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Essential Role of Yl*MPO1*, a Novel *Yarrowia lipolytica* Homologue of *Saccharomyces cerevisiae MNN4*, in Mannosylphosphorylation of N- and O-Linked Glycans▽†

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Mannosylphosphorylation of N- and O-glycans, which confers negative charges on the surfaces of cells, requires the functions of both *MNN4* and *MNN6* in *Saccharomyces cerevisiae*. To identify genes relevant to mannosylphosphorylation in the dimorphic yeast *Yarrowia lipolytica*, the molecular functions of five *Y. lipolytica* genes showing significant sequence homology with *S. cerevisiae MNN4* and *MNN6* were investigated. A set of mutant strains in which *Y. lipolytica MNN4* and *MNN6* homologues were deleted underwent glycan structure analysis. In contrast to *S. cerevisiae MNN4* (Sc*MNN4*), the *Y. lipolytica MNN4* homologue, *MPO1* (Yl*MPO1*), encodes a protein that lacks the long KKKKEEEE repeat domain at its C terminus. Moreover, just a single disruption of Yl*MPO1* resulted in complete disappearance of the acidic sugar moiety in both the N- and O-linked glycan profiles. In contrast, even quadruple disruption of all Sc*MNN6* homologues, designated Yl*KTR1*, Yl*KTR2*, Yl*KTR3*, and Yl*KTR4*, resulted in no apparent reduction in acidic sugar moieties. These findings strongly indicate that YlMpo1p performs a significant role in mannosylphosphorylation in *Y. lipolytica* with no involvement of the Mnn6p homologues. Mutant strains harboring the Yl*MPO1* gene disruption may serve as useful platforms for engineering *Y. lipolytica* glycosylation pathways for humanized glycans without any yeast-specific acidic modifications.

Yeast species are important hosts for the production of therapeutic secretory proteins owing to their ability to secrete and glycosylate proteins, their rapid growth to high cell density, and the ease with which they can be genetically manipulated. However, the glycosylation pathway of yeast cells is known to be different from that of mammalian cells, and yeast glycans could induce immunological responses in humans (5). In the traditional yeast *Saccharomyces cerevisiae*, the typical characteristics of the Asn (N)-linked glycan include hypermannosylation ($Man_{50-150}GlcNAc_2$), mannosylphosphorylation in the core and outer regions, and termination with the α1,3-linked mannose residue. These yeast-specific sugar moieties could prove problematic in the production of therapeutic glycoproteins; hypermannosylation can impair protein activity, and mannosylphosphate residues and terminal α1,3-linked mannoses may elicit an antigenic response (14).

Several nonconventional yeast species, including the methylotrophic species *Pichia pastoris* and *Hansenula polymorpha*, have emerged as alternative systems for the production of recombinant therapeutic proteins. Advantages over *S. cerevisiae* include reduced hypermannosylation ($Man_{8-14}GlcNAc_2$), no terminal α1,3-linked mannose of N-linked oligosaccharides, and efficient heterologous protein secretion (18, 20, 21, 22). As another alternative expression system for the production of human-derived therapeutic glycoproteins, the dimorphic yeast *Yarrowia lipolytica* has recently drawn attention since this yeast naturally secretes several enzymes, including proteases, lipases, esterases, and RNases, at elevated levels and its posttranslational modification ability is similar to that of mammalian systems (3, 26, 29).

Recently, we reported that the N-linked glycans of *Y. lipolytica* are composed of neutral and acidic sugars lacking a terminal α1,3-linked mannose (38). The neutral sugars of N-linked glycans are high-mannose oligosaccharides, principally $Man_{7-12}GlcNAc_2$, and the acidic sugars of N-linked glycans are composed of monomannosylphosphorylated $Man_{7-9}GlcNAc_2$ sugars. In the case of *S. cerevisiae*, at least four mannosylphosphorylation loci have been detected in the core and outer chain regions of N-linked glycans, and the mannosylphosphorylation of N-linked glycans in the Golgi apparatus requires both *S. cerevisiae* Mnn4p (ScMnn4p) and ScMnn6p (19). Deletion of Sc*MNN4* or Sc*MNN6* induced a significant reduction in alcian blue intensity, which reflects the presence of negatively

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TABLE 1. Strains used in this study

Strain	Genotype	Reference
Y. lipolytica		
SMS397A	*MATA ade1 ura3 xpr2*	38
Yl*mpo1*Δ	*MATA ade1 ura3 xpr2 mpo1::tc*	This study
Yl*mpo1*Δ::Yl*MPO1*	*MATA ade1 ura3 xpr2 mpo1::tc MPO1::URA3*	This study
Yl*ktr1*Δ	*MATA ade1 ura3 xpr2 ktr1::tc*	This study
Yl*ktr2*Δ	*MATA ade1 ura3 xpr2 ktr2::tc*	This study
Yl*ktr3*Δ	*MATA ade1 ura3 xpr2 ktr3::tc*	This study
Yl*ktr4*Δ	*MATA ade1 ura3 xpr2 ktr4::tc*	This study
Yl*ktr1,2,3,4*Δ	*MATA ade1 ura3 xpr2 ktr1::tc ktr2::tc ktr3::tc ktr4::tc*	This study
Yl*och1*Δ	*MATA ade1 ura3 xpr2 och1::tc*	38
Yl*och1*Δ Yl*mpo1*Δ	*MATA ade1 ura3 xpr2 och1::tc mpo1::tc*	This study
Yl*och1*Δ Yl*ktr1,2,3,4*Δ	*MATA ade1 ura3 xpr2 och1::tc ktr1::tc ktr2::tc ktr3::tc ktr4::tc*	This study
Yl*och1*Δ Yl*mpo1*Δ Yl*ktr1,2,3,4*Δ	*MATA ade1 ura3 xpr2 och1::tc mpo1::tc ktr1::tc ktr2::tc ktr3::tc ktr4::tc*	This study
Yl*och1*Δ Yl*mpo1*Δ::Yl*MPO1*	*MATA ade1 ura3 xpr2 och1::tc mpo1::tc MPO1::URA3*	This study

charged molecules such as mannosylphosphate. However, the level of acidic oligosaccharides in N-linked core oligosaccharides is not reduced in the Sc*mnn6*Δ strain, which implies the existence of additional genes required for core oligosaccharide phosphorylation. Moreover, the Sc*mnn4*Δ strain also retains approximately 30% of its acidic oligosaccharide content, thereby indicating the presence of an Mnn4p-independent mannosylphosphorylation pathway (19).

