P. pastoris are responsible for the addition of O-glycans on secreted protein, we created strains with individual knockouts for PMT1, PMT2, and PMT4, and a strain with a double knockout of both PMT5 and PMT6 as described in Materials and Methods, and shown in the schematic in Figure 4A. It has been reported that in C. albicans, S. pombe and even P. pastoris an individual knockout of the PMT2 gene is lethal [22,24]; however we were able to construct single knockout strains of all five Pichia pastoris PMT genes, but not double knock-outs Δpmt1/Δpmt2, Δpmt1/Δpmt4 or Δpmt2/Δpmt4. Not unexpectedly, we also found that the Δpmt1 and Δpmt4 strains displayed a slight and the Δpmt2 strain a significant slow growth phenotype (see Figure 5). In order to determine the feasibility of PMT knockouts in N-linked glycosylation engineered strains, we also attempted the PMT deletions in a Δact1 background with reduced N-glycan complexity. We were able to generate the PMT1, PMT4, PMT5 and PMT6 single knockouts as well as the PMT5/PMT6 double knockouts, but we were unable to obtain a Δpmt2 strain (for a list of strains and their corresponding genotype please see Table 1). The PMT1, PMT2, and PMT4 single knockouts, as well as the PMT5/PMT6 double knockout strains were then transformed with a plasmid expressing a His-tagged reporter protein consisting of human plasminogen domains Kringle 1, Kringle 2 and Kringle 3 (K1-3) under the control of the P. pastoris alcohol oxidase 1 (AOX1) promoter. Since the Kringles domain contains an N-glycosylation site at position 308, we changed this asparagine residue to a serine by site directed mutagenesis in order to make O-glycosylation the only post-translational modification of the secreted protein. The protein was then expressed in shake flasks and analyzed by SDS-gel analysis followed by Western blotting for size. Furthermore, we removed the bound O-glycans by beta elimination and analyzed them by high pH anion-exchange chromatography coupled with pulsed amperometric detection-DIONEX HPLC (HPAEC-PAD) for O-glycan site occupancy determination (see Materials and Methods). As shown in Figure 4B, only the PMT1 and PMT2 knockouts lead to a significant reduction in the extent of O-glycosylation, corresponding to a reduction in O-glycan site occupancy from 20 down to 3 to 4 moles of O-linked glycan per mole of protein (Table 2). By contrast, the PMT4 single knockout and the PMT5/PMT6 double knockout only displayed a very slight reduction of about 10% in O-glycan site occupancy (Table 2). We therefore conclude that in P. pastoris Pmt1p and Pmt2p are the main transferases responsible for O-glycan attachment to secreted protein.

3): Pmt inhibitors block the activity of multiple PMTs in Pichia pastoris

In search for novel antifungal agents, Orchard and colleagues described in 2004 a group of rhodanine-3-acetic acid derivatives that inhibit Pmt1p activity in C. albicans [8]. The fact that these inhibitors are indeed specific for Pmt1p in C. albicans was later confirmed by Cantero and coworkers [9]. In S. cerevisiae, however, they have been shown to affect members of all three subclasses [10]. In order to test whether these compounds could be used instead of PMT knockout strains and thereby circumvent the accompanying slow growth phenotype, we tested the effect these inhibitors have in wild type P. pastoris and a subset of the PMT knockout strains described above. To this end, we grew strains yJC53 (wild type
for PMT), yJC55 (Δpmt1), yJC66 (Δpmt2), yJC65 (Δpmt4) and yJC51 (Δpmt5/Δpmt6) in the absence or presence of 2 µM PMTi-1 (described in [8]) and analyzed the purified K1-3 protein for O-glycan site occupancy (see Table 2). Whereas PMTi-1 leads to a substantial reduction in O-glycan site occupancy in PMT wild type, and the Δpmt4 single and Δpmt5/Δpmt6 double knockout strains, it had no apparent effect on the Δpmt1 and Δpmt2 strains. In a related experiment we determined the growth characteristics of single PMT knockout strains on media containing varying amounts of a slightly modified compound, PMTi-3, with approximately ten times higher specific activity (for details please refer to Materials and Methods), using a drop dilution assay. As is evident from Figure 5, the Δpmt4 strain is hypersensitive to PMTi-3, as it shows a significant growth defect already at low concentrations. At higher PMTi-3 concentrations the Δpmt1 strain also is unable to grow and the Δpmt6 strain displays a slow growth phenotype, whereas the growth of the Δpmt2 and Δpmt5 strains are unaffected. Taken together, this suggests that in P. pastoris, rhodanine-derived Pmt inhibitors affect multiple PMTs, and that different inhibitors are able to affect different PMTs (PMTi-1 inhibiting PMT1 and PMT2, and PMTi-3 inhibiting PMT2 and PMT5).

