

Essential Role of *YIMPO1*, a Novel *Yarrowia lipolytica* Homologue of *Saccharomyces cerevisiae* *MNN4*, in Mannosylphosphorylation of N- and O-Linked Glycans^{∇†}

Jeong-Nam Park,^{1,2} Yunkyong Song,³ Seon Ah Cheon,¹ Ohsuk Kwon,² Doo-Byoung Oh,² Yoshifumi Jigami,⁴ Jeong-Yoon Kim,^{3*} and Hyun Ah Kang^{1*}

Department of Life Science, Chung-Ang University, Seoul 156-756, South Korea¹; Integrative Omics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, South Korea²; Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 305-764, South Korea³; and Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan⁴

Received 30 September 2010/Accepted 13 December 2010

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* (*ScMNN4*), the *Y. lipolytica* *MNN4* homologue, *MPO1* (*YIMPO1*), encodes a protein that lacks the long KKKKEEEE repeat domain at its C terminus. Moreover, just a single disruption of *YIMPO1* 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 *ScMNN6* homologues, designated *YIKTR1*, *YIKTR2*, *YIKTR3*, and *YIKTR4*, resulted in no apparent reduction in acidic sugar moieties. These findings strongly indicate that *YIMPO1p* performs a significant role in mannosylphosphorylation in *Y. lipolytica* with no involvement of the *Mnn6p* homologues. Mutant strains harboring the *YIMPO1* 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 ($\text{Man}_{50-150}\text{GlcNAc}_2$), mannosylphosphorylation in the core and outer regions, and termination with the α 1,3-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 α 1,3-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 ($\text{Man}_{8-14}\text{GlcNAc}_2$), no terminal α 1,3-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 α 1,3-linked mannose (38). The neutral sugars of N-linked glycans are high-mannose oligosaccharides, principally $\text{Man}_{7-12}\text{GlcNAc}_2$, and the acidic sugars of N-linked glycans are composed of monomannosylphosphorylated $\text{Man}_{7-9}\text{GlcNAc}_2$ 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 *ScMNN4* or *ScMNN6* induced a significant reduction in alcian blue intensity, which reflects the presence of negatively

* Corresponding author. Mailing address for Hyun Ah Kang: Department of Life Science, College of Natural Science, Chung-Ang University, Seoul 156-756, South Korea. Phone: 82-2-820-5863. Fax: 82-2-825-5206. E-mail: hyunkang@cau.ac.kr. Mailing address for Jeong-Yoon Kim: Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 305-764, South Korea. Phone: 82-42-821-6419. Fax: 82-42-822-7367. E-mail: jkim@cnu.ac.kr.

† Supplemental material for this article may be found at <http://aem.asm.org/>.

∇ Published ahead of print on 23 December 2010.

TABLE 1. Strains used in this study

Strain	Genotype	Reference
<i>Y. lipolytica</i>		
SMS397A	<i>MATA ade1 ura3 xpr2</i>	38
<i>Ylmpo1Δ</i>	<i>MATA ade1 ura3 xpr2 mpo1::tc</i>	This study
<i>Ylmpo1Δ::YIMPO1</i>	<i>MATA ade1 ura3 xpr2 mpo1::tc MPO1::URA3</i>	This study
<i>Ylptr1Δ</i>	<i>MATA ade1 ura3 xpr2 ptr1::tc</i>	This study
<i>Ylptr2Δ</i>	<i>MATA ade1 ura3 xpr2 ptr2::tc</i>	This study
<i>Ylptr3Δ</i>	<i>MATA ade1 ura3 xpr2 ptr3::tc</i>	This study
<i>Ylptr4Δ</i>	<i>MATA ade1 ura3 xpr2 ptr4::tc</i>	This study
<i>Ylptr1,2,3,4Δ</i>	<i>MATA ade1 ura3 xpr2 ptr1::tc ptr2::tc ptr3::tc ptr4::tc</i>	This study
<i>Yloch1Δ</i>	<i>MATA ade1 ura3 xpr2 och1::tc</i>	38
<i>Yloch1Δ Ylmpo1Δ</i>	<i>MATA ade1 ura3 xpr2 och1::tc mpo1::tc</i>	This study
<i>Yloch1Δ Ylptr1,2,3,4Δ</i>	<i>MATA ade1 ura3 xpr2 och1::tc ptr1::tc ptr2::tc ptr3::tc ptr4::tc</i>	This study
<i>Yloch1Δ Ylmpo1Δ Ylptr1,2,3,4Δ</i>	<i>MATA ade1 ura3 xpr2 och1::tc mpo1::tc ptr1::tc ptr2::tc ptr3::tc ptr4::tc</i>	This study
<i>Yloch1Δ Ylmpo1Δ::YIMPO1</i>	<i>MATA ade1 ura3 xpr2 och1::tc mpo1::tc MPO1::URA3</i>	This study

charged molecules such as mannosylphosphate. However, the level of acidic oligosaccharides in N-linked core oligosaccharides is not reduced in the *Scmnn6Δ* strain, which implies the existence of additional genes required for core oligosaccharide phosphorylation. Moreover, the *Scmnn4Δ* 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 *ScMNN4* and *ScMNN6* 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) (*YIMPO1*) without the involvement of other *MNN6* homologues. Thus, the results of this study lead us to suggest that a *Yloch1Δ Ylmpo1Δ* 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 *YIMPO1* gene, primer set YIMPO1-NF and YIMPO1-NR and primer set YIMPO1-CF and YIMPO1-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 *YIMPO1* gene, respectively. The PCR fragments amplified by *Taq* polymerase (Bioneer, Daejeon, South Korea) were fused via a linker sequence (AGATCTACGGATCCATGG) using the

