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Protein glycosylation pathways in filamentous fungi

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Glycosylation of proteins is important for protein stability, secretion, and localization. In this study, we have investigated the glycan synthesis pathways of 12 filamentous fungi including those of medical/agricultural/industrial importance for which genomes have been recently sequenced. We have adopted a systems biology approach to combine the results from comparative genomics techniques with high confidence information on the enzymes and fungal glycan structures, reported in the literature. From this, we have developed a composite representation of the glycan synthesis pathways in filamentous fungi (both *N*- and *O*-linked). The *N*-glycosylation pathway in the cytoplasm and endoplasmic reticulum was found to be highly conserved evolutionarily across all the filamentous fungi considered in the study. In the final stages of *N*-glycan synthesis in the Golgi, filamentous fungi follow the high mannose pathway as in *Saccharomyces cerevisiae*, but the level of glycan mannosylation is reduced. Highly specialized *N*-glycan structures with galactofuranose residues, phosphodiesters, and other insufficiently trimmed structures have also been identified in the filamentous fungi. *O*-Linked glycosylation in filamentous fungi was seen to be highly conserved with many mannosyltransferases that are similar to those in *S. cerevisiae*. However, highly variable and diverse *O*-linked glycans also exist. We have developed a web resource for presenting the compiled data with user-friendly query options, which can be accessed at www.fungalglycans.org. This resource can assist attempts to remodel glycosylation of recombinant proteins expressed in filamentous fungal hosts.

Keywords: comparative genomics/filamentous fungi/glycan synthesis/recombinant proteins/systems biology

Introduction

Glycosylation is the most universal and structurally diverse form of posttranslational modification. It occurs by the attachment of a glycan to a protein either at an asparagine residue (N-X-S/T motif where X denotes any amino acid except proline) (Bause 1983), termed *N*-glycosylation, or at a hydroxylysine, hydroxyproline, serine, or threonine, called *O*-glycosylation (Carraway and Hull 1989). Glycosylation can contribute to protein secretion, stability, and immunogenicity (Lee et al. 2003) and in the case of membrane glycoproteins, these sugars or glycans can mediate a cell's communication with the outside world (Crocker and Feizi 1996). Glycans are synthesized by the coordinated expression of numerous genes that code for glycosyltransferases, glycosidases, and other enzymes that synthesize and remodel glycan chains, as well as accessory enzymes involved in the synthesis and transport of nucleotide sugars. The *N*-linked glycosylation pathway of proteins has been extensively characterized in mammalian systems (Kornfeld R and Kornfeld S 1985) and the yeast, *Saccharomyces cerevisiae* (Herscovics 1999b). However, the work carried out in filamentous fungi remains focused on a few isolates of particular genera, mainly *Aspergillus* spp. and *Trichoderma* spp. (Maras, van Die et al. 1999).

Filamentous fungi like *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei* display a natural ability to secrete large amounts of native proteins (>40 g/L), such as hydrolytic enzymes, into the growth medium (Iwashita 2002). Like other microbial expression systems, filamentous fungi display shorter process times than, for example, mammalian cell cultures. Hence, attempts have been made for extensive use of filamentous fungi as expression hosts for heterologous proteins of industrial/pharmaceutical significance (Devchand and Gwynne 1991). However, the yield of foreign proteins in fungal hosts, especially those of mammalian origin, has been much less than expected. In part, this may be due to the fungal glycosylation of the mammalian proteins that may further lead to incorrect folding and subsequent elimination of foreign proteins by protein quality control mechanisms in the endoplasmic reticulum (ER). In addition, unusual fungal glycan structures on the heterologous protein may affect its activity, stability, immunogenicity, and performance. Hence the therapeutic use of glycoproteins produced in filamentous fungi has been quite limited to date (Maras, van Die et al. 1999).

Filamentous fungi are known to carry small, high-mannose *N*-glycans (Maras, van Die et al. 1999). Like in other eukaryotes, the sugar linked to asparagine is GlcNAc in a beta configuration. The initial steps for the formation of the lipid-linked precursor oligosaccharide and its *en bloc* transfer to the polypeptide in the rough endoplasmic reticulum are conserved. Hyperglycosylation as observed in *S. cerevisiae* has not been documented as a typical feature of filamentous fungi although there are reports pointing to the occurrence of high mannose glycans in specific strains (Goto et al. 1997). Unusual glycosylation modifications like the presence of phosphate residues and single, nonsubstituted GlcNAc residues on *N*-glycans of cellobiohydrolase I have been detected on potential *N*-glycosylation sites of high cellulase-secreting mutant strains of *T. reesei* (Harrison et al. 1998). *O*-Linked oligosaccharides on glycoproteins are

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diverse among the eukaryotic organisms with respect to both their monosaccharides and the mode of sugar linkage to the protein. *O*-Mannosylation is commonly found in glycoproteins of many higher eukaryotes as well as in most fungi (Strahl-Bolsinger et al. 1999), including the filamentous fungi (Goto 2007). *O*-Mannosylation has been extensively studied in the unicellular yeast *S. cerevisiae*. In *S. cerevisiae*, the oligosaccharides are attached to the protein by a mannosyl residue unlike the *O*-glycosylation usually seen in mammalian cells, in which an *N*-acetyl-galactosaminyl unit is linked to Ser/Thr. The mannose residue from dolicholphosphate mannose is transferred to Ser/Thr in the endoplasmic reticulum by protein-*O*-D-mannosyltransferase. The oligosaccharide is then linearly elongated in the Golgi by up to seven mannose residues by mannosyltransferases that utilize guanosine diphosphate mannose (GDP-mannose) as the mannosyl donor for the elongation process (Lussier et al. 1997).

Samuelson and co-workers have worked on a phylogenetic approach to find common ancestory and differences for specific glycosyltransferases over a wide family of eukaryotes. This included a thorough bioinformatic analysis followed by experimental identification of *N*-glycans (Samuelson et al. 2005). In our present work, we elucidate the diversity of *N*- and *O*-glycan biosynthesis in filamentous fungi using an integrated systems biology approach. The known information about glycosylation from the genome, proteome, and glycome is compared between 12 filamentous fungi, including fungi of medical and industrial importance for which genomes have been recently sequenced. The well-characterized yeast, *S. cerevisiae*, is used as a reference control. Results are discussed in the context of *N*- and *O*-linked glycosylation pathways in filamentous fungi and their relevance to recombinant glycoprotein production.

Materials and methods

Data source

The raw genomic sequence data and putative gene transcript and protein sequences for the 12 filamentous fungi were obtained from the fungal sequencing organizations including The Fungal Genome Initiative (FGI) Broad Institute (http://www.broad.mit.edu/) and DOE Joint Genome Institute (JGI) (http://genome.jgi-psf.org/euk_home.html). Complete details of all data sources are given in Table I in the Supplementary Data. Budding yeast *S. cerevisiae* represents an excellent model system as many of the metabolic pathways, including glycan synthesis, have been extensively studied using functional genomics, genetics, and molecular biology (Barr 2003). *S. cerevisiae* was hence used as the reference species for sequence-based comparative analysis in the present work.