4): Pmt inhibitors block O-mannosylation at concentrations much below those at which growth is inhibited

For the industrial application of Pmt inhibitors in the production of therapeutic proteins it is of great importance that the inhibitors not only reduce O-mannosylation but also have a negligible effect on growth of the culture. Since relatively high concentrations of PMTi-1 are needed to consistently reduce O-glycan site occupancy in P. pastoris, we again employed PMTi-3 to test the window between reduction in O-glycan site occupancy and growth inhibition. To this end we grew strain YGLY4280 which expresses a recombinant IgG1 (for genotype refer to Table 1) in non-inducing glycerol medium in 96 well plates overnight, and then induced the cultures in media containing PMTi-3 at increasing concentrations. After induction overnight, the supernatant was then analyzed by Western blot for the presence of O-glycosylated heavy and light chains. As can be seen in Figure 6, hyperglycosylated heavy chain (marked by an asterisk) is clearly visible in the control lane without inhibitor, but cannot be detected anymore in the sample incubated in the presence of 0.15 µg/ml of PMTi-3. Incidentally, PMTi-3 does not inhibit growth of P. pastoris at concentrations well above 1 µg/ml (see Figures 5 and 6). This means that there is about a 10 fold window in which PMTi-3 can be used to reliably reduce O-glycan site occupancy in P. pastoris.
5): PMT knockouts and Pmt inhibitors not only reduce O-glycan site occupancy but also O-glycan chain length in *P. pastoris*

For the production of therapeutic glycoproteins in the glycoengineered *P. pastoris* platform, it is essential to understand the O-glycosylation profile of secreted proteins fully. To this end, in addition to characterizing the O-glycan site occupancy following PMT manipulations, we also profiled the O-glycosylation structures under the same conditions. Table 3 shows that a *PMT2* knockout, and to a lesser extent a *PMT1* knockout, in addition to a reduction in O-glycan site occupancy also lead to shortened O-glycan chain lengths. When we compared these two parameters on protein that had been expressed in a wild type strain with PMTi-3 addition with those of protein expressed in *PMT1* and especially *PMT2* knockouts without PMTi-3 addition, we saw that this effect could also be accomplished by the addition of inhibitor during the induction phase (compare Table 3 line 2 with lines 3 and 5). Furthermore, the effect seems to be additive, as a Δ*pmt2* strain induced in the presence of PMTi-3 expressed IgG1 with only single mannose O-glycan chains (Table 3 line 6).

**Discussion**

In the current report we have identified and characterized the PMT family of *Pichia pastoris*. Comparing each member of the *P. pastoris* PMT family to those of several other yeast and fungal organisms, we have elucidated that the *P. pastoris* family bears close homology to that of *C. albicans* [10,22–25]. Both *C. albicans* and *P. pastoris* possess five PMT members. Like *C. albicans*, *P. pastoris* possesses homologues to *PMT1*, *2*, *4*, *5* and *6* but no homologues to *PMT3* or *PMT7* [22]. PMTs function as a dimeric complex. Typically a member of the PMT1 sub-family (which includes *PMT1*, *PMT5* and *PMT7*) will dimerize with a member of the PMT2 sub-family (which includes *PMT2*, *PMT3* and *PMT6*) to form a heterodimeric complex, or *PMT4* will self-dimerize to form a homodimeric complex [7]. As such, this implies that *P. pastoris* possesses a redundant member of both the PMT1 and PMT2 gene sub-families. To elucidate if each member of the PMT1 and PMT2 sub-families contributed equally to O-mannosylation, an Agilent RNA expression microarray was employed to determine the relative expression of all of the PMT genes. The normalized intensity values indicated that *PMT2* was predominantly expressed, while *PMT1* and *PMT4* were expressed to moderate extents and *PMT5* and *PMT6* expression were relatively the lowest. This was the case with strains utilizing either glycerol or methanol as a carbon source. Cantero and Ernst reported that *PMT2* and *PMT4* transcript levels were upregulated more than two fold in the presence of a Pmt1 inhibitor in *C. albicans* [28]. However we could not detect any significant change in expression level of any of the PMTs when

