

Engineering of the Yeast *Yarrowia lipolytica* for the Production of Glycoproteins Lacking the Outer-Chain Mannose Residues of N-Glycans[∇]

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In an attempt to engineer a *Yarrowia lipolytica* strain to produce glycoproteins lacking the outer-chain mannose residues of N-linked oligosaccharides, we investigated the functions of the *OCH1* gene encoding a putative α -1,6-mannosyltransferase in *Y. lipolytica*. The complementation of the *Saccharomyces cerevisiae och1* mutation by the expression of *YIOCH1* and the lack of in vitro α -1,6-mannosyltransferase activity in the *Yloch1* null mutant indicated that *YIOCH1* is a functional ortholog of *S. cerevisiae OCH1*. The oligosaccharides assembled on two secretory glycoproteins, the *Trichoderma reesei* endoglucanase I and the endogenous *Y. lipolytica* lipase, from the *Yloch1* null mutant contained a single predominant species, the core oligosaccharide $\text{Man}_8\text{GlcNAc}_2$, whereas those from the wild-type strain consisted of oligosaccharides with heterogeneous sizes, $\text{Man}_8\text{GlcNAc}_2$ to $\text{Man}_{12}\text{GlcNAc}_2$. Digestion with α -1,2- and α -1,6-mannosidase of the oligosaccharides from the wild-type and *Yloch1* mutant strains strongly supported the possibility that the *Yloch1* mutant strain has a defect in adding the first α -1,6-linked mannose to the core oligosaccharide. Taken together, these results indicate that *YIOCH1* plays a key role in the outer-chain mannosylation of N-linked oligosaccharides in *Y. lipolytica*. Therefore, the *Yloch1* mutant strain can be used as a host to produce glycoproteins lacking the outer-chain mannoses and further developed for the production of therapeutic glycoproteins containing human-compatible oligosaccharides.

Yeast can secrete a variety of proteins in much the same way that mammalian cells do. The presence of yeast-specific outer-chain mannosylation, however, has been a primary hindrance to the exploitation of yeast for therapeutic glycoprotein production, because glycoproteins decorated with yeast-specific glycans are immunogenic and show poor pharmacokinetic properties in humans (1, 24). In the budding yeast *Saccharomyces cerevisiae*, the N-linked oligosaccharides assembled on glycoproteins include hypermannose structures with outer chains that may contain up to 200 mannose units (6). Elongation of the outer chain is initiated by the Och1 protein, which adds the first α -1,6-linked mannose to the core N-linked oligosaccharides upon their arrival in the Golgi apparatus in *S. cerevisiae* (17). Following the addition of the first α -1,6-mannose by Och1p, the core oligosaccharide is elongated by additional α -1,6-mannosyltransferases, Mnn9p and Van1p, which extend the α -1,6-linked polymannose backbone, and the core oligosaccharide is further branched by the addition of α -1,2- and α -1,3-linked mannoses (5, 8). Other yeast species, including *Pichia pastoris*, *Hansenula polymorpha*, and *Schizosaccharomyces pombe*, also use the Och1 protein to extend the mannose outer chain of N-glycans (14, 24, 26, 28). Therefore, the

elimination of the Och1 protein was performed to block the yeast-specific outer-chain mannosylation, followed by further engineering of yeast N-glycosylation pathways for the production of glycoproteins with human-compatible oligosaccharides (14, 17, 26).