Enzyme, glycan, and pathway analysis

We focused our study on catalytic subunits of glycan synthesis enzymes. The protein sequences from the reference species, *S. cerevisiae*, representing the various enzymes in the glycan synthesis pathway were retrieved from the online repositories like NCBI and Kyoto Encyclopedia of Genes and Genomes (KEGG). Where glycan synthesis enzymes of interest were not present in *S. cerevisiae*, relevant gene sequences from other species (e.g., *Trypanosoma cruzi, Leishmania major*) were also used. BLAST (Altschul et al. 1997) searches of genome sequences and ESTs were used to identify genes encoding glycan synthesis enzymes and multiple alignment of enzyme homologs was undertaken with ClustalW (Thompson et al. 1994). Homologs were further examined with SMART (Gavin et al. 2002) and Pfam (Bateman et al. 2004) to determine if they carried conserved functional domains. BLAST2GO (Conesa et al. 2005) was used for gene ontology classification where required. Glycan structures identified and/or characterized in filamentous fungi were collected from repositories like KEGG glycan (Hashimoto et al. 2006), the CFG glycan database (http://www.functionalglycomics.org/static/index.shtml), and highly curated repositories like GlycoSuiteDB (Cooper et al. 2003). Where nonstandard monosaccharides or linkages were seen in glycans, searches for genes encoding relevant enzymes were undertaken. Finally, composite pathway maps for glycan synthesis pathways were drawn by combining sequence analysis results, experimental evidence, and filamentous fungi-specific glycan structure data. Details of various techniques adopted and tools used for data curation are shown in Figure "Data analysis: tools and techniques" on the web resource homepage at www.fungalglycans.org.

User interface and visualization tools

The results of this analysis are made available online at www.fungalglycans.org. The resource was developed using open source technologies Zope (V2.8.1) (http://www.zope.org), Python (V2.4.3) (http://www.python.org), and the MySQL relational database (V4.1.10a) (http://www.mysql.com). An easy to use web-based query interface allows the user to choose from a range of options and query the underlying filamentous fungi-specific glycan synthesis datasets. The query interface and output datasets are shown in the Figure "User query options and output data-sets" on the web resource homepage at www.fungalglycans.org. Detailed guidelines for using the query tool are also provided.

Results

The primary objective of this work was to compare and understand the protein-associated glycan synthesis pathways in filamentous fungi. Twelve species of filamentous fungi were chosen for analysis as they were of medical or industrial interest, with genomic sequences available in the public domain. *S. cerevisiae* and *Schizosaccharomyces pombe* were included in the analyses as reference species. Pathways were constructed using a systems biology approach, by analyzing the genes encoding the various enzymes involved in all steps of *N*- and *O*-linked oligosaccharide structure synthesis together with all known glycan structures from the filamentous fungi.

N-Glycan synthesis

The evolutionary conservation of *N*-glycan synthesis in the filamentous fungi was investigated. In most cases and for most species, genes for all steps of *N*-glycan synthesis were found (Figure 1). Genes were absent for some species and are shown as 0% sequence identity. These are shown by gray rectangles in Figure 1. There are two possible explanations for this. Firstly, the gene is not present in the genome of the species. This is likely to be the case for the gene *Mnn5* in *Sch. pombe* whose genome is well understood. The second reason could be that the

Table I. Species-specific variations in the *N*-glycan synthesis pathway. Enzymes involved are placed in a sequential order of action and categorized by subcellular localization

	Gene	EC number	Molecular function	Product	Organisms: Human	Yeast: sce	Yeast: spo	Filamentous fungi: anu	ani	ncr	tre	fgr	mgr	chg	coi	sci	stn	ure	usm
Cytoplasm	*Mpg1*	2.7.7.13	Alpha-D-mannose-1-phosphate guanylyltransferase	GDP-mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Dpm1	2.4.1.83	Dolichol phosphate mannosyltransferase	Dolicholphosphate mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Sec59	2.7.1.108	Dolichol kinase	Dolichol phosphate	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
	Alg7	2.7.8.15	UDP-N-acetyl-D-glucosamine:dolichol phosphate	GlcNAcPP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alg13 /Alg14	2.4.1.141	Beta-1,4-N-acetylglucosaminyltransferase	$GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	Alg1	2.4.1.142	Beta-1,4-mannosyltransferase	$ManGlcNAc_2$ PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	Alg2	2.4.1.132	Alpha-1,3/alpha-1,6-mannosyltransferase	$Man_3GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alg11	2.4.1.-	Alpha-1,6-mannosyltransferase	$Man_5GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Endoplasmic reticulum	*Rft1*	-	Oligosaccharidyl-lipid flippase family	$Man_5GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alg3	2.4.1.130	Alpha-1,3-mannosyltransferase	$Man_6GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
	Alg9	2.4.1.-	Alpha-1,2-mannosyltransferase	$Man_7GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alg12	2.4.1.130	Alpha-1,3/alpha-1,6-mannosyltransferase	$Man_8GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alg9	2.4.1.-	Alpha-1,2-mannosyltransferase	$Man_9GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alg6	2.4.1.-	Alpha-1,3-glucosyltransferase	$GlcMan_9GlcNAc_2$PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alg8	2.4.1.-	Alpha-1,2-glucosyltransferase	$Glc_2Man_9GlcNAc_2$ PP-Dol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alg10	2.4.1.-	Alpha-1,2-glucosyltransferase	$Glc_3Man_9GlcNAc_2$ PP-Dol	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
	Ost1-alpha	2.4.1.119	Oligosaccharyltransferase	$Glc_3Man_9GlcNAc_2$ Asn	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Wbp1-beta	2.4.1.119	Oligosaccharyltransferase	$Glc_3Man_9GlcNAc_2$ Asn	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Stt3	2.4.1.119	Oligosaccharyltransferase	$Glc_3Man_9GlcNAc_2$ Asn	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Gls1	3.2.1.106	Glucosidase I	$Glc_2Man_9GlcNAc_2$ Asn	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Gls2	3.2.1.84	Glucosidase II	$Man_9GlcNAc_2$ Asn	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Mns1	3.2.1.113	Alpha-1,2-mannosidase	$Man_5GlcNAc_2$ Asn	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Mns1	3.2.1.113	Alpha-1,2-mannosidase	$Man_8GlcNAc_2$ Asn	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	Och1	2.4.1.232	Alpha 1,6-mannosyltransferase	$Man_9GlcNAc_2$ Asn	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	Mnn9	2.4.1.-	Mannan polymerase complex Mnn9 subunit	$Man_{15}GlcNAc_2$ Asn	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-
	Van1	2.4.1.-	Mannan polymerase complex I Van1 subunit	$Man_{15}GlcNAc_2$ Asn	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-
	Anp1	2.4.1.-	Mannan polymerase II complex Anp1 subunit	$Man_{15\text{-}N}GlcNAc_2$ Asn	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-
	Mnn10	2.4.1.-	Mannan polymerase II complex Mnn10 subunit	$Man_{15\text{-}N}GlcNAc_2$ Asn	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-
	Mnn11	2.4.1.-	Mannan polymerase II complex Mnn11 subunit	$Man_{15\text{-}N}GlcNAc_2$ Asn	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-
	Mnn2	2.4.1.-	Alpha 1,2-mannosyltransferase	$Man_{15\text{-}N}GlcNAc_2$ Asn	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-
	Mnn5	2.4.1.-	Alpha 1,2-mannosyltransferase	$Man_{15\text{-}N}GlcNAc_2$ Asn	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	Mnn6	2.4.1.-	Mannosyltransferase	$Man_{15\text{-}N}GlcNAc_2$ Asn	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-
	Mnn1	2.4.1.-	Alpha 1,3-mannosyltransferase	$Man_{13}GlcNAc_2$ Asn	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Golgi	*MGAT1*	2.4.1.101	Beta-1,2-N-acetylglucosaminyltransferase	$Man_5GlcNAc_3$ Asn	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MAN2A1	3.2.1.114	Alpha-mannosidase II	$Man_3GlcNAc_3$ Asn	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MGAT2	2.4.1.143	Beta-1,2-N-acetylglucosaminyltransferase	$Man_3GlcNAc_4$ Asn	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	FUT8	2.4.1.68	Alpha (1,6) fucosyltransferase	$LFucMan_3GlcNAc_4$ Asn	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B4GALT1	2.4.1.38	Beta-1,4-galactosyltransferase	$Gal_2LFucMan_3GlcNAc_4$ Asn	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	ST6GAL1	2.4.99.1	Alpha-2,6-sialyltransferase	$Neu5AcGal_2LFucMan_3GlcNAc_4Asn$	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1 Conservation of enzymes across cytoplasm and ER in all eukaryotes