In this study, we have identified and deleted *Y. lipolytica* homologues to Sc*MNN4* and Sc*MNN6* in various combinations, and analyzed the N- and O-linked glycan profiles in the mutants in order to identify the gene(s) responsible for the mannosylphosphorylation of *Y. lipolytica* glycans. We demonstrate that mannosylphosphorylation of *Yarrowia* N- and O-linked glycans is mediated primarily by a single gene, *Y. lipolytica MPO1* (mannosyl phosphorylation of oligosaccharides 1) (Yl*MPO1*) without the involvement of other *MNN6* homologues. Thus, the results of this study lead us to suggest that a Yl*och1*Δ Yl*mpo1*Δ double mutant strain might be a useful host for the production of glycoproteins lacking yeast-specific hypermannosylation and mannosylphosphorylation.

MATERIALS AND METHODS

Strains, media, and culture conditions. The *Y. lipolytica* strains employed in this study are described in Table 1. The yeast strains were grown routinely in YPD medium (1% yeast extract [BD, Franklin Lakes, NJ], 2% peptone [BD], 2% glucose [Junsei Chemical Co., Tokyo, Japan]) and synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids [BD], 2% glucose, dropout amino acid mixture, including all of the required amino acids [Sigma, St. Louis, MO]) at 28°C. When necessary, 0.625 mg/ml 5′-fluoroorotic acid (5′-FOA; MP Bio, Solon, OH) was added to the YPD agar medium for the selection of strains auxotrophic for Ura (Ura$^-$). Phenotypic analysis was conducted on YPD solid medium containing 10 μg/ml calcofluor white (CFW; Sigma), 50 μg/ml Congo red (CR; Sigma), 7.5 mM sodium orthovanadate (Van; Sigma), or 40 μg/ml hygromycin B (Hyg B; Sigma). YPDm medium (38) was used for the expression of *Trichoderma reesei* endoglucanase I (EGI).

Recombinant DNA techniques and gene disruption. Recombinant DNA techniques and one-step transformation of *Y. lipolytica* were conducted as previously described by Sambrook and Russell (37) and Chen et al. (6), respectively. The oligonucleotides used for this study are listed in Table S1 in the supplemental material. The *Y. lipolytica* target genes were disrupted via the PCR-based gene disruption method described by Song et al. (38). To disrupt the Yl*MPO1* gene, primer set YlMPO1-NF and YlMPO1-NR and primer set YlMPO1-CF and YlMPO1-CR were designed to amplify the 5′ and 3′ flanking regions (571 bp for the 5′ region and 477 bp for the 3′ region) of the Yl*MPO1* gene, respectively. The PCR fragments amplified by *Taq* polymerase (Bioneer, Daejeon, South Korea) were fused via a linker sequence (AGATCTACGGATCCATGG) using the YlMPO1-NF and YlMPO1-CR primers, and the product (1,066 bp) was subcloned into the pDrive vector (Qiagen, Hilden, Germany). The BamHI/BglII-treated *TcR*-Yl*URA3*-*TcR* cassette from pYIUB (38) was inserted into the BglII site of the linker sequence of the fused PCR product. This disruption cassette was linearized via digestion with BamHI/HindIII and used to transform the *Y. lipolytica* SMS397A strain. Correct disruption was confirmed by PCR using the primer set YlMPO1-NCF and YlMPO1-CCR. The Yl*URA3* gene of the integrated *TcR*-Yl*URA3*-*TcR* cassette was deleted from the Yl*mpo1*Δ (*URA3*) strain by growth on YPD medium containing 5′-FOA at 28°C for 3 days. Additionally, mutants with the Yl*KTR* (KRE two-related) gene deletion were constructed using a strategy that was the same as that used for the detection of the Yl*MPO1* gene.

In order to reintegrate the Yl*MPO1* gene into the Yl*mpo1*Δ mutant, the Yl*MPO1* gene, including its own promoter (1,000 bp) and terminator (426 bp) regions, was amplified via PCR using *TaKaRa Ex Taq* polymerase (Takara Bio, Shiga, Japan) with the appropriate primers (primers YlMPO1_prom and YlMPO1_term) and inserted into the pDrive cloning vector. The Yl*URA3* gene was used as an auxotrophic marker and was amplified from the pIMR53-AUX plasmid as a template (38) and then inserted into the EcoRI/BamHI sites in pUC18 (Invitrogen, Carlsbad, CA), generating pUC18-YlURA3. The cloned Yl*MPO1* DNA fragment was then cut by XbaI and ligated with XbaI-treated pUC18-YlURA3, yielding pUC18-YlURA3-YlMPO1. The recombinant plasmid linearized by DraIII was introduced into the Yl*mpo1*Δ and Yl*och1*Δ Yl*mpo1*Δ strains. Correct transformants were selected on Ura$^-$ minimal medium and confirmed by PCR using gene-specific primers.

Analysis of N-linked oligosaccharides. The secretory recombinant EGI protein was purified as described previously by Song et al. (38), and the crude cell wall mannoproteins (CWPs) were obtained and purified using hot citrate buffer and concanavalin A (ConA)-Sepharose beads (GE Healthcare, Uppsala, Sweden) as previously described (7). Two hundred micrograms of the purified EGI and 100 μg of the CWPs were treated with 3 μl of peptide:*N*-glycosidase F (500 U/μl; NEB, Ipswich, MA) for 16 h at 37°C, and the N-linked glycans were purified using a Carbograph Extract-Clean column (150 mg; Alltech, Deerfield, IL). The isolated sugars were then labeled with 2-aminopyridine (PA) or 2-aminobenzoic acid (2-AA). Labeled glycans were analyzed by normal-phase high-pressure liquid chromatography (NP-HPLC). Detailed procedures of sample preparation and analysis are described in the Materials and Methods section in the supplemental material. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) analysis of the N-linked oligosaccharides from the CWPs of the Yl*och1*Δ and Yl*och1*Δ Yl*mpo1*Δ mutant strains was conducted as previously described (38).

Analysis of O-linked glycans. O-linked glycans were obtained by a modified hydrazinolysis reaction as previously described (30). In brief, dried CWPs (100 to 200 μg) were completely dissolved in 10 to 20 μl of hydrazine monohydrate (Tokyo Chemical Industry, Tokyo, Japan), and the mixture was incubated in a heat block (60°C) for 6 h. After the reactants were dried without heating, they were dissolved in 10 μl of saturated $NaHCO_3$, mixed with 4 μl of $(CH_3CO)_2O$, and incubated on ice for 30 min. Next, other contaminants, such as peptides and salts, were removed using Sepabeads SP-20SS (Supelco, Bellefonte, PA) and Dowex 50WX8-400 (H^+ form; Sigma) resins, and the purified O-glycans were dried in a vacuum concentrator. The 2-AA labeling, purification, and HPLC analysis of the O-glycans were conducted as described in the Materials and

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Methods section in the supplemental material. To identify the acidic O-glycans, the glycans underwent mild acid hydrolysis with 0.02 M HCl and dephosphorylation with alkaline phosphatase (1 U/μl; Promega, Madison, WI). The processed glycan was analyzed by HPLC, as described in the Materials and Methods section in the supplemental material.