Yarrowia lipolytica, a heterothallic yeast, is presently considered to be a good potential host for heterologous gene expression due to its ability to secrete large amounts of extracellular proteins (4, 12, 21) and the simplicity with which it is cultivated to a high cell density (13). Despite the potential of *Y. lipolytica* as a valuable host, little information on the structural characteristics of N-linked oligosaccharides of *Y. lipolytica* glycoproteins is available. It was recently reported that *Y. lipolytica OCH1* (*YIOCH1*), the homolog of *S. cerevisiae OCH1* encoding an α -1,6-mannosyltransferase, may have a minor role in N-glycosylation (3). In our study, however, glycoproteins secreted from a *Yloch1* mutant strain showed more homogeneous band patterns upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than the wild-type strain. Therefore, we have investigated the function of *YIOCH1* by systematically analyzing the structures of N-linked oligosaccharides assembled on recombinant proteins secreted from the *Yloch1* null mutant strain, and we present several lines of evidence that *YIOch1p* is a key enzyme responsible for adding the first α -1,6-mannose residue onto the core oligosaccharide, $\text{Man}_8\text{GlcNAc}_2$, in *Y. lipolytica*. The *Yloch1* mutant strain, which secretes glycoproteins lacking the outer-chain mannoses, can be used as a starting host for further glycoengineering to produce human-compatible oligosaccharides.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
<i>Y. lipolytica</i>		
SMS397A	<i>MATA ade1 ura3 xpr2</i>	11
<i>och1Δ (URA3)</i>	<i>MATA ade1 ura3 xpr2 och1::tc-URA3-tc</i>	This study
<i>och1Δ (ura3)</i>	<i>MATA ade1 ura3 xpr2 och1::tc</i>	This study
SMS(pAUX-EGI)	SMS397A harboring pAUX-EGI	This study
<i>och1Δ</i> (pAUXEGI)	<i>Yloch1Δ</i> harboring pAUX-EGI	This study
SMS(pAUX-YILIP2)	SMS397A harboring pAUX-YILIP2	This study
<i>och1Δ</i> (pAUX-YILIP2)	<i>Yloch1Δ</i> harboring pAUX-YILIP2	This study
<i>S. cerevisiae</i>		
W303-1A	<i>MATa leu2 ura3 trp1 ade2 his3</i>	Lab collection
<i>och1Δ</i>	<i>MATa leu2 ura3 trp1 ade2 his3 och1Δ::TRP1</i>	14
TOY137	<i>MATa leu2 ura3 trp1 ade2 his3 och1Δ::hisG mnn1Δ::hisG mnn4Δ::hisG</i>	14
W303-1A(pYEp352GAPII)	W303-1A harboring pYEp352GAPII	This study
<i>och1Δ</i> (pYEp352GAPII)	<i>Scoch1Δ</i> harboring pYEp352GAPII	This study
TOY137(pYEp352GAPII)	TOY137 harboring pYEp352GAPII	This study
W303-1A(pYEp352GAPII-YIOCH1)	W303-1A harboring pYEp352GAPII-YIOCH1	This study
<i>och1Δ</i> (pYEp352GAPII-YIOCH1)	<i>Scoch1Δ</i> harboring pYEp352GAPII-YIOCH1	This study
TOY137(pYEp352GAPII-YIOCH1)	TOY137 harboring pYEp352GAPII-YIOCH1	This study

MATERIALS AND METHODS

Strains, plasmids, and media. The *Y. lipolytica* and *S. cerevisiae* strains used in this study are described in Table 1. The yeast strains were routinely grown in YPD (1% yeast extract, 2% Bacto peptone, and 2% glucose) at 28°C (*Y. lipolytica*) or 30°C (*S. cerevisiae*). Synthetic complete medium was composed of 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, and a dropout amino acid mixture including all of the amino acids required. When required, 0.625 mg of 5'-fluoroorotic acid/ml was added to solid medium for the selection of Ura auxotrophic strains. Drug sensitivity was assayed by spotting serially diluted yeast cultures onto YPD solid medium containing 30 µg of hygromycin B (Sigma)/ml, 20 µg of calcofluor white (Sigma)/ml, 100 µg of Congo red (Junsei, Japan)/ml, 0.05% SDS (Sigma), or 7 mM sodium orthovanadate (Sigma).