YIMPO1-NF and YIMPO1-CR primers, and the product (1,066 bp) was subcloned into the pDrive vector (Qiagen, Hilden, Germany). The BamHI/BglII-treated *TcR-YIURA3-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 YIMPO1-NCF and YIMPO1-CCR. The *YIURA3* gene of the integrated *TcR-YIURA3-TcR* cassette was deleted from the *Ylmpo1Δ* (*URA3*) strain by growth on YPD medium containing 5'-FOA at 28°C for 3 days. Additionally, mutants with the *YIKTR* (*KRE* two-related) gene deletion were constructed using a strategy that was the same as that used for the detection of the *YIMPO1* gene.

In order to reintegrate the *YIMPO1* gene into the *Ylmpo1Δ* mutant, the *YIMPO1* 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 YIMPO1_prom and YIMPO1_term) and inserted into the pDrive cloning vector. The *YIURA3* 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-YIURA3. The cloned *YIMPO1* DNA fragment was then cut by XbaI and ligated with XbaI-treated pUC18-YIURA3, yielding pUC18-YIURA3-YIMPO1. The recombinant plasmid linearized by DraIII was introduced into the *Ylmpo1Δ* and *Yloch1Δ Ylmpo1Δ* 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 *Yloch1Δ* and *Yloch1Δ Ylmpo1Δ* 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 NaHCO₃, mixed with 4 μl of (CH₃CO)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

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/ μ l; 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 μ l of 8-aminopyrene-1,3,6-trisulfonic acid (APTS; Invitrogen, Carlsbad, CA) labeling solution (1 μ l of 100 mM APTS, 4 μ l of 1.2 M citric acid, and 5 μ l of 1 M sodium cyanoborohydride dissolved in dimethyl sulfoxide) and incubated overnight at 37°C. The reaction was halted by adding 4 μ l of water. The APTS-labeled glycans were purified using Sephadex G-10 resin (GE Healthcare), dried, and dissolved in 5 μ l 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 μ l 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 μ l 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 ScMNN4 and ScMNN6. 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.

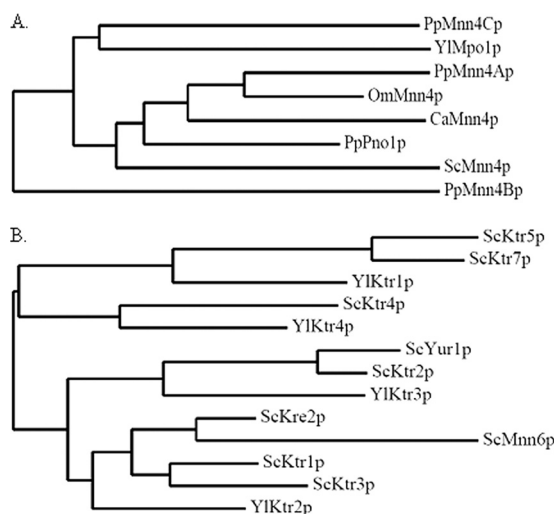


FIG. 1. Phylogenetic analysis of yeast Mnn4p and Mnn6p homologues. (A) Phylogenetic tree analysis of YIMpo1p and other yeast Mnn4p homologues was carried out as previously described (13) using the PhyML (version 3.0) and TreeDyn 198 program tools (<http://www.phylogeny.fr/>). (B) Phylogenetic tree of the *Y. lipolytica* Mnn6p homologues and ScKTR family. OmMnn4p, *Ogataea minuta* Mnn4p.

However, the four *Yarrowia* homologues showed higher sequence identity with the other ScKTR 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 YIKTR1, YIKTR2, YIKTR3, and YIKTR4, respectively.

YIMPO1 is responsible for mannosylphosphorylation of N-linked oligosaccharides in *Y. lipolytica*. In an effort to evaluate the functions of the *Y. lipolytica* Mnn4p homologue YIMpo1p in the mannosylphosphorylation of N-linked glycans, we deleted the YIMPO1 gene from the wild-type and *Yloch1Δ* strains, generating mutants *Ylmpo1Δ* and *Yloch1Δ Ylmpo1Δ*, 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 *Yloch1Δ* strain displayed an elevated acidic glycan peak (monomannosylphosphorylated Man₈GlcNAc₂) 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 *Yloch1Δ* strain, reflecting the general presence of phosphorylated N-glycans in endogenous *Y. lipolytica* glycoproteins (Fig. 2B, panels a and b). Notably, the deletion of YIMPO1 in the wild-type or *Yloch1Δ* strain completely eliminated the acidic glycan