2 Enzymes in Golgi in high mannose pathway common to *S. cerevisiae* and filamentous fungi

3 Enzymes in Golgi involved in hypermannosylation in *S. cerevisiae*

4 Enzymes leading to complex and hybrid oligosaccharides in humans

ani, *aspergillus niger*; anu, *aspergillus nidulans*; chg, *chaetomium globosum*; coi, *coccidioides immitis*; fgr, fusarium graminearum; mgr, *magnaporthe grisea*; ncr, neurospora crassa; sce, *saccharomyces cerevisiae*; sci, sclerotinia sclerotiorum; spo, *schizosaccharomyces pombe*; stn, stagonospora nodorum; tre, *trichoderma reesei*; ure, uncinocarpus reesei; usm, ustilago maydis.
+: ortholog present in the species and −: ortholog absent in the species.

genomic information was incomplete and the gene is yet to be sequenced. This is likely to be the case for *Sec59* in *Coccidioides immitis*, *Alg3* in *Chaetomium globosum*, *Alg10* in *Magnaporthe grisea*, and *Alg14* in *Ustilago mayadis*. For *U. maydis*, which has a newly sequenced genome, a number of genes are absent. These genes include *Alg14* and *Alg1* encoding corresponding enzymes in the cytoplasm and also the genes *Mnn9*, *Van1*, *Anp1*, and *Mnn11* encoding mannosyltransferases in the Golgi.

The degree of sequence conservation between the yeast and filamentous fungal species is also shown in Figure 1. For all genes, the two yeasts *Saccharomyces paradoxes* and *S. cerevisiae* showed very high sequence identity. The filamentous fungi, however, showed large but consistent sequence divergence from the two yeast species. Interestingly, a cross-comparison of sequences between the filamentous fungi themselves showed divergence within this group, with sequences having 25–30% difference between species (data not shown).

There are a few genes, particularly associated with glycan mannosylation, which were found to have very low sequence conservation in filamentous fungi in comparison to the reference

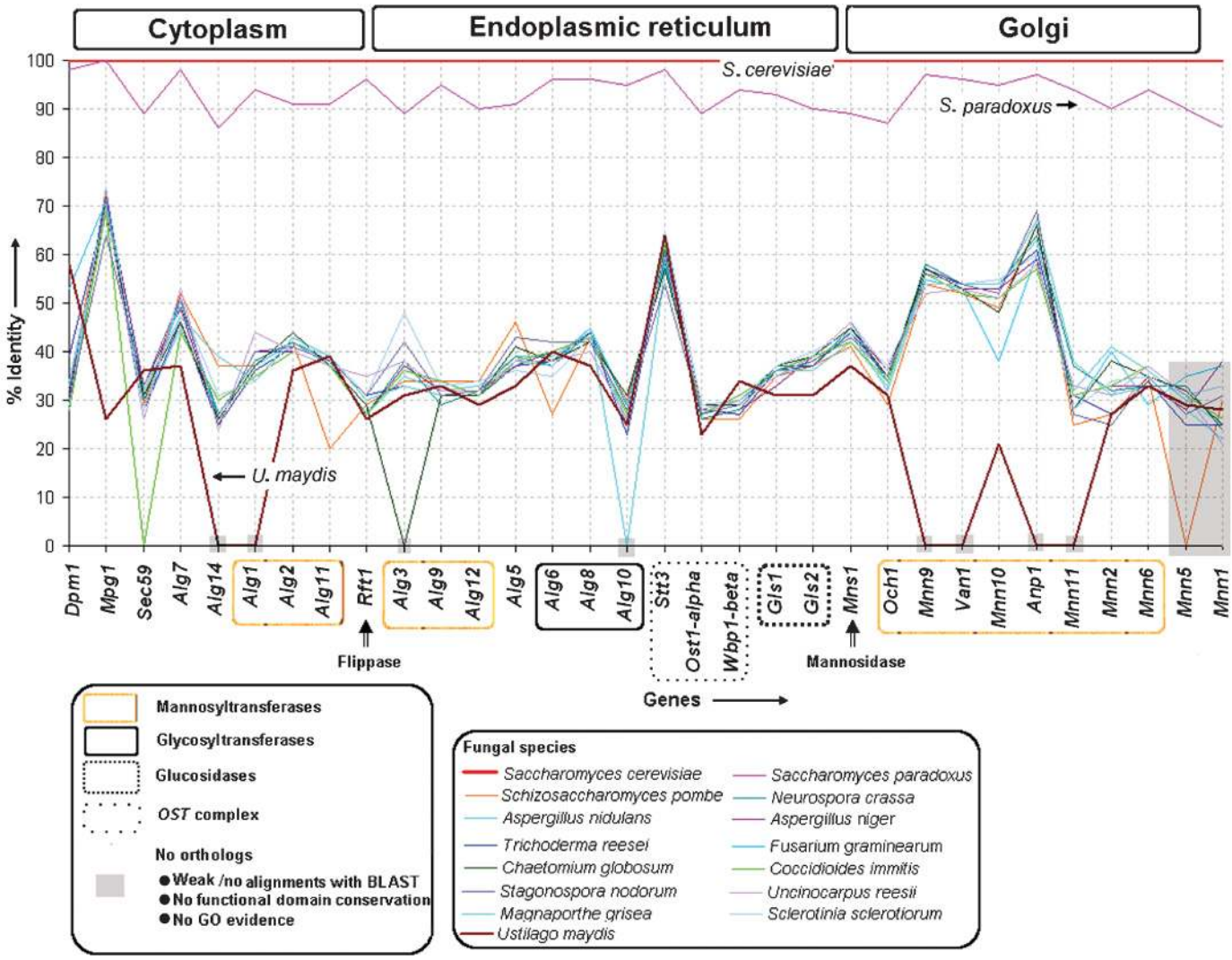

Fig. 1. Identity plot displaying evolutionary conservation/divergence of the *N*-glycan synthesis pathway across fungal species. Species included are yeasts (*S. cerevisiae*, *S. paradoxus*, *Sch. pombe*) and 12 filamentous fungal species with *S. cerevisiae* as reference. Domain conservation and gene ontologies are also used where necessary in defining probable orthologs. Enzymes on the *x*-axis are sorted by their order in the *N*-glycan synthesis pathway and are described by their gene names.

yeast, *S. cerevisiae* (Figure 1). Examples of this are *Mnn1* and *Mnn5*. Putative orthologs were identified from BLAST analyses for the above two genes with weak alignments. However, we found no evidence to suggest that they are functionally identical to the *S. cerevisiae* genes encoding these proteins. Searches for domains in these proteins did not reveal strongly conserved functional regions. In the absence of other additional evidence, we view these proteins as unlikely to be direct orthologs that are functioning in *N*-glycan synthesis.

Many genes expressed in the ER, such as *Alg3*, *Alg9*, *Alg12, Ost1-alpha,* and *Wbp1-beta* and a few others expressed in the Golgi, like *Mnn11* and *Mnn6*, displayed sequence identity in the range of 25–40%, but retained their functional domains over evolution. These were categorized as probable orthologs.