Capillary electrophoresis. The O-glycans isolated from the yeast strains were labeled with 1 μl of 8-aminopyrene-1,3,6-trisulfonic acid (APTS; Invitrogen, Carlsbad, CA) labeling solution (1 μl of 100 mM APTS, 4 μl of 1.2 M citric acid, and 5 μl of 1 M sodium cyanoborohydride dissolved in dimethyl sulfoxide) and incubated overnight at 37°C. The reaction was halted by adding 4 μl of water. The APTS-labeled glycans were purified using Sephadex G-10 resin (GE Healthcare), dried, and dissolved in 5 μl of water. After the above purification step was repeated, the glycans were diluted with water (1:10, vol/vol) and analyzed using an ABI GA3130 DNA sequencer and GeneMapper software (Applied Biosystems, Foster City, CA) as previously described (24).

Alcian blue staining. Yeast colonies were seeded and cultivated in YPD medium for 16 h at 28°C in a shaking incubator. The cells were inoculated at an optical density at 600 nm (OD_{600}) of 0.1 and incubated for 24 h at 28°C. Following the incubation, the cells were harvested, adjusted to an OD_{600} of 10, washed in 1 ml of washing solution A (0.9% NaCl), and mixed with 100 μl of an alcian blue staining solution (0.1% alcian blue 8GX [Sigma] dissolved in 0.02 N HCl [pH 3.0]). After the yeast cells were allowed to stand for 10 min at room temperature, they were harvested and washed twice with 100 μl of washing solution B (0.02 N HCl, pH 3.0). The cells were transferred into test tubes and analyzed for color as previously described (10).

RESULTS

Sequence analysis of *Y. lipolytica* homologues to Sc*MNN4* and Sc*MNN6*. We initially searched for *Y. lipolytica* homologues of *S. cerevisiae MNN4* in the *Yarrowia* genome database (http://cbi.labri.fr/Genolevures/elt/YALI/). A single *Y. lipolytica* homologue (YALI0D24101g) was predicted to encode a type II membrane protein showing an overall 40% identity with ScMnn4p that would localize to the Golgi apparatus (http://www.cbs.dtu.dk/services/TMHMM/, http://bioapps.rit.albany.edu/pTARGET/; see Fig. S1 and Table S2 in the supplemental material). The predicted product of YALI0D24101g has a region with significant homology to the LicD domain found in the LICD protein family (PF04991), a member of recently identified groups of nucleotidyltransferase fold proteins. The LICD protein family includes ScMnn4p and bacterial LicD phosphotransferase which use GDP-mannose and CDP-choline as substrates, respectively (23). However, the *Y. lipolytica* homologue does not have at its C terminus the long KKKK EEEE repeat region, which has been reported to be an important domain for the function of ScMnn4p (32). Moreover, phylogenetic analysis with other yeast ScMnn4p homologues revealed that the *Y. lipolytica* homologue was distantly related to ScMnn4p and other yeast homologues (Fig. 1A). Therefore, we designated the *Y. lipolytica MNN4* homologue *MPO1*.

It has been proposed that ScMnn6p may be a main enzyme involved in mannosylphosphorylation and ScMnn4p may be a regulator of ScMnn6p (31, 32, 40). ScMnn6p, which is also referred to as ScKtr6p, belongs to the ScKTR family (characterized by mannosyltransferase activity), playing roles in the outer chain elaboration of N-glycans and/or O-glycans (25). Our BLAST search for homologues to *S. cerevisiae MNN6* identified four putative *Y. lipolytica* open reading frames (YALI0F25817g, YALI0A06589g, YALI0B01672g, and YALI0E01892g) (see Table S3 in the supplemental material). As shown in Fig. S2 in the supplemental material, the *Yarrowia* Mnn6p homologues, with the exception of YALI0A06589g, encoded proteins with a type II transmembrane topology.

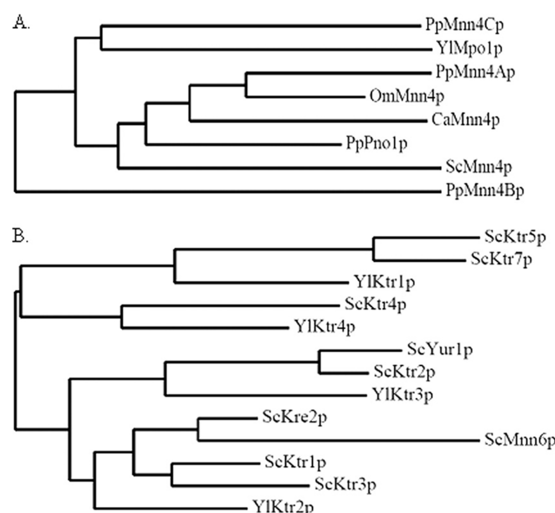

FIG. 1. Phylogenetic analysis of yeast Mnn4p and Mnn6p homologues. (A) Phylogenetic tree analysis of YlMpo1p and other yeast Mnn4p homologues was carried out as previously described (13) using the PhyML (version 3.0) and TreeDyn 198 program tools (http://www.phylogeny.fr/). (B) Phylogenetic tree of the *Y. lipolytica* Mnn6p homologues and ScKTR family. OmMnn4p, *Ogataea minuta* Mnn4p.

However, the four *Yarrowia* homologues showed higher sequence identity with the other Sc*KTR* family of genes than with the *S. cerevisiae MNN6* gene. The predicted proteins of *Yarrowia* homologues shared 41% to 54% identities with the ScKTR proteins, including ScKre2p, ScKtr1p, ScKtr2p, ScKtr3p, ScKtr4p, ScKtr5p, ScKtr7p, and ScYur1p. On the other hand, the four *Yarrowia* proteins shared 28% to 33% identities with ScMnn6p (see Table S3 in the supplemental material). On the basis of the phylogenetic tree analysis (Fig. 1B) as well as the higher similarity of the four *Y. lipolytica* homologues to the ScKTR proteins than to ScMnn6p, we designated the four *Y. lipolytica* homologues, YALI0F25817g, YALI0A06589g, YALI0B01672g, and YALI0E01892g, as Yl*KTR1*, Yl*KTR2*, Yl*KTR3*, and Yl*KTR4*, respectively.