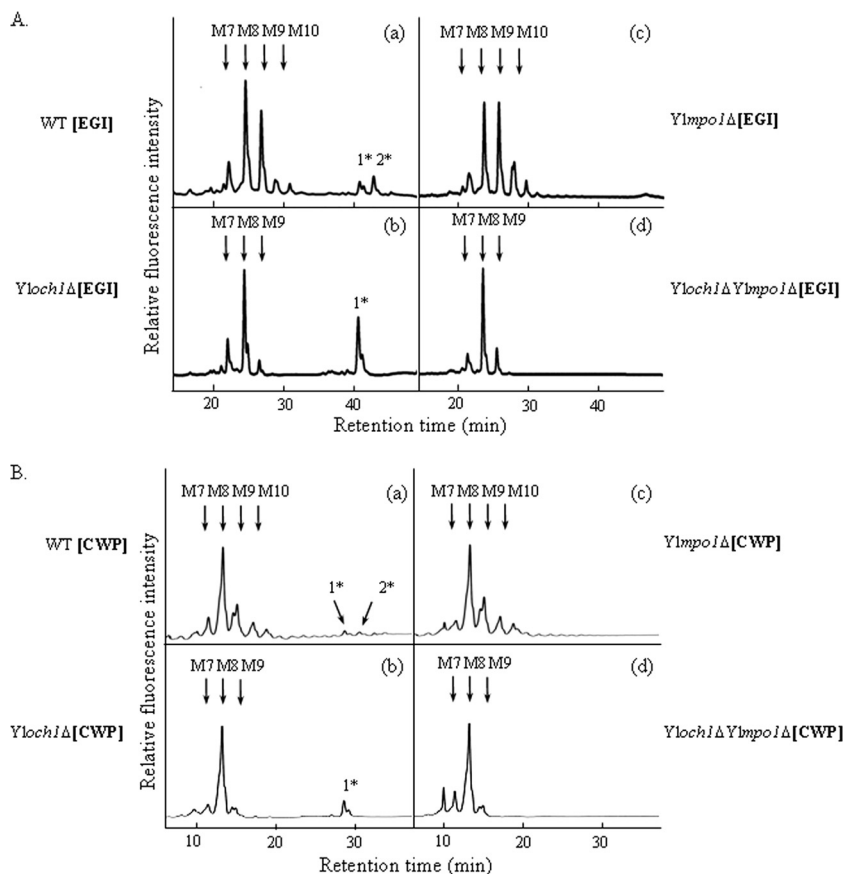


FIG. 2. HPLC analysis of the N-linked oligosaccharides of EGI and cell wall mannoproteins from wild-type (WT) and *Ylmpo1Δ* mutant strains. (A) N-linked oligosaccharides from the recombinant EGI secreted from wild-type (a), *YlochlΔ* (b), *Ylmpo1Δ* (c), and *YlochlΔ Ylmpo1Δ* (d) strains were labeled with PA and analyzed by NP-HPLC. (B) N-linked oligosaccharides from the CWPs of wild-type (a), *YlochlΔ* (b), *Ylmpo1Δ* (c), and *YlochlΔ Ylmpo1Δ* (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 (Man₇GlcNAc₂-AA and Man₈GlcNAc₂-AA) of known structure (indicated by arrows): M7, Man₇GlcNAc₂-PA; M8, Man₈GlcNAc₂-PA; M9, Man₉GlcNAc₂-PA; M10, Man₁₀GlcNAc₂-PA; 1*, monomannosylphosphorylated Man₈GlcNAc₂-PA; 2*, monomannosylphosphorylated Man₉GlcNAc₂-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 *YIMPO1* was confirmed via MALDI-TOF mass spectrometry analysis. The N-linked oligosaccharides derived from the CWPs of the *YlochlΔ* and *YlochlΔ Ylmpo1Δ* 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 *YlochlΔ* mutant detected in the negative mode corresponded to mannosylphosphorylated Man₇GlcNAc₂ and Man₈GlcNAc₂ (Man₈PGlcNAc₂ and Man₉PGlcNAc₂, respectively) species, with their molecular masses exceeding those of the neutral sugars. These peaks were clearly eliminated via the disruption of the *YIMPO1* gene. The results clearly demonstrate the crucial function of YIMpo1p in the mannosylphosphorylation of N-linked glycans in *Y. lipolytica*.

We also attempted to determine whether the *Y. lipolytica* *MNN6* homologues, *YIKTR1*, *YIKTR2*, *YIKTR3*, and *YIKTR4*, are involved with the mannosylphosphorylation of N-glycans in

Y. lipolytica. Interestingly, all mutant strains from which each *YIKTR* 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 *YlochlΔ* background strain, which showed a more definite acidic sugar profile (Fig. 4). Additionally, even the quadruple deletion of all *YIKTR* genes (*YIKTR1*, *YIKTR2*, *YIKTR3*, and *YIKTR4*; strain *YlochlΔ YIkt1,2,3,4Δ*) did not influence mannosylphosphorylation of N-glycans (Fig. 4C). Moreover, no changes in the overall glycan profiles of the *Ylkt* mutant strains were observed, thereby indicating that these genes do not participate in the elongation of N-glycans. The reintroduction of *YIMPO1* resulted in the reappearance of the acidic glycan peak (Fig. 4E), demonstrating that *YIMPO1* may perform an essential function in the mannosylphosphorylation of N-linked oligosaccharides without the involvement of *YIKTR1*, *YIKTR2*, *YIKTR3*, and *YIKTR4*.