The gene *Mnn2*, encoding an alpha-1,2-mannosyltransferase, displayed sequence conservation of 25–35%. It did not contain any functional domains either in the reference species, *S. cerevisiae*, or its putative ortholog sequences in filamentous fungi. In such instances, gene ontology definitions (Ashburner et al. 2000) were used as additional parameters to define the validity of orthologs. Prominent mannosyltransferase activity from GO definitions assisted the inclusion of *Mnn2* gene as a possible candidate for the *N*-glycan synthesis pathway.

In order to better understand the relative importance of the enzymes associated with *N*-glycan synthesis, an alternative representation of the above data was generated. Figure 2 shows that the most critical genes in the *N*-glycan synthesis pathway like *Mpg1*, the gene *Stt3* encoding for a major subunit of the *Ost* complex, and the genes encoding for various mannosyltransferases involved in the elongation of the high mannose glycan, viz. *Anp1*, *Van1*, and *Mnn9*, are most highly conserved across the filamentous fungal species. Clearly, the function of these enzymes is highly specialized and they are essential for the correct development or transfer of the *N*-glycans. These genes are represented by the uppermost lines in the graph and show

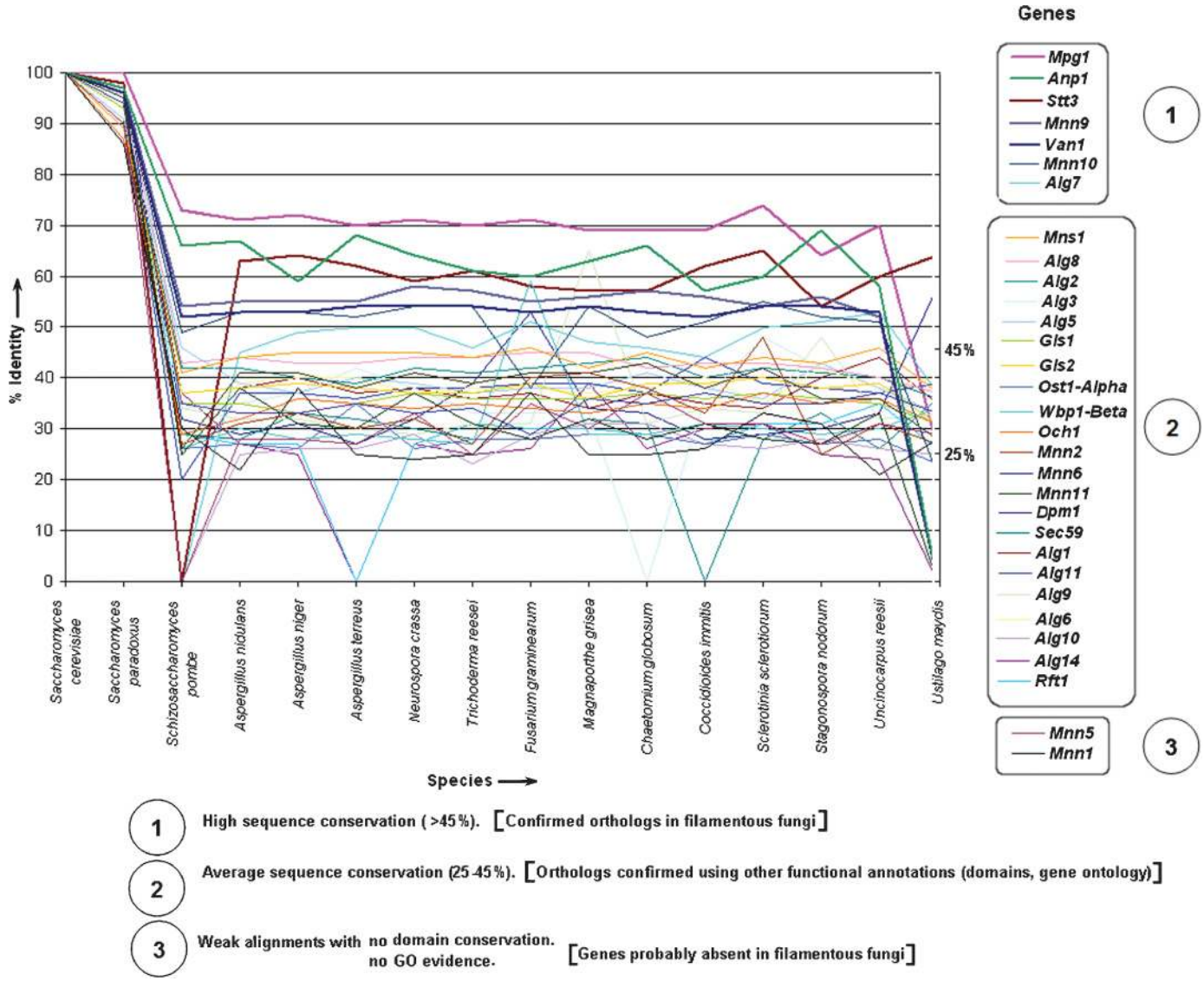

Fig. 2. Identity plot displaying conservation of genes encoding enzymes involved in *N*-glycan synthesis across filamentous fungi species. Genes are classified into three separate groups. Group 1, which displays high sequence conservation (with functional domain evidence), group 2 with moderate sequence conservation (with functional domains preserved and/or with gene ontology verification), and group 3 with the genes that are unlikely to be functional orthologs (weak alignments with no additional evidence found). Enzymes are described by their gene names.

greater than 45% sequence identity, forming group 1 in Figure 2. A second group of genes could be seen and form group 2 in the figure. These include genes like *Alg3* and *Alg9* encoding mannosyltransferases which are involved in addition of initial mannose molecules and some genes like *Alg6*, *Alg8*, and *Alg10* encoding glycosyltransferases which add glucose molecules to the glycan. This group also includes the genes *Ost1-alpha*, *Wbp1-beta*, *Gls1*, *Gls2*, *Mnn2*, and *Mnn6*. They displayed sequence identity of 25–40% in comparison to the reference, *S. cerevisiae*. However, we were able to confirm the sequences as being functional orthologs by using additional evidence such as highly conserved functional domains and gene ontology classification data. Group 3 comprised enzymes encoded by the genes *Mnn5* and *Mnn1* which displayed weak sequence alignments and no domain conservation or gene ontology evidence in filamentous fungi. We can thus conclude that these genes are probably absent in the filamentous fungal species. (Also refer to the results for Figure 1 above.)

O-Glycan synthesis

The evolutionary conservation of the *O*-glycan synthesis pathway was also investigated. All the filamentous fungal species displayed a consistent sequence divergence from the reference species *S. cerevisiae*, with *U. maydis* displaying the lowest conservation (Figure 3). The family of alpha-1,2-Mnt-encoding genes (*Mnt1*, *Ktr1*, *Ktr3*) are the most conserved with highest percent identity of around 40–65% with *S. cerevisiae*, followed by the protein mannosyl transferase gene family (*Pmt1–6*), which shows identity in the range of 35–55%. Functional domains were also seen to be preserved over evolution. The family