![Figure 3. PMT expression profile by microarray analysis.](https://doi.org/10.1371/journal.pone.0068325.g003)
Figure 4. Overview of PMT knockout experiment. (A) The PCR overlap and PMT knockout procedure. First a linear gene deletion allele was constructed the following way: a region 5' of the PMT ORF was amplified using primers 1 and 2, the selectable marker was amplified using primers 3 and 4, and a region 3' of the PMT ORF was amplified using primers 5 and 6. The primers in parentheses were added to the schematic to illustrate that primers 2 and 3, as well as primers 4 and 5, are complementary to each other (thereby creating the overlaps). The amplified products were then used as templates together with primers 1 and 6 in the overlap reaction. The linear deletion allele was then integrated into the P. pastoris genome via homologous recombination, and knockouts were selected on plates supplemented with the appropriate antibiotic. (B) Western blot analysis of Kringle 1-3 protein (K1-3) expressed in wild type and PMT knockout strains. Strains JC53 (wild type), JC55 (Δpmt1), JC66 (Δpmt2), JC65 (Δpmt4), and JC51 (Δpmt5/6) were grown and induced in shake flasks. Seven µl of each supernatant was separated by polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto nitrocellulose membranes. K1-3 protein was detected using an anti-His antibody. O-glycan site occupancy (Occ.) was determined as described in Materials and Methods. The expected mass of the fully deglycosylated protein is 44.2 kDa, and the band at 36 kDa probably represents proteolytic cleavage.

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grown in the presence of PMTi-3 (data not shown). The relative contribution of each PMT was further validated by generating knockouts of the five genes. Western blot analysis of protein produced in these knockouts indicated that both PMT1 and PMT2 were predominantly involved in O-linked mannose addition to the Kringle reporter protein, whereas PMT4 knockout and the double knockout of both PMT5 and PMT6 showed only minor reduction in O-glycan site occupancy. Furthermore, PMT2 was the only PMT that was demonstrated to be essential in a Δoch1 N-glycan modified background. This fact was also one of the main reasons why a PMT2 knockout was not a viable option to control O-glycosylation in glycoengineered strains. However, through the use of Pmt inhibitors it is possible to overcome this issue.

The observation that PMT2 is a highly essential gene has been previously reported for several yeast and fungi, including C. albicans, S. pombe, A. fumigatus and Cryptococcus neoformans, where PMT2 knockout is lethal [22,24,29,30]. However, as in S. cerevisiae [25], no single PMT knockout is lethal in a wild type P. pastoris background.

During the revision of this manuscript, Govindappa and coworkers published a study on the role of the PMT1 gene in O-mannosylation of insulin precursor in P. pastoris [31]. In agreement with our data they identified the same P. pastoris homologues of S. cerevisiae PMT1, 2, 4, 5 and 6. In contrast to our results, however, they were unable to knock out the PMT2 gene and therefore suggested it to be an essential gene. The reason for their inability to isolate a PMT2 knockout despite screening approximately 1000 colonies is not obvious. The insertional inactivation method by which they tried to accomplish their PMT2 knockout is based on a single crossover, or roll-in, integration into the genome [32]. This method leads to the duplication of part of the gene, which makes the integration potentially reversible. Our knock out method, however, is based on a double crossover integration, which leads to stable removal of the open reading frame of the gene to be knocked out [13]. It is also possible that the slow growth phenotype of the PMT2 knockouts made the authors miss the much slower growing knocked out transformants, which in our case appeared several days after the wild type background colonies.

The data reported in our study indicates that Pmt1p and Pmt2p play the major role in modification of the Kringle reporter protein and that they most likely contribute to O-mannosylation of important cell house-keeping proteins. By contrast, PMT4

Table 1. P. pastoris strains used in this study.