***YIMPO1* 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 YIMpo1p or YIKtr protein in the

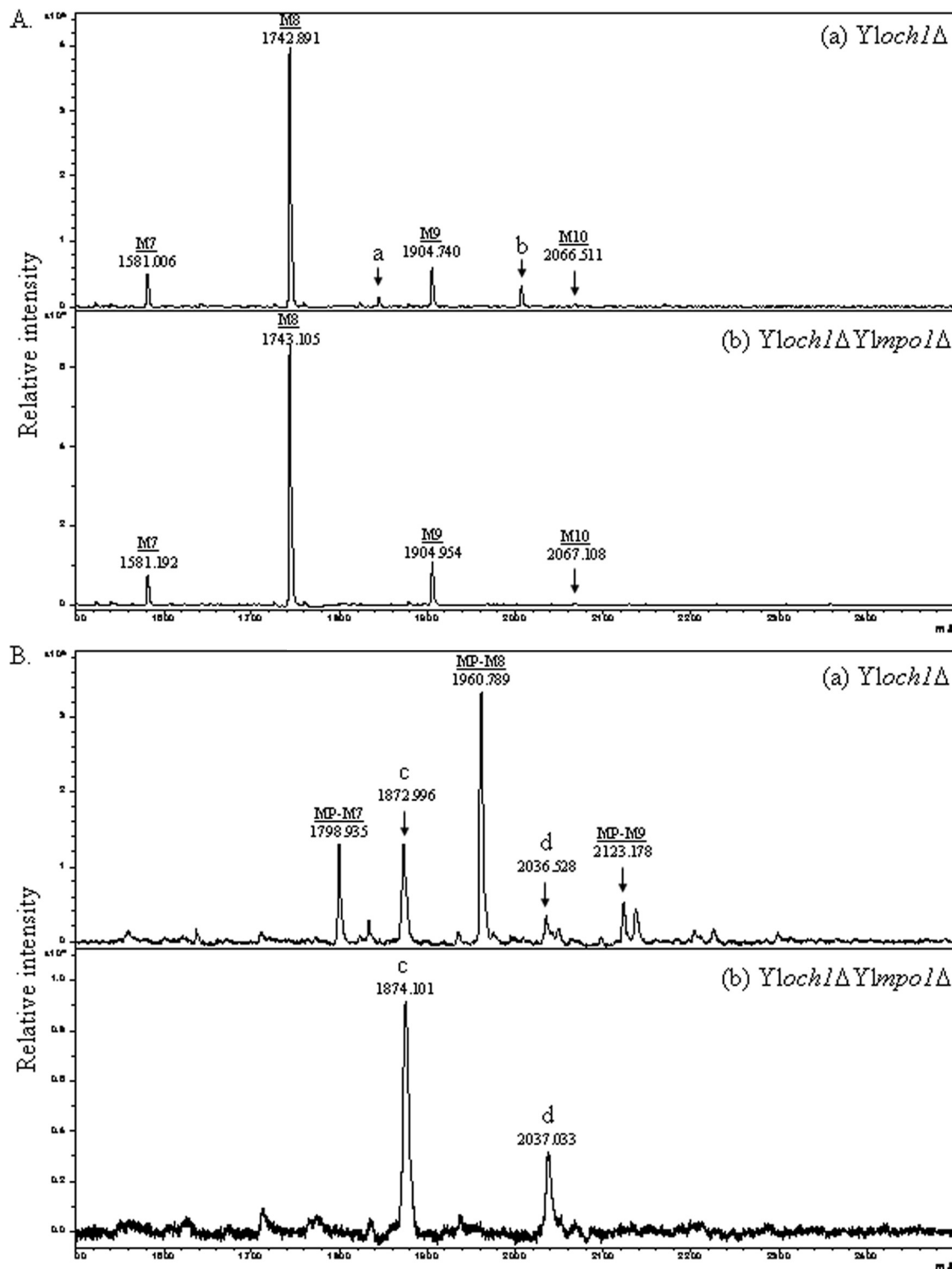


FIG. 3. MALDI-TOF mass spectrometry of the N-linked oligosaccharides from CWPs of *Yloch1Δ* and *Yloch1Δ Ylmpo1Δ* mutant strains. (A) Mass spectra analyzed in the positive reflector mode for the detection of neutral sugars of *Yloch1Δ* (a) and *Yloch1Δ Ylmpo1Δ* (b) strains. The intermediate peaks, designated a and b, are assumed to represent the monomannosylphosphorylated forms of $\text{Man}_7\text{GlcNAc}_2$ and $\text{Man}_8\text{GlcNAc}_2$ on the basis of their m/z values of 1844.583 and 2006.425, respectively. M7, $\text{Man}_7\text{GlcNAc}_2$; M8, $\text{Man}_8\text{GlcNAc}_2$; M9, $\text{Man}_9\text{GlcNAc}_2$; M10, $\text{Man}_{10}\text{GlcNAc}_2$. (B) Mass spectra analyzed in the linear negative mode for the detection of acidic sugars of the *Yloch1Δ* (a) and *Yloch1Δ Ylmpo1Δ* (b) strains. The intermediate peaks, designated c and d, are assumed to represent $\text{Man}_9\text{GlcNAc}_2$ and $\text{Man}_{10}\text{GlcNAc}_2$, respectively. MP-M7, monomannosylphosphorylated $\text{Man}_7\text{GlcNAc}_2$; MP-M8, monomannosylphosphorylated $\text{Man}_8\text{GlcNAc}_2$; MP-M9, monomannosylphosphorylated $\text{Man}_9\text{GlcNAc}_2$.