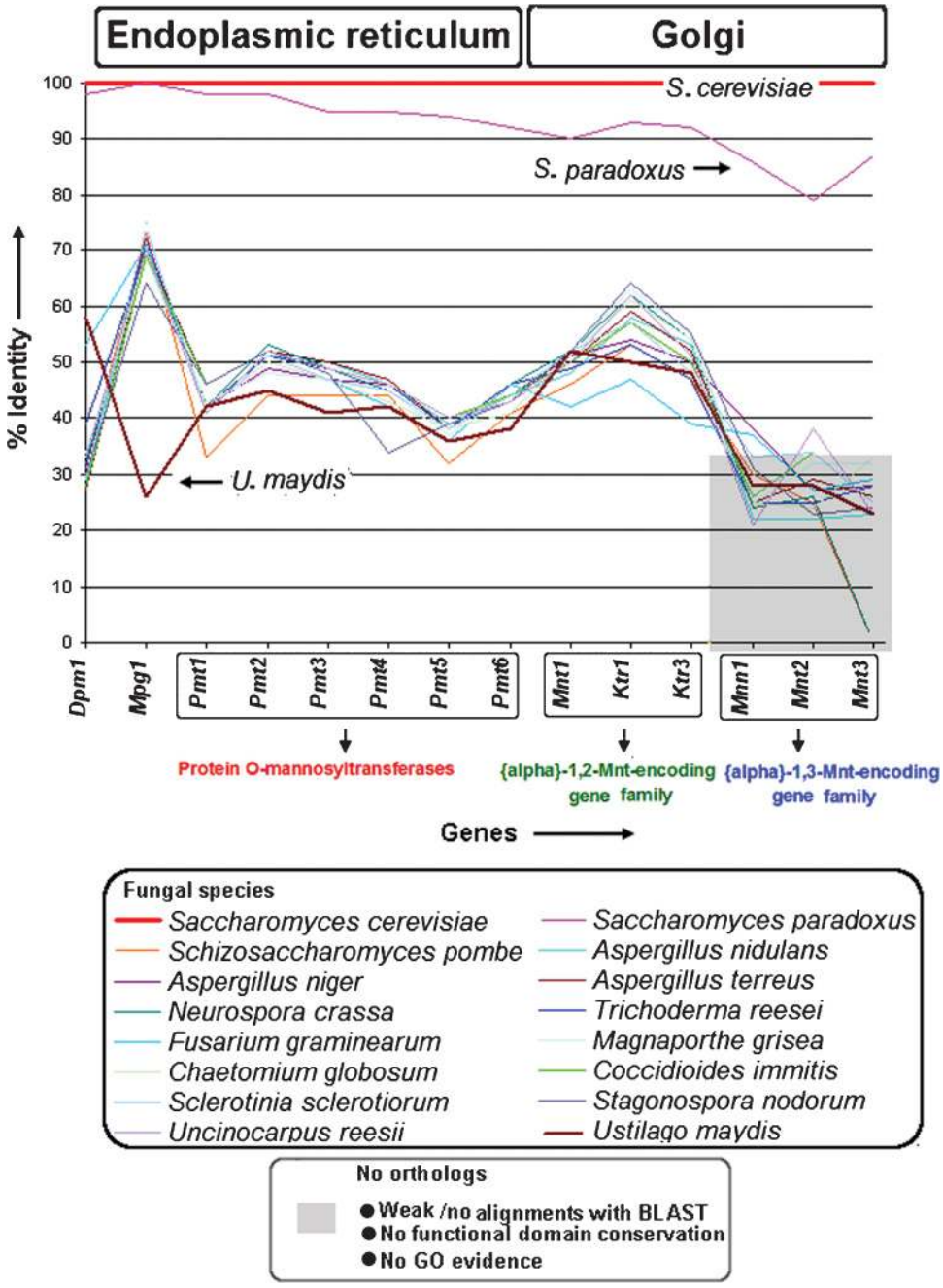

Fig. 3. Identity plot displaying evolutionary conservation/divergence of the *O*-glycan synthesis pathway across fungal species. Species included are yeasts (*S. cerevisiae, S. paradoxus, Sch. pombe*) and 12 filamentous fungal species with *S. cerevisiae* as reference. Domain conservation and gene ontologies are also used where necessary in defining probable orthologs. Enzymes on the *x*-axis are sorted by their order in the *O*-glycan synthesis pathway and are described by their gene names.

of alpha-1,3-Mnt-encoding genes (*Mnn1*, *Mnt2*, *Mnt3*), known to be involved in protein mannosylation in *S. cerevisiae*, are the least conserved and likely to be absent in filamentous fungi and fission yeast *Sch. pombe*.

Glycan synthesis pathways for filamentous fungi

Glycan synthesis pathways for filamentous fungi were constructed by combining gene sequence ortholog data, reference pathway information from KEGG (Hashimoto et al. 2006), as well as other literature sources and information about glycan structures identified in filamentous fungi. The *N*- and *O*-glycan synthesis pathways we developed are shown in Figures 4 and 5, respectively. The individual steps in glycan synthesis have been categorized by subcellular localizations. The genes which are involved in various steps of glycan synthesis and for which orthologs were identified in filamentous fungi are represented by shaded boxes. The glycan structures identified in filamentous fungi have been positioned appropriately in the pathways. Additional details for all glycan structures are provided in Table II of Supplementary Data. The *N*-glycan synthesis pathway in filamentous fungi follows the evolutionarily conserved path in the cytoplasm and endoplasmic reticulum. It then

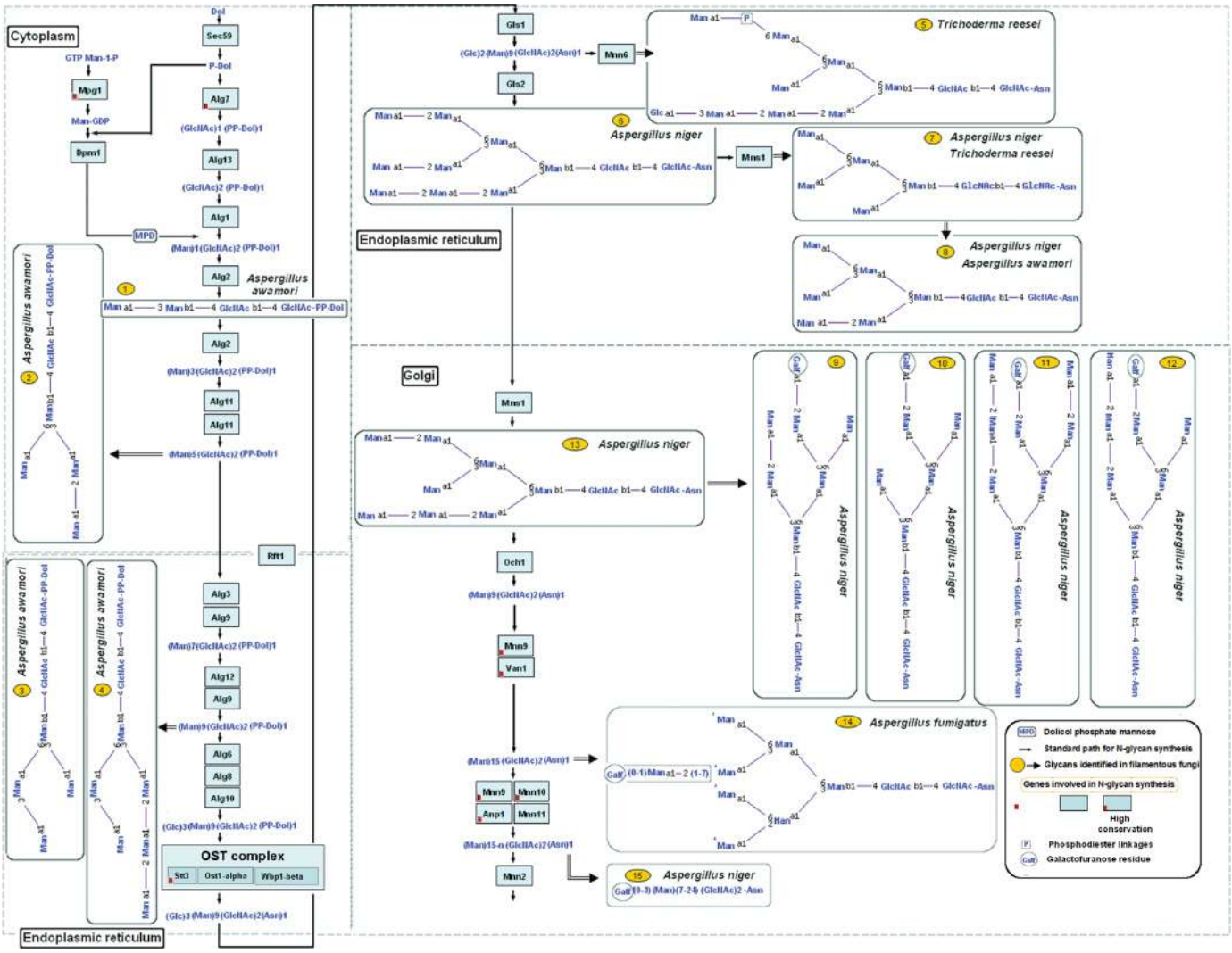

Fig. 4. *N*-Glycan synthesis pathway in filamentous fungi. The shaded rectangles represent enzymes involved in various steps of glycan synthesis. Glycan structures characterized in filamentous fungal species are represented in rounded boxes. See Supplementary Data for details.