| Strain          | Relevant genotype              |
|-----------------|--------------------------------|
| NRRL-Y11430     | wild type                       |
| YGLY4280        | TRP2::ZeoIlgG1                  |
| YGLY4140        | Δoch1::lacZ                     |
| YGLY6001        | Δoch1::lacZ Δpmt1::NatR         |
| YGLY4329        | Δoch1::lacZ Δpmt4::NatR         |
| YGLY733         | Δoch1::lacZ                     |
| YGLY7377        | Δoch1::lacZ Δpmt5::lacZ-URA5-LacZ|
| YGLY5968        | Δoch1::lacZ Δpmt6::lacZ-URA5-lacZ|
| YGLY35032       | Δpmt2::HygR                     |
| YJC53           | AOX1::ZeoI.onreadystatechangeK1-3 Δoch1::lacZ-URA3-lacZ |
| YJC55           | Δpmt1::HygR AOX1::ZeoI.onreadystatechangeK1-3 Δoch1::lacZ-URA3-lacZ |
| YJC66           | Δpmt2::NatR AOX1::ZeoI.onreadystatechangeK1-3 |
| YJC65           | Δpmt4::NatR AOX1::ZeoI.onreadystatechangeK1-3 |
| YJC51           | Δpmt5::NatR Δpmt6::KanR AOX1::ZeoI.onreadystatechangeK1-3 |
| YJC204          | AOX2::ZeoI.onreadystatechangeG1   |
| YJC200          | Δpmt1::HygR AOX2::ZeoI.onreadystatechangeG1 |
| YJC210          | Δpmt2::NatR AOX2::ZeoI.onreadystatechangeG1 |

Figure 5. Inhibitor sensitivity of PMT knockouts. For dilution assays, yeast cells were grown overnight in YSD and then adjusted to an OD600 of 1.0 using YSD, diluted in 10-fold increments, and then 3 µl of each dilution were spotted onto solid YSD media with varying concentrations of PMTi-3. The picture of the plate without PMTi-3 was taken after 48 hours, and the picture of the plates containing PMTi-3 were taken after 96 hours of incubation at 25°C. YGLY4140 is the parent strain of YGLY6001 and YGLY4329, YGLY733 is the parent strain of YGLY3773 and YGLY5968, and NRRL-Y11430 is the parent strain of YGLY35032.

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demonstrated relatively little effect on Kringle modification but appeared to be essential for cell viability in either a Δpmt1 or Δpmt2 background. Therefore, it appears that while Pmt4p does not play a major role in O-mannosylation in a wild type strain background, it does play a vital role in the absence of Pmt1p or Pmt2p. This finding agrees with observations that have been made in other yeast and fungal organisms. In both C. albicans and A. fumigatus, the concerted knockout of PMT1 and PMT4 were lethal, like the single PMT2 knockout in both of these organisms [22,29]. However, in A. nidulans, where PMT2 was not essential, knockout of PMT4 in combination with either PMT1 or PMT2 proved lethal [23], as in P. pastoris. Likewise, both PMT5 and PMT6, being paralogues of PMT2 and PMT1 respectively, may play more prominent roles in the absence of their sub-family members or under different stress conditions. Alternatively, both Pmt5p and Pmt6p may O-mannosylate specific proteins. For example, S. cerevisiae Pmt4p has been shown to O-glycosylate the protein gp115/Gas1p but not chitinase, thus indicating that protein O-glycosyltransferases differ in their specificity towards protein substrates [25].

Since many yeasts and fungi are pathogenic organisms, attempts have been made to produce anti-fungal agents to restrict their growth. In 2004, Orchard et al. identified a group of anti-fungal agents that were rhodanine-3-acetic acid (RAA) derivatives which targeted members of the PMT family [8]. In that study, the authors targeted C. albicans Pmt1p for anti-fungal development. A subsequent study in C. albicans concluded that the inhibitors preferentially blocked Pmt1p activity but could not exclude that the inhibitors had a minor effect on the other PMT family members [9]. By contrast, in S. cerevisiae, the RAA derivatives were shown to inhibit all three PMT sub-families, and not to be specific for Pmt1p [10]. In P. pastoris we demonstrated that RAA derivatives appear to target either Pmt1p and Pmt2p (in the case of PMT1-1) or Pmt2p and Pmt5p (in the case of PMT1-3) and have little effect on the other PMTs. As such, it was not unexpected to observe that Δpmt4 cells were hyper-sensitive to RAA derivatives, a similar phenotype to what was observed in C. albicans [9]. Furthermore, the hypersensitivity of this combination of inhibitor and knockout is consistent with the lethality of the double Δpmt1Δpmt4 mutants in Candida [22] and the fact that we

Table 2. O-glycan site occupancy of PMT knockout strains (w or w/o PMTi).

| Strain     | Relevant genotype | PMTi | O-glycan site occupancy |
|------------|------------------|------|-------------------------|
| NRRL-Y11430 | wild type        | –    | 20 (+ 9)                |
| YJC55      | Δpmt1            | –    | 3 (+ 2)                 |
| YJC66      | Δpmt2            | –    | 4 (+ 4)                 |
| YJC65      | Δpmt4            | –    | 16 (+ 7)                |
| YJC51      | Δpmt5 Δpmt6      | –    | 17 (+ 6)                |

O-glycan site occupancy of K1-3 protein expressed in wild type and PMT knockout strains in the presence or absence of 2 µM PMT1. Occupancy is expressed as number of O-mannose chains per molecule of K1-3.