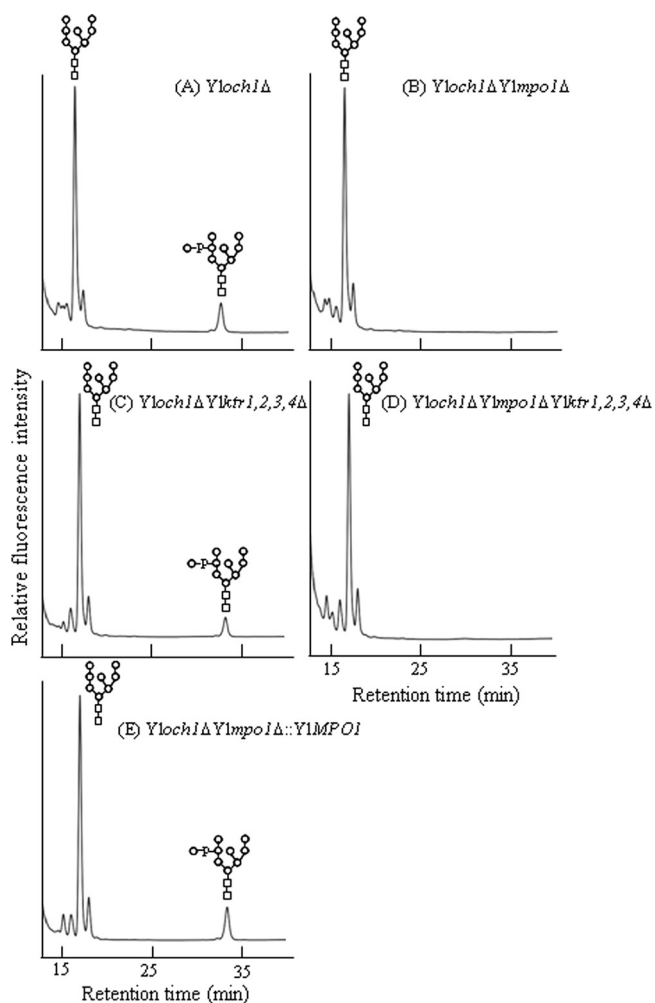


FIG. 4. HPLC analysis of the N-linked oligosaccharide of CWPs from the *Ylptr*Δ mutant strains. The N-linked oligosaccharide profiles of CWPs from *Yloch1*Δ (A), *Yloch1*Δ *Ylmpo1*Δ (B), *Yloch1*Δ *Ylptr1,2,3,4*Δ (C), *Yloch1*Δ *Ylmpo1*Δ *Ylptr1,2,3,4*Δ (D), and *Yloch1*Δ *Ylmpo1*Δ::*YIMPO1* (E) mutant strains were compared. $\text{Man}_5\text{GlcNAc}_2\text{-AA}$ was used as a standard AA-sugar chain.

mannosylphosphorylation of O-glycans in *Y. lipolytica*. O-glycans of CWPs from the wild-type and *Ylmpo1*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 *Ylmpo1*Δ strain (Fig. 5A, trace c) but reappeared following the reintroduction of the *YIMPO1* gene into the *Ylmpo1*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_{2-4} , as suggested by the DNA sequencer analysis (Fig. 5A, trace b). The peak at 20.5 min in

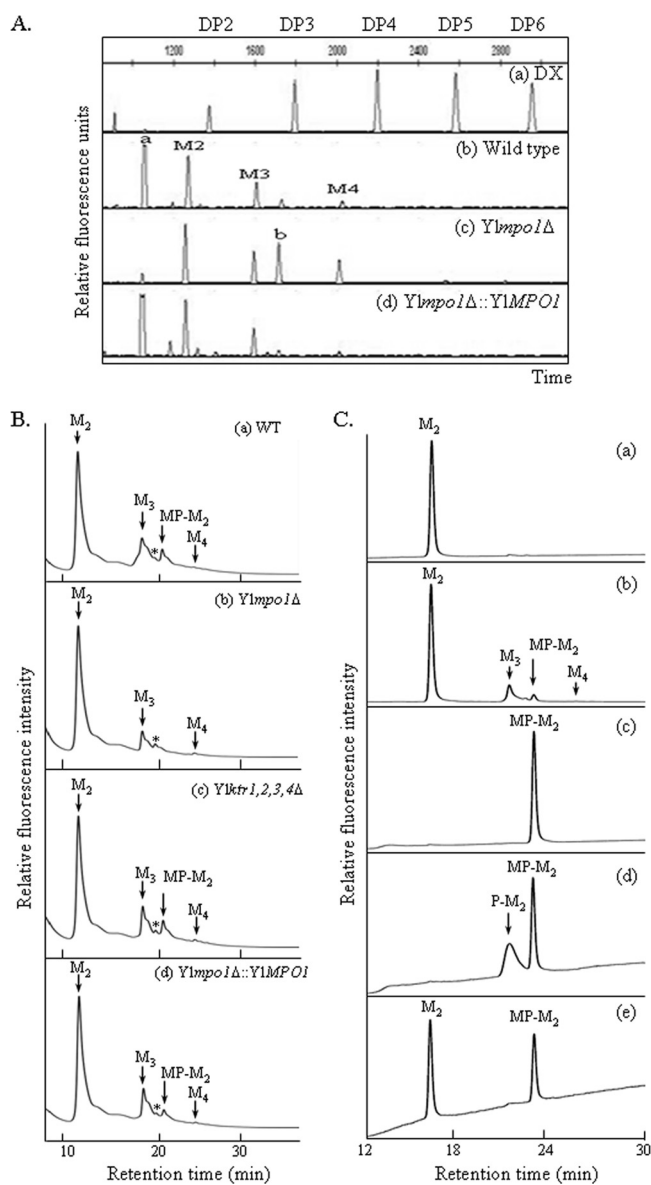


FIG. 5. Analysis of the O-linked oligosaccharides from *Ylmpo1*Δ and *Ylptr*Δ mutant strains. (A) O-linked oligosaccharides of CWPs from wild-type (b), *Ylmpo1*Δ (c), and *Ylmpo1*Δ::*YIMPO1* (d) strains were analyzed with a DNA sequencer. (a) Profile of the APTS-maltooligosaccharide ladder, which served 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), *Ylmpo1*Δ (b), *Ylptr1,2,3,4*Δ (c), and *Ylmpo1*Δ::*YIMPO1* (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. α 1,2-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.