deviates toward the high mannose path as in the reference, *S. cerevisiae*, though with much lower level of mannosylation. The presence of complex mannosyltransferases and high mannose glycan structures characterized in specific filamentous fungal species substantiate these results.

Table I shows the clear distinction between the pathway for *N*-glycan synthesis in the Golgi of the higher eukaryotes (humans), yeasts (*S. cerevisiae*, *Sch. pombe*), and the filamentous fungi. Humans form hybrid and complex oligosaccharides with the addition of GlcNAc, galactose, fucose, and sialic acid residues. Fungal species deviate to the high mannose pathway. The level of mannosylation is found to be limited in the filamentous fungi (when compared to *S. cerevisiae*) due to the absence of terminal mannosyltransferases such as Mnn5 and Mnn1.

Similarly we have constructed *O*-linked glycan synthesis pathways, based on reference data for genes and pathways from *S. cerevisiae* (Figure 5). Filamentous fungi display *O*-mannosylation; however, the family of alpha-1,3-Mnt-encoding genes (*Mnn1*, *Mnt2*, *Mnt3*) which add terminal mannose residues in *S. cerevisiae* are probably absent. Unique linear as well as branched glycan structures have been documented for various filamentous fungi.

Discussion

Genomic data have recently become available for numerous evolutionarily related species of medically and industrially significant filamentous fungi. This makes possible the analysis of generic as well as more specific features of proteins involved in glycan synthesis pathways. To our knowledge, this work is the first detailed report and resultant web resource, describing the nature and extent of glycosylation pathways in the filamentous fungal species.

N-Glycan synthesis

The initial steps of *N*-glycan synthesis in the cytoplasm and the process of transfer of the core oligosaccharide

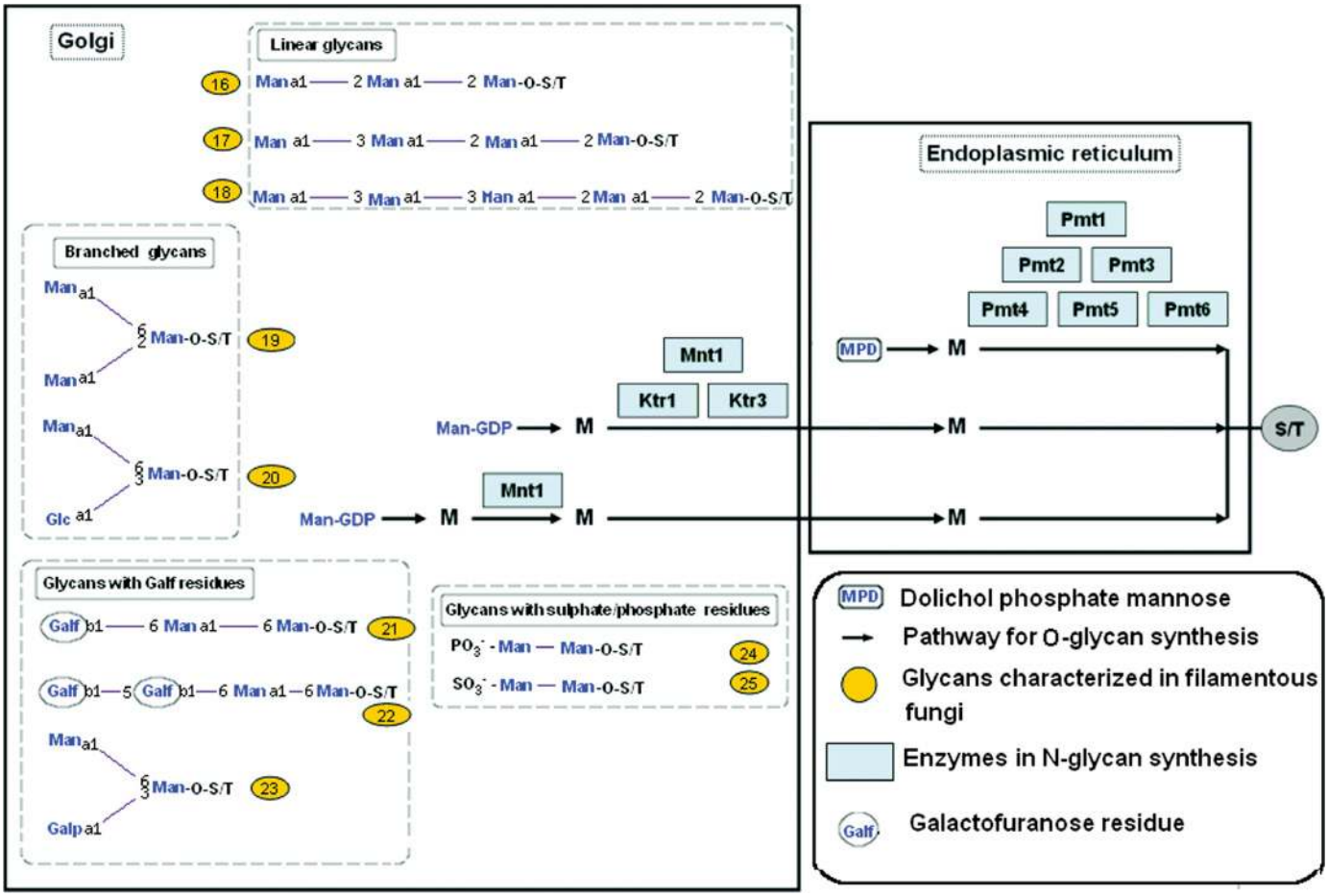

Fig. 5. *O*-Glycan synthesis pathway in filamentous fungi. The shaded rectangles represent enzymes involved in various steps of glycan synthesis. Glycan structures characterized in filamentous fungal species are marked by dotted lines and categorized either according to composition (containing galactofuranose (Gal*f*)/phosphate/sulfate residues) or structure of the glycan (linear/branched) if known.

($Glc_3Man_9GlcNAc_2$) in the ER to newly synthesized polypeptides are conserved almost completely across all eukaryotes (Helenius and Aebi 2004). Higher eukaryotes like mammals posses multiple alpha-1,2-mannosidases, localized separately in the ER and Golgi, with common specificity for linkage but differences in activity. This eventually leads to trimming of the core oligosaccharide to $Man_5GlcNAc_2$ and serves as the precursor for complex, hybrid, and high-mannose *N*-glycans. Model yeasts like *S. cerevisiae* do not form these structures but undergo hypermannosylation. Below we discuss the details of our composite *N*-glycan synthesis pathway for the 12 filamentous fungal species considered in this analysis.