Yl*MPO1* is responsible for mannosylphosphorylation of N-linked oligosaccharides in *Y. lipolytica*. In an effort to evaluate the functions of the *Y. lipolytica* Mnn4p homologue YlMpo1p in the mannosylphosphorylation of N-linked glycans, we deleted the Yl*MPO1* gene from the wild-type and Yl*och1*Δ strains, generating mutants Yl*mpo1*Δ and Yl*och1*Δ Yl*mpo1*Δ, respectively. The N-linked oligosaccharides from a model secretory glycoprotein, the recombinant *Trichoderma reesei* EGI, and from CWPs were obtained from the wild-type and mutant strains for structural analysis with NP-HPLC. Similar to a previous report (38), the N-linked oligosaccharides of the recombinant EGI secreted from the Yl*och1*Δ strain displayed an elevated acidic glycan peak (monomannosylphosphorylated $Man_8GlcNAc_2$) compared to that generated from the wild-type strain (Fig. 2A, panels a and b). The same pattern of glycan profiles with an increased acidic glycan peak was also observed in the N-linked oligosaccharides of CWPs from the Yl*och1*Δ strain, reflecting the general presence of phosphorylated N-glycans in endogenous *Y. lipolytica* glycoproteins (Fig. 2B, panels a and b). Notably, the deletion of Yl*MPO1* in the wild-type or Yl*och1*Δ strain completely eliminated the acidic glycan

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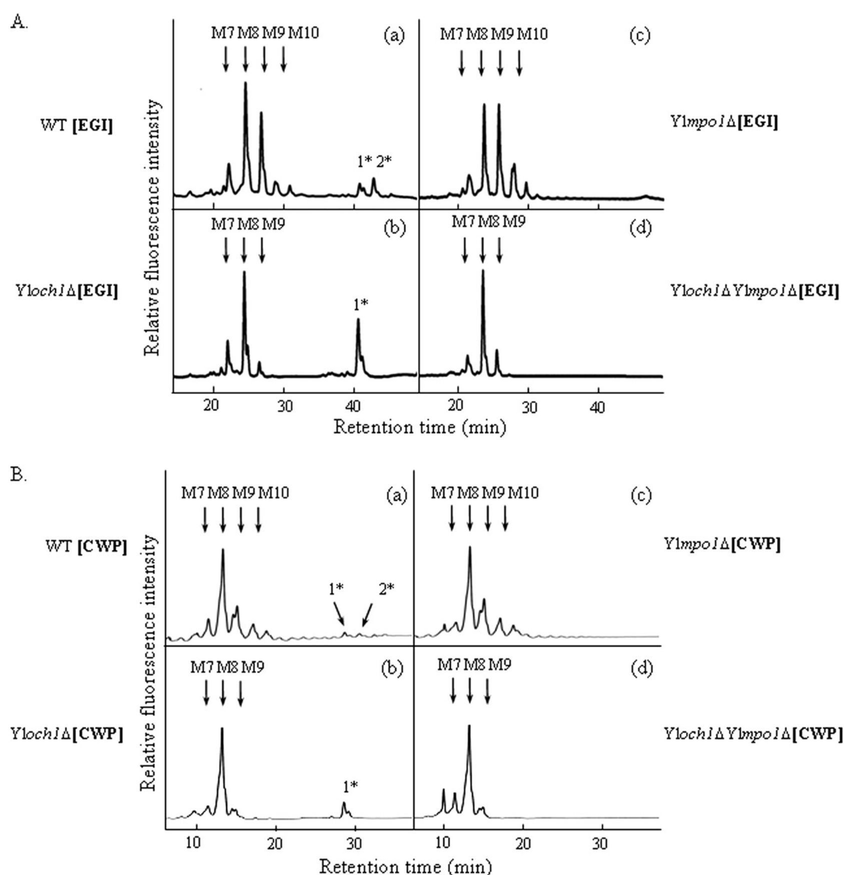

FIG. 2. HPLC analysis of the N-linked oligosaccharides of EGI and cell wall mannoproteins from wild-type (WT) and Yl*mpo1*Δ mutant strains. (A) N-linked oligosaccharides from the recombinant EGI secreted from wild-type (a), Yl*och1*Δ (b), Yl*mpo1*Δ (c), and Yl*och1*Δ Yl*mpo1*Δ (d) strains were labeled with PA and analyzed by NP-HPLC. (B) N-linked oligosaccharides from the CWPs of wild-type (a), Yl*och1*Δ (b), Yl*mpo1*Δ (c), and Yl*och1*Δ Yl*mpo1*Δ (d) strains were labeled with 2-AA and analyzed by NP-HPLC. The elution times of the peaks were compared with those of authentic PA- or AA-sugar chains ($Man_7GlcNAc_2$-AA and $Man_8GlcNAc_2$-AA) of known structure (indicated by arrows): M7, $Man_7GlcNAc_2$-PA; M8, $Man_8GlcNAc_2$-PA; M9, $Man_9GlcNAc_2$-PA; M10, $Man_{10}GlcNAc_2$-PA; 1*, monomannosylphosphorylated $Man_8GlcNAc_2$-PA; 2*, monomannosylphosphorylated $Man_9GlcNAc_2$-PA.

peaks of the N-glycan profiles in the secreted EGI (Fig. 2A, panels c and d) and in the cell wall proteins (Fig. 2B, panels c and d).

The dramatic disappearance of acidic glycan moieties resulting from the deletion of Yl*MPO1* was confirmed via MALDI-TOF mass spectrometry analysis. The N-linked oligosaccharides derived from the CWPs of the Yl*och1*Δ and Yl*och1*Δ Yl*mpo1*Δ mutant strains were analyzed using MALDI-TOF, both in the positive reflector mode for the detection of neutral sugars (Fig. 3A) and in the negative linear mode for the detection of acidic sugars (Fig. 3B). The major peaks of the Yl*och1*Δ mutant detected in the negative mode corresponded to mannosylphosphorylated $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ ($Man_8PGlcNAc_2$ and $Man_9PGlcNAc_2$, respectively) species, with their molecular masses exceeding those of the neutral sugars. These peaks were clearly eliminated via the disruption of the Yl*MPO1* gene. The results clearly demonstrate the crucial function of YlMpo1p in the mannosylphosphorylation of N-linked glycans in *Y. lipolytica*.

We also attempted to determine whether the *Y. lipolytica MNN6* homologues, Yl*KTR1*, Yl*KTR2*, Yl*KTR3*, and Yl*KTR4*, are involved with the mannosylphosphorylation of N-glycans in *Y. lipolytica*. Interestingly, all mutant strains from which each Yl*KTR* gene was deleted showed mannosylphosphorylation in N-glycans similar to that for the wild-type strain (see Fig. S3 in the supplemental material). Comparable results were observed in the Yl*och1*Δ background strain, which showed a more definite acidic sugar profile (Fig. 4). Additionally, even the quadruple deletion of all Yl*KTR* genes (Yl*KTR1*, Yl*KTR2*, Yl*KTR3*, and Yl*KTR4*; strain Yl*och1* ΔYl*ktr1,2,3,4*Δ) did not influence mannosylphosphorylation of N-glycans (Fig. 4C). Moreover, no changes in the overall glycan profiles of the Yl*ktr* mutant strains were observed, thereby indicating that these genes do not participate in the elongation of N-glycans. The reintroduction of Yl*MPO1* resulted in the reappearance of the acidic glycan peak (Fig. 4E), demonstrating that Yl*MPO1* may perform an essential function in the mannosylphosphorylation of N-linked oligosaccharides without the involvement of Yl*KTR1*, Yl*KTR2*, Yl*KTR3*, and Yl*KTR4*.