the wild-type strain (Fig. 5B, trace a), which was also detected in the *Ylptr1,2,3,4*Δ strain (Fig. 5B, trace c), disappeared in the *Ylmpo1*Δ strain (Fig. 5B, trace b). This peak reappeared in the *Ylmpo1*Δ::*YIMPO1* strain into which the functional *YIMPO1*

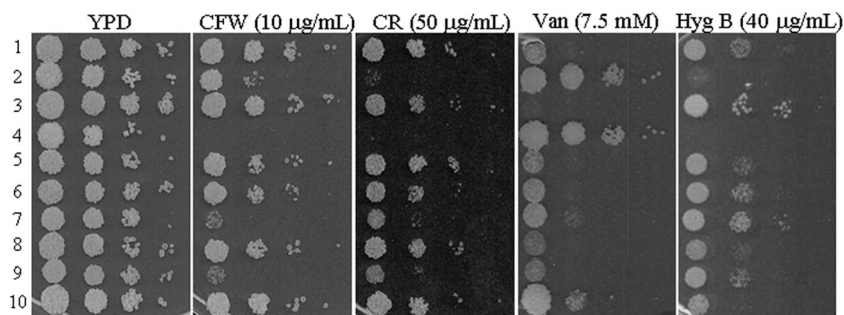


FIG. 6. Phenotypic analyses of the *Ylmpo1Δ* and *YlctrΔ* mutant strains. Wild-type (row 1), *Yloch1Δ* (row 2), *Ylmpo1Δ* (row 3), *Yloch1Δ Ylmpo1Δ* (row 4), *Ylctr1Δ* (row 5), *Ylctr2Δ* (row 6), *Ylctr3Δ* (row 7), *Ylctr4Δ* (row 8), *Ylctr1,2,3,4Δ* (row 9), and *Ylmpo1Δ::YIMPO1* (row 10) cells were spotted onto YPD medium plates containing 10 $\mu\text{g/ml}$ of CFW, 50 $\mu\text{g/ml}$ of CR, 7.5 mM Van, or 40 $\mu\text{g/ml}$ 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-M₂). 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-M₂) (Fig. 5C, trace d). If P-M₂ resulted from hydrolysis of MP-M₂, then treatment with alkaline phosphatase would be anticipated to yield a peak corresponding to α -linked mannobiose (M₂). 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 α 1,2-Man₂ (Fig. 5C, trace a). These results clearly indicate that the peak missing from the *Ylmpo1Δ* strain is an acidic O-glycan, a monomannosylphosphorylated mannobiose form, and that the *YIMPO1* gene participates in the mannosylphosphorylation of O-glycans as well as N-glycans in *Y. lipolytica*. However, the *Ylctr1,2,3,4Δ* 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 *Ylmpo1Δ* and *YlctrΔ* 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 β 1,3- and β 1,6-glucans (34, 35). CR binds preferentially to β 1,3-glucan and to chitin to a lesser degree. To evaluate the roles of the *YIMPO1* and *YIKTR* genes in cell wall integrity, each mutant strain was spotted onto YPD plates containing CFW or CR. The *Yloch1Δ* strain, which is sensitive to CFW and CR, was used as a positive control in the assay. As shown in Fig. 6, the *Ylmpo1Δ* strain was slightly more sensitive to CFW and CR than the wild-type strain, and the deletion of *YIMPO1* in the *Yloch1Δ* mutant increased the sensitivity to CFW and CR. These results indicate that *YIMPO1p* is required, but not to a great extent, for cell wall integrity in *Y. lipolytica*. Another interesting finding from this analysis was that the *Ylctr3Δ* strain was as sensitive to CFW and CR as the *Yloch1Δ* mutant. The data imply that although *YIKTR3* does not appear to be involved in N- or O-linked

glycosylation, the functions of *YIKTR3* 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 *Yloch1Δ* 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 *Ylmpo1Δ* strain displayed phenotypes opposite those of the *Yloch1Δ* strain; it became rather sensitive to vanadate, but the level of hygromycin B resistance increased. Reintroduction of *YIMPO1* into the *Ylmpo1Δ* 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 *Ylmpo1Δ* to vanadate further supports the notion that *YIMPO1p* 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 *Ylmpo1Δ* 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 *YIKTR4* (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	<i>xpr2</i>	+++
<i>Yloch1Δ</i>	<i>xpr2 och1</i>	++++
<i>Ylmpo1Δ</i>	<i>xpr2 mpo1</i>	-
<i>Yloch1Δ Ylmpo1Δ</i>	<i>xpr2 och1 mpo1</i>	+
<i>Ylmpo1Δ::YIMPO1</i>	<i>xpr2 mpo1 MPO1</i>	+++
<i>Ylctr1Δ</i>	<i>xpr2 ktr1</i>	+++
<i>Ylctr2Δ</i>	<i>xpr2 ktr2</i>	+++
<i>Ylctr3Δ</i>	<i>xpr2 ktr3</i>	+++
<i>Ylctr4Δ</i>	<i>xpr2 ktr4</i>	+++