Two genes, *mpg1*, which encodes the enzyme Mpg1 transferase and *dpm1*, which encodes Dpm synthase, have been isolated from the filamentous fungus *T. reesei* (Kruszewska et al. 1998, 2000). The enzymes encoded by these two genes play a significant part in the initial stages of glycan synthesis. The enzyme Mpg1 transferase is involved in the synthesis of GDP-mannose from GTP and mannose-1-phosphate while DPM synthase acts as a catalyst in the transfer of mannose from GDP-mannose to dolichol phosphate, forming dolicholphosphate mannose (MPD). In filamentous fungi and yeast, MPD is also known to be involved in initial steps of *O*-glycosylation and glycosylphosphatidyl inositol (GPI) anchor processing. Experiments have shown overexpression of the *mpg1* gene in *T. reesei* to result in a substantial increase in the GDP-mannose level, resulting in hypermannosylation in both *N*- and *O*-linked glycosylation of secreted proteins, thus suggesting a regulatory role of GDP-mannose. The high sequence homology and conservation of glycosyl transferase domains emphasize the importance and elementary role of these enzymes across the filamentous fungi. The gene *gptA* encoding the enzyme UDP *N*-acetylglucosamine:dolichol phosphate *N*-acetylglucosaminylphosphoryl transferase has been isolated from the filamentous fungus *A. niger*. This enzyme catalyzes the first step in the assembly of dolichol-linked oligosaccharides by adding GlcNAc-1-P from UDP-GlcNAc onto Dol-P to form GlcNAc-PP-dolichol (Sorensen et al. 2003). The gene *gptA* displays high sequence conservation across the fungal species with around 50% identity at an amino acid level to *S. cerevisiae* and about 40% with mammals.

Genes representing the various enzymes present on the cytosolic face of the ER membrane and involved in the addition of the first seven monosaccharides, two GlcNac residues and five mannoses were found to be conserved across the filamentous fungi. Evidence for gene expression was found in the dbEST

database (Boguski et al. 1993) for the gene *Alg2* which encodes an alpha-1,3/alpha-1,6-mannosyltransferase in *Aspergillus nidulans* and the *Alg11* encoding the enzyme alpha-1,2-mannosyltransferase in *T. reesei*. The glycan structure $Man_2GlcNAc_2(PP\text{-}Dol)_1$ (Figure 4, structure 1) is identified in *Aspergillus awamori* which would be a product of the above enzymes and is known to be present in the standard *N*-glycan synthesis pathway.

The gene *Rft1* encoding a membrane protein, a flippase, is involved in translocating oligosaccharides across the membrane into the ER lumen. It is known to be evolutionarily conserved in eukaryotes and the same was found to be true for most of the filamentous fungal species with functional Rft-1 domain conserved. Further evidence for the existence of this gene was provided by ESTs in *A. niger*. The genes *Alg3*, *Alg12*, and *Alg9*, encoding for alpha-1,2-mannosyltransfereses which further add four mannose residues, are also seen to be conserved across the fungal species. Two genes A*lg6* and A*lg8* encoding alpha-1,3-glycosyltransferases and the gene *Alg10* encoding an alpha-1,2-glycosyltransferase add three glucose residues in the filamentous fungi *N*-glycan synthesis pathway. In addition to sequence homology, EST evidence was verified for the gene *Alg8* in *T. reesei* and for A*lg9* in *T. reesei* and *A. niger*.

We conducted a detailed comparative genomics analysis of the oligosaccharyl transferase enzyme complex. The complex catalyses the *en bloc* transfer of a pre-assembled high-mannose oligosaccharide ($Glc_3Man_9GlcNAc_2$) from the dolichol-linked pyrophosphate donor to the side chain of asparagines in the N-X-S/T motif. Five genes in this complex, *Ost1*, *Ost2*, *Stt3*, *Wbp1*, and *Swp1* from *S. cerevisiae*, have been shown to be essential for the viability of the cell. Out of these, *Ost1-alpha*, *Stt3,* and *Wbp1-beta* are seen to be highly conserved across the filamentous fungi. The genes *Ost3* and *Ost6*, which are essential for recruiting a fully active complex necessary for efficient *N*-glycosylation, seem to have retained the functional catalytic domain, though with a much lower sequence conservation. Homology studies with domain analysis did not unambiguously confirm the existence of homologs for *Ost2*, *Swp1*, and *Ost4* in the filamentous fungi. The genes *Gls1* and *Gls2*, encoding Glucosidase I and Glucosidase II, which further trim the oligosaccharide in filamentous fungi, displayed average sequence homology over the entire sequence length but with the functional domains highly conserved. The glycan structure $Man_9GlcNAc_2$ (Figure 4, structure 6), known to be present before trimming by Mns1 (alpha-1,2-mannosidase), has been identified in *A. niger.*

The unicellular budding yeast *S. cerevisiae* has a single ER-specific alpha-1,2-mannosidase, which removes one mannose residue from the core oligosaccharide producing $Man_8GlcNAc_2$ (Herscovics 1999a). However filamentous fungi have two kinds of alpha-1,2-mannosidases. The first alpha-1,2-mannosidase, which is localized in ER, removes all four alpha-1,2-linked mannose residues from $Man_9GlcNAc_2$ to synthesize $Man_5GlcNAc_2$ and has been isolated from *A. oryzae* and *Aspergillus saitoi* (Ichishima et al. 1999; Akao et al. 2006). A second kind of alpha-1,2-mannosidase in *A. oryzae* has been shown to clip a single mannose molecule from the core oligosaccharide in Golgi and form $Man_8GlcNAc_2$ (Yoshida et al. 2000). Glycan structures (Figure 4, structure 7) in the ER (*A. niger*, *T. reesei*) and (Figure 4, structure 13) in the Golgi (*A. niger*) represent the intermediate glycans following the action of each of the above two mannosidases and thus validate the presence of both enzymes. The processing of the final glycan in the Golgi in different eukaryotes is known to be highly diverse, leading to different glycan structures (Munro 2001).

The availability of $Man_8GlcNAc_2$ as a substrate and the presence of alpha-1,6-mannosyltransferase encoded by the gene *Och1* in the Golgi of the filamentous fungi are likely to result in high mannose *N*-glycan structures. Two routes for maturation of the glycans have been proposed by Herscovics and Orlean (1993) in *S. cerevisiae*, both with high mannose structures but with different number of mannose units and linkages. Our analysis has shown the presence of complex mannosyltransferases encoded by the genes *Mnn9*, *Van1*, *Mnn10*, *Mnn11*, and *Anp1* in the filamentous fungal genomes. This clearly points to glycan maturation involving the Mnn enzymes, producing high levels of mannosylation. A few other glycan structures have been identified in specific filamentous fungi with high mannose content and are discussed in detail in later sections.

O-Glycan synthesis

The study of *O*-glycans (*O*-mannosylation) in the yeast *S. cerevisiae* is well documented (Gentzsch and Tanner 1996, 1997). It has been suggested that *O*-mannosylation is closely associated with protein secretion and stability in *T. reesei* (Kruszewska et al. 1999). Many industrially important extracellular enzymes such as cellobiohydrolase I (CBH I) in *T. reesei* are *O*-glycosylated. Genes encoding the protein-*O*-mannosyltransferase (PMT) enzyme have been isolated from *T. reesei* (*pmt1*) (Oka et al. 2004), *A. nidulans (AnPmtA)* (Oka et al. 2005), and *A. awamori (AnPmtA)* (Zakrzewska et al. 2003). All three enzymes possess characteristic PMT domains but the translated *T. reesei* protein sequence shows higher divergence from *Aspergillus* species (31% amino acid identity) and other filamentous fungi. The *T. reesei* gene sequence displayed highest sequence similarity to the genes *Pmt* from *Sch. pombe* and *Pmt4p* of *S. cerevisiae*. Although no other enzymes in the *O*-glycosylation pathway have been identified in filamentous fungi, our study suggests a hypothetical pathway. Sequence homology combined with domain analysis which shows strong preservation over evolution provides proof for reasonable conservation of the three alpha-1,2-Mnt-encoding genes (*Mnt1*, *Ktr1*, *Ktr3*) with *S. cerevisiae* as the reference. However, the same approach was inconclusive to determine if orthologs exists for the three alpha-1,3-Mnt-encoding genes *Mnn1*, *Mnt2*, *Mnt3*, as in the *S. cerevisiae* *O*-glycosylation pathway.