Yl*MPO1* is also involved in the mannosylphosphorylation of O-linked glycans in *Y. lipolytica*. ScMnn4p and ScMnn6p are required for the mannosylphosphorylation of both N- and O-oligosaccharides in *S. cerevisiae* (19). Thus, we evaluated the possible functions of the YlMpo1p or YlKtr protein in the

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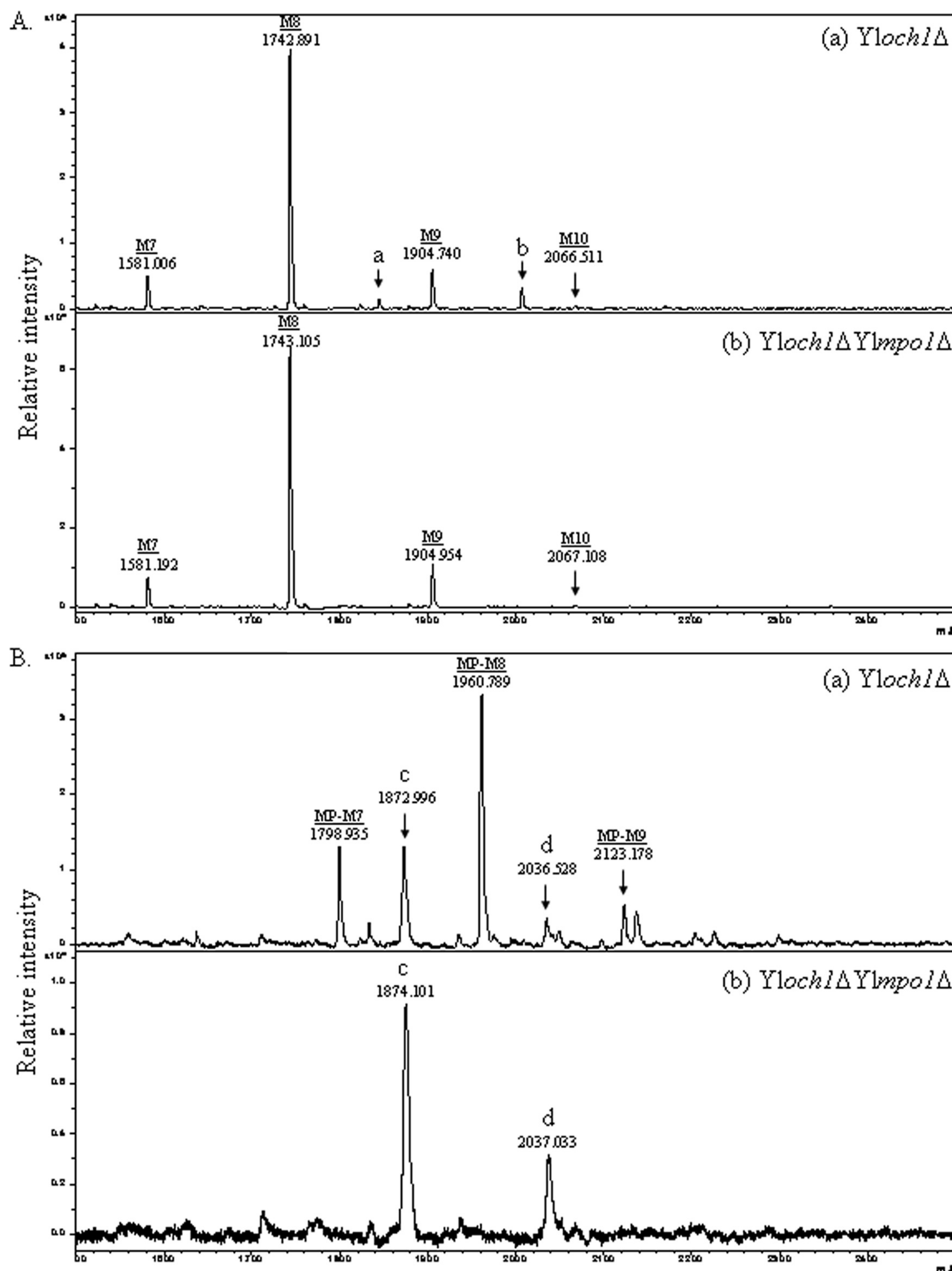

FIG. 3. MALDI-TOF mass spectrometry of the N-linked oligosaccharides from CWPs of Yl*och1*Δ and Yl*och1*Δ Yl*mpo1*Δ mutant strains. (A) Mass spectra analyzed in the positive reflector mode for the detection of neutral sugars of Yl*och1*Δ (a) and Yl*och1*Δ Yl*mpo1*Δ (b) strains. The intermediate peaks, designated a and b, are assumed to represent the monomannosylphosphorylated forms of $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ on the basis of their *m/z* values of 1844.583 and 2006.425, respectively. M7, $Man_7GlcNAc_2$; M8, $Man_8GlcNAc_2$; M9, $Man_9GlcNAc_2$; M10, $Man_{10}GlcNAc_2$. (B) Mass spectra analyzed in the linear negative mode for the detection of acidic sugars of the Yl*och1*Δ (a) and Yl*och1*Δ Yl*mpo1*Δ (b) strains. The intermediate peaks, designated c and d, are assumed to represent $Man_9GlcNAc_2$ and $Man_{10}GlcNAc_2$, respectively. MP-M7, monomannosylphosphorylated $Man_7GlcNAc_2$; MP-M8, monomannosylphosphorylated $Man_8GlcNAc_2$; MP-M9, monomannosylphosphorylated $Man_9GlcNAc_2$.

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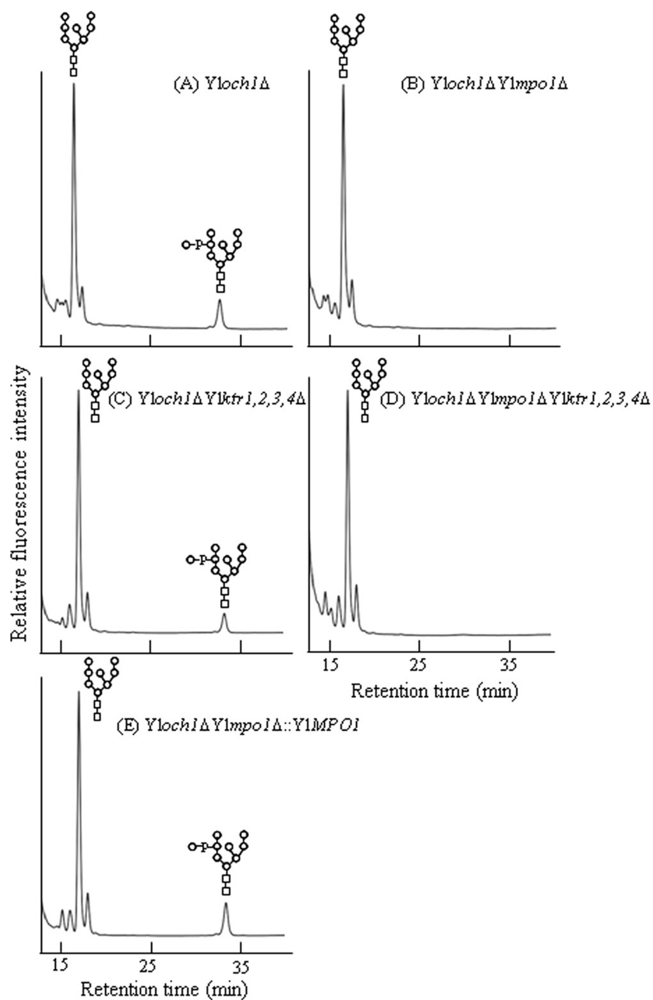