Figure 6. Western blot analysis of an IgG1 expressed in the presence of PMTi-3. The PMT wild type strain YGLY4280 was grown in 96 well deep-well plates and induced in medium containing no PMTi-3 (C) or increasing concentrations of PMTi-3 (0.0187, 0.0375, 0.075, 0.15, 0.3, 0.625, 1.25, 2.5, 5, 10 µg/ml). After overnight induction, 7 µl of each supernatant was separated by polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto nitrocellulose membranes. IgG1 was detected using an anti-human IgG (heavy and light chain) antibody. Hyperglycosylated heavy-chain (indicated by an asterisk) is barely detectable at PMTi-3 concentrations of 0.15 µg/ml and higher, however strain growth and fitness (strain lysis) was still unaffected at PMTi-3 concentrations of up to 1.25 µg/ml. A representation of the PMTi-3 increase in concentration and the relative reduction in cell fitness are illustrated by light and dark gray triangles respectively.

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were unable to generate either the Δpmt1/Δpmt2, the Δpmt1/Δpmt4, or the Δpmt2/Δpmt4 double knockouts in *P. pastoris*.

Over the last decade significant advances have been made in engineering the N-linked glycosylation pathway of *P. pastoris* to produce glycoproteins possessing human-like N-glycans [5,18,33–35]. This process not only involves the generation of human-like N-glycans but also the elimination of non-desirable yeast glycans [36]. Likewise, it is desirable to limit yeast-type O-glycans on therapeutic proteins produced in this organism, and that the process utilized is scalable. Since the knockout of PMTs can lead to undesirable slow growth phenotypes, in the current study we have used a number of Pmt inhibitors which have demonstrated marked differences in their potencies. Although PMTi-1 was efficient at reducing O-glycan site occupancy, it also retarded growth at the dose required. A second inhibitor used, PMTi-3, proved much more potent and demonstrated significantly reduced O-mannosylation on a therapeutic antibody with little effect on cell growth. We used this therapeutic antibody as a second, commercially more relevant, test protein and to guard against the possibility that O-glycan reduction of K1-3 had been protein specific like has been shown for human insulin-like growth factor in *S. cerevisiae* [37]. RAA derivatives were also used to reduce the degree of O-mannosylation on a human anti-TRAIL-R antibody produced in the methylotrophic yeast *Ogataea minuta* [38]. Interestingly, these authors additionally observed an increase in the amount of assembled antibody secreted, and that antigen binding was also increased. The latter observation may have been attributable to the significant reduction in O-mannosylation observed on the light chain, which plays a key role in antigen binding. In contrast to this, a recombinant IgG1 produced in *P. pastoris* in the current report did not possess detectable O-mannosylation on the light chain, and incidentally no observable difference was detected in antigen binding when RAA derivatives were used (data not shown). In a parallel report by our laboratory a more potent RAA derivative (PMTi-4) was assessed for antibody production in *P. pastoris* [39]. Like the Kuroda et al. study, a dramatic increase in antibody titer was observed in the presence of RAA derivatives. Incidentally, unlike PMTi-3 which appears to target Pmt2p and Pmt5p, PMTi-4 preferentially inhibited Pmt2p [39].