The plasmid pYEp352GAPII, containing the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene promoter and terminator (18), was used as a backbone vector for the expression of the *Y. lipolytica* *OCH1* gene in *S. cerevisiae*. The pIMR53-AUX vector (11), containing the *XPR2* promoter and terminator and the *URA3* gene, was used for the secretory expression of *Trichoderma reesei* endoglucanase I (EGI) and *Y. lipolytica* lipase tagged with six histidine residues. To express EGI and lipase, the recombinant *Y. lipolytica* cells were cultivated on YPDm medium (1% yeast extract, 1% proteose peptone, 1% glucose, and 50 mM phosphate-buffered saline, pH 6.8) at 28°C.

Recombinant DNA techniques and gene disruption. Recombinant DNA techniques, Southern blot hybridization, and transformation were carried out essentially as described by Sambrook and Russell (23). PCRs were performed with ExTaq polymerase (Takara, Japan) using the GeneAmp PCR system 2400 (PerkinElmer).

Two pairs of primer sets, YO1F1 (5'-ACTTTTTCATCTGCGGAC-3') and YO1R1 (5'-CCATGGATCCGCTAGACTAGGAGTTCGAAGACGTTG-3') and YO1F2 (5'-AGATCT ACGGATCCATGGGACCGACTGTCTTCGA-3') and YO1R2 (5'-CATCCTCCTGATATACGC-3'), were designed to amplify the 5' and 3' flanking regions of the *YIOCH1* gene. The amplified PCR fragments were fused by performing PCR using the YO1F1 and YO1R2 primers, and the product was subcloned into the pGEMT-Easy vector (Promega). The *tc-URA3-tc* cassette (11) was inserted into the linker site of the fused PCR product. This disruption cassette was made linear by digestion with NotI and used to transform *Y. lipolytica*. Correct disruption was confirmed by PCR and Southern blot analysis. *URA3* of the integrated *tc-URA3-tc* cassette was popped out of the *Yloch1Δ (URA3)* strain by growing the strain on synthetic complete medium containing 0.0625% 5'-fluoroorotic acid at 28°C for 5 days. The resulting *Yloch1Δ (ura3)* strain was again transformed with the EGI and lipase expression vectors, pAUX-EGI and pAUX-YILIP2.

Purification and analysis of EGI and lipase. Culture supernatants containing His₆-tagged EGI and lipase were concentrated by ultrafiltration (YM30 membrane, Millipore). The 100-fold-concentrated culture supernatants were dialyzed against 50 mM sodium phosphate (pH 6.0) and 300 mM NaCl, and His₆-tagged EGI and lipase were purified by a His₆-tagged affinity column using the ÄKTA Prime chromatography system (Amersham Pharmacia Biotech AB, Sweden).

Western blot analysis of the purified His₆-tagged EGI was performed with the polyclonal immunoglobulin G Penta-His antibodies (QIAGEN, Germany), and the EGI was detected using the DIG kit (Roche, Germany). Deglycosylation with endoglycosidase H (endo H) was performed in accordance with the instructions of the manufacturer (New England Biolabs). Activity staining of *T. reesei* EGI was carried out as follows. After the cells expressing EGI were grown in 3 ml of YPDm medium for 30 h, the culture supernatant was collected by centrifugation. Each sample (16 µl of supernatant) was subjected to electrophoresis on a 10% PAGE gel. After the completion of electrophoresis, the substrate gel, which contained 50 mM sodium citrate (pH 5.6), 1% carboxyl methyl cellulose, and 1.5% agarose, was overlaid with the PAGE gel. Both gels were wrapped and incubated at 30°C for 3 h. The substrate gel was stained with 1% Congo red solution for 10 min and then washed several times with 1 M sodium chloride. For invertase activity staining, cells were grown in 3 ml of YPD medium to an optical density at 600 nm of 6, transferred into YPsuc medium (2% yeast extract, 2% Bacto peptone, and 1% sucrose) in order to derepress invertase expression, and then incubated at 25°C for 3 h. The cell pellets were homogenized with acid-washed glass beads (425 to 600 µm in diameter) in PAGE sample buffer (125 mM Tris-HCl [pH 6.8], 1% β-mercaptoethanol, 15% glycerol, 3% SDS, 0.1% bromophenol blue, 2 mM phenylmethylsulfonyl fluoride) and were then subjected to 5% PAGE. The gel was incubated in a solution containing 0.1 M sodium acetate (pH 5.1) and 0.1 M sucrose for 30 min at 37°C, washed with H₂O, and then boiled in a 0.5 N NaOH solution containing 2,3,5-triphenyltetrazolium chloride for the visualization of proteins with invertase activity.