FIG. 4. HPLC analysis of the N-linked oligosaccharide of CWPs from the Yl*ktr*Δ mutant strains. The N-linked oligosaccharide profiles of CWPs from Yl*och1*Δ (A), Yl*och1*Δ Yl*mpo1*Δ (B), Yl*och1*Δ Yl*ktr1,2,3,4*Δ (C), Yl*och1*Δ Yl*mpo1*Δ Yl*ktr1,2,3,4*Δ (D), and Yl*och1*Δ Yl*mpo1*Δ::Yl*MPO1* (E) mutant strains were compared. $Man_8GlcNAc_2$-AA was used as a standard AA-sugar chain.

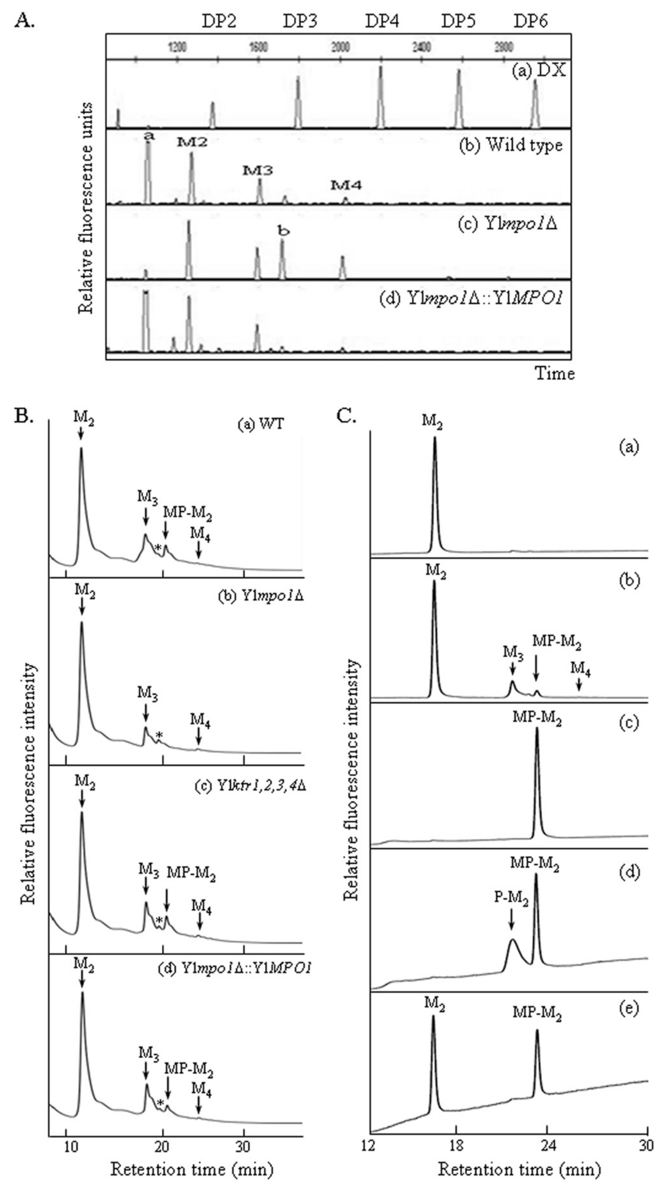

FIG. 5. Analysis of the O-linked oligosaccharides from Yl*mpo1*Δ and Yl*ktr*Δ mutant strains. (A) O-linked oligosaccharides of CWPs from wild-type (b), Yl*mpo1*Δ (c), and Yl*mpo1*Δ::Yl*MPO1* (d) strains were analyzed with a DNA sequencer. (a) Profile of the APTS-maltooligosaccharide ladder, which served as a size reference. The number of glucose units (degrees of polymerization [DP]) is indicated along the *x* axis. (B) O-linked oligosaccharides of CWPs from the wild-type (WT) (a), Yl*mpo1*Δ (b), Yl*ktr1,2,3,4*Δ (c), and Yl*mpo1*Δ::Yl*MPO1* (d) strains were labeled with 2-AA and analyzed by NP-HPLC. O-linked oligosaccharides were acquired via hydrazinolysis, labeled with 2-AA, and subsequently analyzed via NP-HPLC with a TSKgel Amide-80 column. (C) Analysis of MP-M_2 in acidic O-linked glycans of CWPs. α1,2-Mannobiose-AA was used as a standard (a). O-glycans of CWPs from the wild-type strain were analyzed (b). The peak corresponding to the putative MP-M_2 was collected (c), hydrolyzed with mild acid (d), and treated with alkaline phosphatase (e). M_3, α-linked mannotriose; M_4, α-linked mannotetraose.

mannosylphosphorylation of O-glycans in *Y. lipolytica*. O-glycans of CWPs from the wild-type and Yl*mpo1*Δ strains were labeled with APTS fluorescent dye and analyzed with a DNA sequencer. As seen in Fig. 5A, the most rapidly moving peak in the wild-type strain (Fig. 5A, trace b) was not detected in the Yl*mpo1*Δ strain (Fig. 5A, trace c) but reappeared following the reintroduction of the Yl*MPO1* gene into the Yl*mpo1*Δ strain (Fig. 5A, trace d). Considering the negative charge properties of APTS, acidic oligosaccharides are expected to move more rapidly than neutral oligosaccharides in the DNA sequencer. Thus, the fast-moving peak detected in the wild-type strain was speculated to represent an acidic O-glycan with mannosylphosphate residues.