Previous studies which have investigated *PMT* knockouts or the use of Pmt inhibitors have assessed cell fitness or reporter protein gel shifts to characterize these manipulations. Since our laboratory is concerned with the engineering of an expression platform for therapeutic protein production, we also characterized the glycan profiles resulting from our manipulations. An unexpected observation in our study was that in addition to reducing O-glycan site occupancy, both the knockout of *PMT1* or *PMT2*, or the use of PMTi-3, were shown to reduce O-glycan chain length. To our knowledge, this is the first characterization of a connection between *PMT* manipulations and O-linked mannose chain-length. Although we did not follow-up on the specific cause of chain-length reduction, there are a number of reasons why this might occur. For instance, the extension of the O-linked mannose chain is initially performed by members of the KTR α-1,2-mannosyltransferase gene family, which includes *KTR1*, *KTR3* and *KRE2* (reviewed for the *S. cerevisiae* homologues in [40]). It is possible that during events of O-glycan stress, the expression of these genes, or those involved in the synthesis of the required sugar intermediates, may be down regulated. Interestingly, transcriptional profiling of *C. albicans* demonstrated that when any of the *PMT* genes were deleted, with the exception of *PMT6*, the transcript levels for *DPM1*, encoding the dolichol-phosphate mannose synthase which generates dolichol phosphomannose from GDP-Man and dolichol, increased [9]. At the same time the transcript for *SRB1*, encoding the GDP-mannose pyrophosphorylase, which generates GDP-Man from mannose-1-phosphate and guanosine triphosphate, showed no significant change [9]. Taken together, an increase in *DPM1* expression with no increase in *SRB1* expression, would reduce the cellular pools of GDP-Man available to the KTR family members, with a foreseeable reduction in O-mannose chain-length extension. Interestingly, Cantero et al. also demonstrated that when wild type *C. albicans* was treated with PMTi the transcript levels of numerous genes were regulated differently to when the *PMT* genes were knocked-out [9]. In the presence of inhibitor no changes were observed in *SRB1* transcript levels, while a similar increase in *DPM1* transcript levels was observed, thus, potentially reducing GDP-Man levels by converting it into Dol-P-Man. Other explanations for why we observed a reduction in O-mannose chain-length may be due to reduced expression or activity (due to reduced O-glycosylation) of: the GDP-mannose transporter (Vrg4p), which translocates GDP-mannose from the cytoplasm into the Golgi where the KTR members reside, the KTR family members themselves, or, some other accessory protein, such as chaperones. Another possible explanation for the apparent occurrence of a higher percentage of shorter chain-length O-linked glycans is that manipulation of the Pmt1p and Pmt2p activities specifically eliminated O-glycan sites which were more accessible to KTR mannosyltransferase activities. As such, by specifically eliminating O-linked glycans with longer chain-lengths, only the shorter, possibly more sterically restricted, glycans remained. A further possibility is that the activities of Pmt4p, Pmt5p or Pmt6p may have been responsible for these shorter glycans and, having eliminated the longer glycans initiated by Pmt1p or Pmt2p, only these shorter glycans remained.

The importance of controlling the O-mannose chain-length on therapeutic proteins has recently been assessed [41].

**Table 3.** Occupancy and O-glycan length of *PMT* knockout strains (w or w/o PMTi).

| Strain  | PMT knockout | PMTi | Occupancy | Man1 | Man2 | Man3 | Man4 |
|---------|--------------|------|-----------|------|------|------|------|
| YJC204  | wild type    | –    | 32        | 14   | 42   | 37   | 6    |
|         |              | +    | 6         | 76   | 24   | 0    | 0    |
| YJC200  | Δpmt1        | –    | 14        | 34   | 26   | 33   | 6    |
|         |              | +    | 4         | 78   | 11   | 11   | 0    |
| YJC210  | Δpmt2        | –    | 4         | 80   | 11   | 8    | 1    |
|         |              | +    | <2        | 100  | 0    | 0    | 0    |

O-glycan site occupancy of an IgG1 expressed in wild type and PMTi knockout strains in the presence or absence of 0.2 μM PMTi-3. Occupancy is expressed as number of O-mannose chains per molecule of antibody. Man1 through Man4 designates the percentage of mannose chains with the respective length.
Cukan and colleagues showed that those proteins expressed in *P. pastoris* with mannnotriose and longer O-glycans bound to Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), whereas proteins with single mannose O-glycans showed DC-SIGN binding that was comparable to that of CHO-produced IgG1, which lacks O-linked mannos [41]. Since DC-SIGN is involved in antigen presentation and the initiation of an immune response, the lack of binding to this receptor is more desirable. Furthermore, recently we have shown that through engineering of O-glycosylation in *P. pastoris*, by the introduction of a human-like O-glycosylation pathway, we can enhance the therapeutic application of proteins expressed in *P. pastoris* [42].

The methylotrophic yeast, *Pichia pastoris*, is an important organism used for the production of therapeutic proteins. Previously our laboratory has described methods for primarily engineering N-glycosylation in this organism. The current study characterizes the *P. pastoris* protein-O-mannosyltransferase family. In doing so, we have demonstrated that O-glycosylation in *P. pastoris* can be manipulated by either knockout of the PMT genes or the use of Pmt inhibitor if knockouts are not viable or lead to undesirable phenotypes. As such, these offer a means to reduce non-desirable yeast-like O-glycans. This reduction of O-glycosylation represents an important step forward for the *Pichia pastoris* production platform as a suitable system for the production of therapeutic glycoproteins.

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**Author Contributions**

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**Author Contributions**

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