Unique *O*-glycans found in filamentous fungi are shown in Figure 5 and their detailed annotations can be found in Supplementary Data Table II. In addition to linear chains of oligosaccharides containing one to five mannose residues (Figure 5 structures 16–18), branched *O*-glycans with mannose residues linked by alpha-1–6, alpha-1–2, and alpha-1–3 linkages have been identified on GAI (Glucoamylase 1) in *A. niger* and *A. awamori* (Figure 5 structures 19 and 20) (Gunnarsson et al. 1984; Goto et al. 1999). Glc, Gal*p* (galactopyranose), and Gal*f* (galactofuranose) residues were found on GAI in a different strain of *A. niger* (Figure 5, structures 21–23) (Pazur et al. 1980; Neustroev et al. 1993). Specialized linear *O*-glycans on the CBHI protein in *T. reesei*, containing up to three mannose

residues and either sulfate (Harrison et al. 1998) (Figure 5, structure 24) or phosphate (Hui et al. 2001) (Figure 5, structure 25), have also been documented.

Unusual glycan structures identified in filamentous fungi

Insufficient Trimming. High-resolution H-NMR and C-NMR techniques have revealed abnormal glycan intermediates with insufficient trimming by Glucosidase II and the presence of phosphodiester linkages (Maras et al. 1997) (Figure 4, structure 5). The gene *Mnn6* encoding a mannosylphosphate transferase has orthologs across the filamentous fungal species. The function for the ManP group has not yet been deciphered. The significance of such rarely detected mono-glycosylated mature glycans is enhanced by their presence in storage proteins of hen albumin, jack bean storage tissue, and egg jelly coat of starfish amongst others (Stals et al. 2004).

Single GlcNAc. The presence of a single GlcNAc on cellobiohydrolase I and cellobiohydrolase II in cultures of *T. reesei* has been reported by multiple groups attributing this to either endogenous endo-H enzyme activity or possible defects in the dolichol pathway (Harrison et al. 1998; Hui et al. 2001, 2002).

Galactofuranose Residues. The filamentous fungus *A. fumigatus* is an opportunistic pathogen that causes the lethal disease invasive aspergillosis. Research on the *Aspergillus* cell wall polysaccharides and glycoproteins has led to the identification of galactomannans with high immunogenicity of the galactofuranose residues. These may be potential structural markers for aspergillosis disease. The galactose residues found at the nonreducing end of *N*-glycans have been proposed to act like a stop signal for further mannose addition (Morelle et al. 2005). UDP-galactopyranose mutase is the only known generator of UDP-galactofuranose, the precursor of Gal*f* residues. The gene *glf* encoding UDP-galactopyranose mutase has been identified and partially characterized from the opportunistic filamentous fungus *A. fumigatus*. Despite showing very low sequence conservation (<20% at the amino acid level) with the prokaryotic UDP-galactopyranose mutase, the dinucleotide-binding motif at the N-terminus (xhxhGxGxxGxxxhxxh(x)8hxhE(D), where h is a hydrophobic residue) and other amino acids involved in the substrate binding motifs are conserved in *A. fumigatus* (Bakker et al. 2005). We used the gene encoding UDP-galactopyranose mutase from *A. fumigatus* in a comparative analysis across the filamentous fungal genomes. Our data showed that the gene is highly conserved across all filamentous fungal genomes considered.

In a separate study, alpha-galactosidase A from *A. niger* showed a heterogeneous mixture of high-molecular mass oligomannose *N*-glycans ($Man_{7-24}GlcNAc_2$) (Figure 4, structure 10) substituted with one, two, or three terminal β-linked Gal*f* monosaccharides (Wallis et al. 2001). A few glycan structures with single Gal*f* residue substitutions in oligosaccharides partially trimmed by alpha-1,2-mannosidases have been identified in *A. niger* (Figure 4, structures 9, 11, 12). The presence of three novel *O*-linked oligosaccharides in the peptidogalactomannan fraction of the *A. fumigatus* mycelial cell wall has been reported with beta-Galf containing molecules. This suggests that these unusual *O*-linked oligosaccharides might account for a significant part of the antigenicity, in addition to the known activity, associated with the galactomannan component (Leitao et al. 2003). However, our efforts to identify in filamentous fungi a putative galactofuranosyl transferase, the enzyme required to transfer UDP-galactofuranose to the glycan, did not yield satisfactory results by homology searches and applying hidden Markov model (HMM) techniques.

Sialic Acid. Attachment of fungal conidia to proteins in the lung tissue is thought to initiate invasive aspergillosis. Sialic acids (here *Neu5Ac*), which constitute negatively charged carbohydrates on the conidial surface, display much higher density in pathogenic *Aspergillus* species when compared to their nonpathogenic counterparts, thus suggesting that sialic acid density may contribute to the virulence of this fungus (Wasylnka et al. 2001). Yet again, detailed searches involving sequence-based homology analysis and HMM techniques with known sialyltransferases revealed no putative gene candidates in filamentous fungi. Existence of novel sialyltransferases or a completely novel pathway similar to that followed by trans-sialidases in a few *Tyrpanosoma* species (Schenkman et al. 1994) could possibly account for the presence of sialic acids in filamentous fungi.

Engineering filamentous fungi for recombinant protein expression

As discussed in previous sections, filamentous fungi are different from the unicellular yeast and unique among lower eukaryotes in having two types of alpha-1,2-mannosidases, localized both in the ER and Golgi. The glycan with a single trimmed mannose (Figure 4, structure 13) produced by a Golgi mannosidase leads to the addition of further mannoses and thus the high mannose structures, while the ER mannosidase allows the trimming of all four mannose residues from the core oligosaccharide thereby potentially producing a suitable substrate for the synthesis of complex glycans (Figure 4, structure 7) similar to those found in human glycoproteins. Hence several attempts have been made to engineer filamentous fungi to produce complex glycans on functional proteins of higher eukaryotic origin. A rat *GnT-I* gene was expressed in *A. oryzae* which resulted in successful in vivo transfer of an *N*-acetylglucosamine residue to the *N*-glycan of alpha-amylase, initiating complex *N*-glycan synthesis (Kasajima et al. 2006). In a similar experiment with *T. reesei*, in vivo transfer of GlcNAc was demonstrated on the *N*-glycans which were synthesized on cellobiohydrolase I (CBHI). A definitive proof of the formation of the glycan $GlcNAcMan_5GlcNAc_2$ was obtained by NMR analysis (Maras, De Bruyn, et al. 1999), thus demonstrating that the initial step of complex glycan synthesis is possible in the filamentous fungi.

In this paper we have integrated genomic, proteomic, and glycomic data to create a knowledge base of glycosylation pathways in filamentous fungi. This will be useful for future studies involving the engineering of pathways in filamentous fungi for recombinant protein production. Furthermore, the work presented here has the potential to be further extended into a comparative glycan pathway analysis tool to help engineer glycosylation for various other hosts of recombinant protein production.

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Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Conflict of interest statement

None declared.

Abbreviations

ER, endoplasmic reticulum; GDP-mannose, guanosine diphosphate mannose; GPI, glycosyl-phosphatidyl inositol; HMM, hidden Markov model; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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