To evaluate this possibility, O-glycans from several mutant strains were analyzed via NP-HPLC with an amide column. As shown in Fig. 5B, O-glycans from the wild-type *Y. lipolytica* strain were composed of $Man_{2\text{-}4}$, as suggested by the DNA sequencer analysis (Fig. 5A, trace b). The peak at 20.5 min in the wild-type strain (Fig. 5B, trace a), which was also detected in the Yl*ktr1,2,3,4*Δ strain (Fig. 5B, trace c), disappeared in the Yl*mpo1*Δ strain (Fig. 5B, trace b). This peak reappeared in the Yl*mpo1*Δ::Yl*MPO1* strain into which the functional Yl*MPO1*

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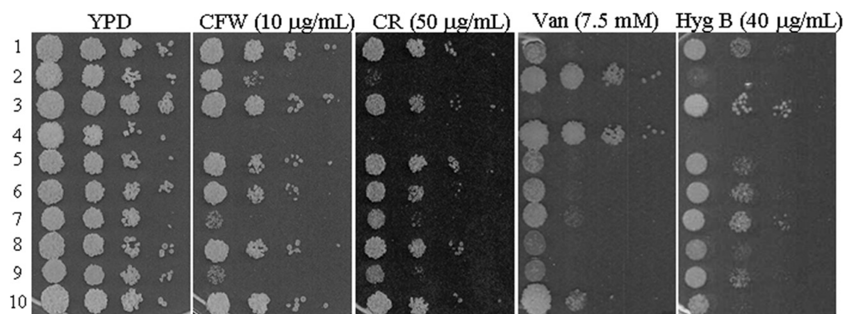

FIG. 6. Phenotypic analyses of the Yl*mpo1*Δ and Yl*ktr*Δ mutant strains. Wild-type (row 1), Yl*och1*Δ (row 2), Yl*mpo1*Δ (row 3), Yl*och1*Δ Yl*mpo1*Δ (row 4), Yl*ktr1*Δ (row 5), Yl*ktr2*Δ (row 6), Yl*ktr3*Δ (row 7), Yl*ktr4*Δ (row 8), Yl*ktr1,2,3,4*Δ (row 9), and Yl*mpo1*Δ::Yl*MPO1* (row 10) cells were spotted onto YPD medium plates containing 10 μg/ml of CFW, 50 μg/ml of CR, 7.5 mM Van, or 40 μg/ml of Hyg B and incubated for 3 to 4 days at 28°C.

was reintegrated (Fig. 5B, trace d), thereby suggesting that it may represent a monomannosylphosphorylated mannobiose (MP-M_2). To confirm the identity, the peak in Fig. 5B was collected, hydrolyzed with mild acid, treated with alkaline phosphatase (31), and analyzed via HPLC (Fig. 5C). Mild acid hydrolysis of the collected peak (Fig. 5C, trace c) generated an additional peak, monophosphorylated mannobiose (P-M_2) (Fig. 5C, trace d). If P-M_2 resulted from hydrolysis of MP-M_2, then treatment with alkaline phosphatase would be anticipated to yield a peak corresponding to α-linked mannobiose (M_2). Indeed, alkaline phosphatase treatment generated a novel peak at a retention time of 16.4 min (Fig. 5C, trace e), corresponding to the retention time of α1,2-Man_2 (Fig. 5C, trace a). These results clearly indicate that the peak missing from the Yl*mpo1*Δ strain is an acidic O-glycan, a monomannosylphosphorylated mannobiose form, and that the Yl*MPO1* gene participates in the mannosylphosphorylation of O-glycans as well as N-glycans in *Y. lipolytica*. However, the Yl*ktr1,2,3,4*Δ strain showed no changes in the O-glycan profiles. This suggests that none of the *Y. lipolytica* Mnn6p homologues are involved in the addition of mannosylphosphate to O-glycans, in contrast to the case in *S. cerevisiae* Mnn6p.

Phenotypic characteristics of Yl*mpo1*Δ and Yl*ktr*Δ mutant strains. It has been demonstrated that glycosylation pathway-defective yeast strains change their sensitivities to cell wall synthesis inhibitors, including CFW and CR (34, 35). CFW functions by binding to the chitin polymer, one of the components of the fungal cell wall, and inhibiting the assembly enzymes that link chitin to β1,3- and β1,6-glucans (34, 35). CR binds preferentially to β1,3-glucan and to chitin to a lesser degree. To evaluate the roles of the Yl*MPO1* and Yl*KTR* genes in cell wall integrity, each mutant strain was spotted onto YPD plates containing CFW or CR. The Yl*och1*Δ strain, which is sensitive to CFW and CR, was used as a positive control in the assay. As shown in Fig. 6, the Yl*mpo1*Δ strain was slightly more sensitive to CFW and CR than the wild-type strain, and the deletion of Yl*MPO1* in the Yl*och1*Δ mutant increased the sensitivity to CFW and CR. These results indicate that YlMpo1p is required, but not to a great extent, for cell wall integrity in *Y. lipolytica*. Another interesting finding from this analysis was that the Yl*ktr3*Δ strain was as sensitive to CFW and CR as the Yl*och1*Δ mutant. The data imply that although Yl*KTR3* does not appear to be involved in N- or O-linked glycosylation, the functions of Yl*KTR3* might be relevant to cell wall integrity.

Glycosylation-defective mutants are generally resistant to Van but sensitive to Hyg B (2, 12). As anticipated, the Yl*och1*Δ strain showed markedly increased resistance to vanadate but reduced sensitivity to an aminoglycoside antibiotic, hygromycin B, compared to the susceptibilities of the wild type. It is worthwhile to note that the Yl*mpo1*Δ strain displayed phenotypes opposite those of the Yl*och1*Δ strain; it became rather sensitive to vanadate, but the level of hygromycin B resistance increased. Reintroduction of Yl*MPO1* into the Yl*mpo1*Δ strain completely restored the expected phenotype but with a slightly altered sensitivity and resistance relative to the susceptibilities of the wild-type strain. It has been proposed that the vanadium contained in sodium orthovanadate has a conformation similar to that of phosphorus and thus competes with phosphorus in phosphorylated compounds (39). Therefore, the increased sensitivity of Yl*mpo1*Δ to vanadate further supports the notion that YlMpo1p plays a role in phosphorylation-related processes.

The *Y. lipolytica* mutant strains were stained with alcian blue dye, which binds via electrostatic forces to negatively charged molecules such as mannosylphosphate (9). The Yl*mpo1*Δ strain exhibited the most dramatic reduction in staining intensity (Table 2), consistent with lowered levels of mannosylphosphate on the cell surfaces. Interestingly, the deletion of Yl*KTR4* (with a yet-undetermined function) also resulted in reduced staining. The stain intensity increased significantly in

TABLE 2. Alcian blue staining of various *Y. lipolytica* strains

Strain	Relevant characteristic(s)	Alcian blue staining[a]
SMS397A	*xpr2*	+++
Yl*och1*Δ	*xpr2 och1*	++++
Yl*mpo1*Δ	*xpr2 mpo1*	−
Yl*och1*Δ Yl*mpo1*Δ	*xpr2 och1 mpo1*	+
Yl*mpo1*Δ::Yl*MPO1*	*xpr2 mpo1 MPO1*	+++
Yl*ktr1*Δ	*xpr2 ktr1*	+++
Yl*ktr2*Δ	*xpr2 ktr2*	+++
Yl*ktr3*Δ	*xpr2 ktr3*	+++
Yl*ktr4*Δ	*xpr2 ktr4*	+++

[a] The colors that developed on the cell pellets in the alcian blue assay were classified as follows: −, white; +, light blue; +++, blue; and ++++, dark blue.