^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.

the *Yloch1Δ* strain compared to that in the wild type, suggesting that the absence of the outer chain might enhance the efficiency of mannosylphosphorylation by YIMpo1p 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 *YIMPO1* 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 YIMpo1p 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 *Scmnn4Δ* 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) (*PpPNO1*) gene and the *PpMNN4B* 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* (*CaMNN4*) gene is the closest homologue to the ScMNN4 gene and harbors a KE repeat region. *CaMNN4* appears to be primarily responsible for mannosylphosphorylation, on the basis of the observation that the *Camnn4Δ* null mutant was unable to bind to alcian blue dye and was devoid of acid-labile β 1,2-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 *Camnn4Δ* mutant have not been performed. It is worth noting that the NCBI BLASTP searches using YIMpo1p as a query identified several hypothetical and putative fungal proteins, besides *C. albicans* Mnn4p (AAL86704), as YIMpo1p 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 YIMpo1p 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 *YIKTR1*, *YIKTR2*, *YIKTR3*, and *YIKTR4* genes, initially detected to be the closest ScMNN6 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 *CaMNT3* and *CaMNT5*, 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 *YIKtr1*, *YIKtr2*, *YIKtr3*, and *YIKtr4* proteins has mannosylphosphate transferase activity as a ScMnn6p functional homologue. Future work will also examine whether YIMpo1p alone can function in mannosylphosphate transfer in association with other multifunctional glycosyltransferases, as has been suggested in *C. albicans*.

Despite the fact that the *YIKTR1*, *YIKTR2*, *YIKTR3*, and *YIKTR4* 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 *YIKtr3Δ* 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 *YIKtr3Δ* mutant strain displayed defective hyphal development (data not shown), we speculate that *YIKTR3* 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 ($\text{Man}_{7-12}\text{GlcNAc}_2$), the absence of a terminal α 1,3-mannose epitope in both N- and O-linked oligosaccharides, and post-translational modification processes similar to those in mammalian systems (26). Therefore, we anticipate that the combined deletion of *YIMPO1* and *YIOCH1*, 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.

ACKNOWLEDGMENTS

We thank Y. Shimma and Y. Chiba at AIST, Japan, for their valuable comments on designing experiments in this study.

This work was supported by grants from the Korean Ministry of Education and Science (Microbial Genomics and Applications R&D Program) and from the Korean Ministry of Knowledge and Economy (Next Generation New Technology Development Program).

REFERENCES

- Akeboshi, H., et al. 2009. Production of human beta-hexosaminidase A with highly phosphorylated *N*-glycans by the overexpression of the *Ogataea minuta* *MNN4* gene. *Glycobiology* **19**:1002–1009.
- Ballou, L., R. A. Hitzeman, M. S. Lewis, and C. E. Ballou. 1991. Vanadate-resistant yeast mutants are defective in protein glycosylation. *Proc. Natl. Acad. Sci. U. S. A.* **88**:3209–3212.
- Barth, G., and C. Gaillardin. 1997. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. *FEMS Microbiol. Rev.* **19**:219–237.
- Bobrowicz, P., T. A. Stadheim, and S. Wildt. August 2007. Methods for eliminating mannosylphosphorylation of glycans in the production of glycoproteins. U.S. patent 7,259,007.
- Böer, E., G. Steinborn, G. Kunze, and G. Gellissen. 2007. Yeast expression platforms. *Appl. Microbiol. Biotechnol.* **77**:513–523.
- Chen, D.-C., J.-M. Beckerich, and C. Gaillardin. 1997. One-step transformation of the dimorphic yeast *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol.* **48**:232–235.
- Chiba, Y., et al. 1998. Production of human compatible high mannose-type ($\text{Man}_5\text{GlcNAc}_2$) sugar chains in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**:26298–26304.
- Chigira, Y., T. Oka, T. Okajima, and Y. Jigami. 2008. Engineering of a mammalian *O*-glycosylation pathway in the yeast *Saccharomyces cerevisiae*: production of *O*-fucosylated epidermal growth factor domains. *Glycobiology* **18**:303–314.
- Conde, R., G. Pablo, R. Cueva, and G. Larrriba. 2003. Screening for new yeast mutants affected in mannosylphosphorylation of cell wall mannoproteins. *Yeast* **20**:1189–1211.
- Conde, R., R. Cueva, and G. Larrriba. 2007. Rsc14-controlled expression of *MNN6*, *MNN4* and *MNN1* regulates mannosylphosphorylation of *Saccharomyces cerevisiae* cell wall mannoproteins. *FEMS Yeast Res.* **7**:1248–1255.
- Corbacho, I., I. Olivero, and L. M. Hernández. 2005. A genome-wide screen for *Saccharomyces cerevisiae* nonessential genes involved in mannosyl phosphate transfer to mannoprotein-linked oligosaccharides. *Fungal Genet. Biol.* **42**:773–790.
- Dean, N. 1995. Yeast glycosylation mutants are sensitive to aminoglycosides. *Proc. Natl. Acad. Sci. U. S. A.* **92**:1287–1291.
- Dereeper, A., et al. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* **36**(Web Server issue):W465–W469.
- Gemmill, T. R., and R. B. Trimble. 1999. Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species. *Biochim. Biophys. Acta* **1426**:227–237.
- Gerngross, T. U. 2004. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nat. Biotechnol.* **22**:1409–1414.
- Hamilton, S. R., et al. 2006. Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science* **313**:1441–1443.
- Hobson, R. P., et al. 2004. Loss of cell wall mannosylphosphate in *Candida albicans* does not influence macrophage recognition. *J. Biol. Chem.* **279**:39628–39635.
- Ilgén, C., J. Lin-Cereghino, and J. M. Cregg. 2005. Production of recombinant proteins, p. 143–162. In G. Gellissen (ed.), *Pichia pastoris*. Wiley-VCH, Weinheim, Germany.
- Jigami, Y., and T. Odani. 1999. Mannosylphosphate transfer to yeast mannan. *Biochim. Biophys. Acta* **1426**:335–345.
- Kang, H. A., and G. Gellissen. 2005. Production of recombinant proteins, p. 111–142. In G. Gellissen (ed.), *Hansenula polymorpha*. Wiley-VCH, Weinheim, Germany.
- Kim, M. W., et al. 2006. Functional characterization of the *Hansenula polymorpha* *HOCl1*, *OCH1*, and *OCR1* genes, as members of the yeast *OCH1* mannosyltransferase family involved in protein glycosylation. *J. Biol. Chem.* **281**:6261–6272.
- Kim, M. W., et al. 2004. Characterization of *N*-linked oligosaccharides assembled on secretory recombinant glucose oxidase and cell wall mannoproteins from the methylotrophic yeast *Hansenula polymorpha*. *Glycobiology* **14**:243–251.
- Kuchta, K., L. Knizewski, L. S. Wyrwicz, L. Rychlewski, and K. Ginalski. 2009. Comprehensive classification of nucleotidyltransferase fold proteins: identification of novel families and their representatives in human. *Nucleic Acids Res.* **37**:7701–7714.
- Lee, K. J., et al. 2009. High-throughput quantitative analysis of plant *N*-glycan using a DNA sequencer. *Biochem. Biophys. Res. Commun.* **380**:223–229.
- Lussier, M., A.-M. Sdicu, and H. Bussey. 1999. The *KTR* and *MNN1* mannosyltransferase families of *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1426**:323–334.
- Madzak, C., C. Gaillardin, and J.-M. Beckerich. 2004. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. *J. Biotechnol.* **109**:63–81.
- Miura, M., M. Hirose, T. Miwa, S. Kuwae, and H. Ohi. 2004. Cloning and characterization in *Pichia pastoris* of *PNO1* gene required for phosphomannosylation of *N*-linked oligosaccharides. *Gene* **324**:129–137.
- Mora-Montes, H. M., et al. 2010. A multifunctional mannosyltransferase family in *Candida albicans* determines cell wall mannan structure and host-fungus interactions. *J. Biol. Chem.* **285**:12087–12095.
- Müller, S., T. Sandai, P. Kamp-Hansen, and H. Dalbøge. 1998. Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. *Yeast* **14**:1267–1283.
- Nakakita, S., W. Sumiyoshi, N. Miyanishi, and J. Hirabayashi. 2007. A practical approach to *N*-glycan production by hydrazinolysis using hydrazine monohydrate. *Biochem. Biophys. Res. Commun.* **362**:639–645.
- Nakayama, K., Y. Feng, A. Tanaka, and Y. Jigami. 1998. The involvement of *mnn4* and *mnn6* mutations in mannosylphosphorylation of *O*-linked oligosaccharides in yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1425**:255–262.
- Odani, T., Y. Shimma, A. Tanaka, and Y. Jigami. 1996. Cloning and analysis of the *MNN4* gene required for phosphorylation of *N*-linked oligosaccharides in *Saccharomyces cerevisiae*. *Glycobiology* **6**:805–810.
- Oh, D.-B., et al. 2008. Glycoengineering of the methylotrophic yeast *Hansenula polymorpha* for the production of glycoproteins with trimannosyl core *N*-glycan by blocking core oligosaccharide assembly. *Biotechnol. J.* **3**:659–668.
- Ram, A. F., A. Wolters, R. Ten Hoopen, and F. M. Klis. 1994. A new approach for isolating cell wall mutants in *Saccharomyces cerevisiae* by screening for hypersensitivity to calcofluor white. *Yeast* **10**:1019–1030.
- Ram, A. F., and F. M. Klis. 2006. Identification of fungal cell wall mutants using susceptibility assays based on calcofluor white and Congo red. *Nat. Protoc.* **1**:2253–2256.
- Ruiz-Herrera, J., P. García-Maceira, L. C. Castillo-Barahona, E. Valentín, and R. Sentandreu. 2003. Cell wall composition and structure of *Yarrowia lipolytica* transposon mutants affected in calcofluor sensitivity. *Antonie Van Leeuwenhoek* **84**:229–238.
- Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Song, Y., et al. 2007. Engineering of the yeast *Yarrowia lipolytica* for the production of glycoproteins lacking the outer-chain mannose residues of *N*-glycans. *Appl. Environ. Microbiol.* **73**:4446–4454.
- Thiebault, F., F. Kiss, R. Bonaly, and J. Coulon. 2002. Effect of sodium orthovanadate on glycosylation of the phosphopeptidomannans involved in the cell-cell aggregation of the yeast *Kluyveromyces bulgaricus*. *Carbohydr. Res.* **337**:1873–1877.
- Wang, X.-H., K. Nakayama, Y. Shimma, A. Tanaka, and Y. Jigami. 1997. *MNN6*, a member of the *KRE2/MNT1* family, is the gene for mannosylphosphate transfer in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**:18117–18124.