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the Yl*och1*Δ strain compared to that in the wild type, suggesting that the absence of the outer chain might enhance the efficiency of mannosylphosphorylation by YlMpo1p or the binding of core N-glycans with the dye.

DISCUSSION

In this study, we evaluated the molecular functions of the *Y. lipolytica MPO1* gene, which encodes a protein that exhibits an overall identity of 40% to ScMnn4p but which lacks a long C-terminal KE repeat domain crucial for the function of ScMnn4p (32). One of the most noticeable results was that, in contrast to *S. cerevisiae* and *P. pastoris*, a single deletion of the Yl*MPO1* gene proved to be sufficient for completely eliminating mannosylphosphorylation of both N- and O-linked glycans in *Y. lipolytica*. Thus, it can be surmised that YlMpo1p controls the majority of the mannosylphosphorylation of N- and O-linked glycans in *Y. lipolytica*.

Many yeast species harbor multiple Mnn4p homologues (or hypothetical proteins) to ScMnn4p. Two Mnn4-like genes exist in *S. cerevisiae* (19), four exist in *P. pastoris* (4, 27) and *Ogataea minuta* (1), and eight exist in *Candida albicans* (17). However, in *Y. lipolytica*, only a single gene (YALI0D24101g) was predicted to encode a type II membrane protein showing homology with ScMnn4p. In *S. cerevisiae*, the inability of the Sc*mnn4*Δ mutant to remove mannosylphosphorylation entirely from glycans (32) implies that an additional gene(s) may participate in mannosylphosphorylation. In *P. pastoris*, both the *P. pastoris PNO1* (phosphomannosylation of N-linked oligosaccharides) (Pp*PNO1*) gene and the Pp*MNN4B* gene perform roles in the mannosylphosphorylation of N-linked oligosaccharides (4). Among the eight *MNN4*-like sequences in the *C. albicans* genome, the *C. albicans MNN4* (Ca*MNN4*) gene is the closest homologue to the Sc*MNN4* gene and harbors a KE repeat region. Ca*MNN4* appears to be primarily responsible for mannosylphosphorylation, on the basis of the observation that the Ca*mnn4*Δ null mutant was unable to bind to alcian blue dye and was devoid of acid-labile β1,2-linked oligomannosaccharides in the outer cell wall (17). However, detailed structural analysis data demonstrating the lack of mannosylphosphorylated residues in the N- and O-glycans derived from the Ca*mnn4*Δ mutant have not been performed. It is worth noting that the NCBI BLASTP searches using YlMpo1p as a query identified several hypothetical and putative fungal proteins, besides *C. albicans* Mnn4p (AAL86704), as YlMpo1p homologues with greater sequence similarities than the sequence similarity of ScMnn4p. Interestingly, most of these homologues possess a predicted LicD region, strongly indicating their possible roles in nucleotidyl transfer.

ScMnn4p is regarded as a positive regulator of ScMnn6p, a mannosylphosphate transferase (19). However, the relationship between the *MNN4* and *MNN6* genes has yet to be clearly elucidated. If YlMpo1p also participates in mannosylphosphorylation together with a putative mannosylphosphate transferase, we anticipate that a gene encoding mannosylphosphate transferase should exist in *Y. lipolytica*. However, the Yl*KTR1*, Yl*KTR2*, Yl*KTR3*, and Yl*KTR4* genes, initially detected to be the closest Sc*MNN6* homologues, are not involved in the mannosylphosphorylation of *Y. lipolytica* glycans (Fig. 4; see also Fig. S3 in the supplemental material). In *C. albicans*, no ScMnn6p homologues were detected in the genome database. However, a very recent study demonstrated that the double deletion of Ca*MNT3* and Ca*MNT5*, which are involved in N-glycosylation, resulted in a reduction in the ability to bind to alcian blue for staining. This suggests the presence of multifunctional mannosyltransferases with redundant activities in both the N-glycosylation and phosphomannosylation of *C. albicans* (28). Aside from phosphomannosyltransferase activity *per se*, the extent of mannosylphosphorylation of glycans might be affected indirectly by several factors. This could include the activities of other mannosyltransferases, which can generate more optimal substrates or compete for a common substrate, as well as vacuolar targeting and actin/cytoskeleton organization in the secretory pathway, which may influence the localization of enzymes (11). Therefore, further studies will attempt to determine which protein(s) besides the YlKtr1, YlKtr2, YlKtr3, and YlKtr4 proteins has mannosylphosphate transferase activity as a ScMnn6p functional homologue. Future work will also examine whether YlMpo1p alone can function in mannosylphosphate transfer in association with other multifunctional glycosyltransferases, as has been suggested in *C. albicans*.

Despite the fact that the Yl*KTR1*, Yl*KTR2*, Yl*KTR3*, and Yl*KTR4* genes appeared to play no role in the mannosylphosphorylation or protein glycosylation of *Y. lipolytica* (Fig. 4; see also Fig. S3 in the supplemental material), the Yl*ktr3*Δ mutant exhibited a profound sensitivity to CFW and CR (Fig. 6). Ruiz-Herrera et al. (36) reported that an increase in either the sensitivity or resistance of *Y. lipolytica* to certain levels of CFW is indicative of alterations in cell wall structure independent of the level of chitin. Considering that the Yl*ktr3*Δ mutant strain displayed defective hyphal development (data not shown), we speculate that Yl*KTR3* might be associated with the glycosylation process of glycolipids which is required for morphogenesis in *Y. lipolytica* (3).

Several yeast and fungal species have been used in the development of secretion hosts producing therapeutic glycoproteins with human complex-type N-glycans (15, 16, 33). Recently, pioneering work on the engineering of the O-glycosylation pathway expressing human proteins in *S. cerevisiae* was reported (8). As one of the nonconventional yeasts, *Y. lipolytica* exhibits some advantages as a potential host for the secretory production of human-derived recombinant glycoproteins, including greatly reduced hypermannosylation ($Man_{7\text{-}12}GlcNAc_2$), the absence of a terminal α1,3-mannose epitope in both N- and O-linked oligosaccharides, and posttranslational modification processes similar to those in mammalian systems (26). Therefore, we anticipate that the combined deletion of Yl*MPO1* and Yl*OCH1*, the factor responsible for yeast-specific outer chain initiation in N-linked glycans (38), should serve as a foundation for engineering a humanized glycosylation pathway in *Y. lipolytica* with no yeast-specific glycan modification.

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