Analysis of N-linked oligosaccharides. N-linked oligosaccharides were released from 200 µg of the purified EGI and lipase by PNGase F (New England Biolabs). The oligosaccharides were labeled at their reducing ends with 2-amino-pyridine (PA) by using the PALSTATION pyridylation reagent kit (Takara Shuzo Co., Japan). After pyridylation, the samples were purified by Sephadex G-15 spin columns (Amersham Pharmacia Biotech AB, Sweden) to remove residual PA. The digestion of PA-labeled oligosaccharides with α-1,2-mannosidase from *Aspergillus saitoi* (Glyko, Japan) or α-1,6-mannosidase from *Xanthomonas manihotis* (New England Biolabs) was carried out according to the instructions of the manufacturer.

Size fractionation high-performance liquid chromatography (HPLC) was per-

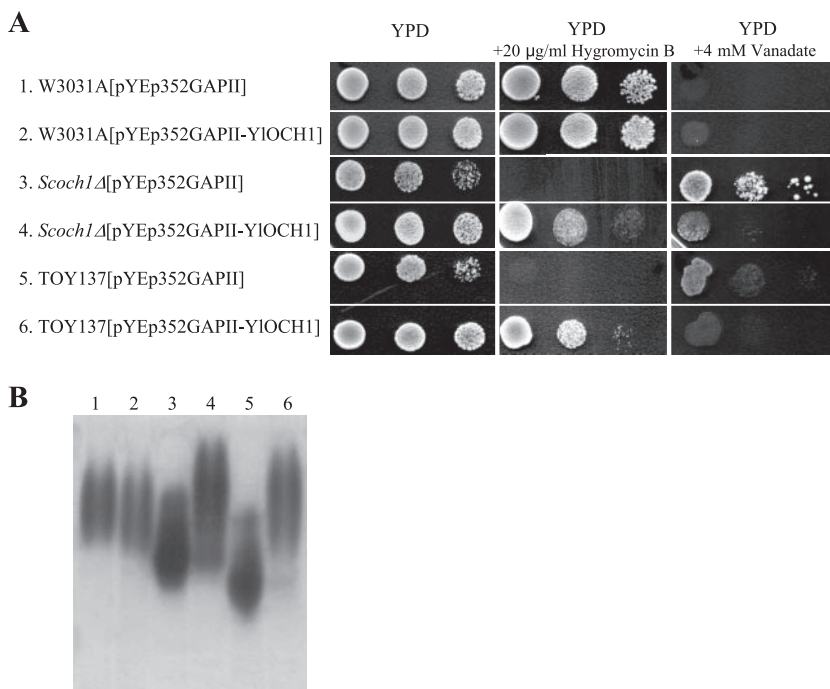


FIG. 1. Functional complementation of *S. cerevisiae och1* mutation by the *Y. lipolytica OCH1* gene. (A) Spotting analysis of growth phenotype. The *S. cerevisiae* wild-type W303-1A, *och1*Δ, and TOY137 (*och1*Δ *mnn1*Δ *mnn4*Δ) strains were transformed with a *YIOCH1* expression vector, pYEp352GAPII-YIOCH1, and a control vector, pYEp352GAPII. Serial (1/10) dilutions of cells were spotted onto plates containing YPD, YPD with 20 µg of hygromycin B/ml, or YPD with 4 mM vanadate. The plates were incubated for 3 days at 25°C. (B) Activity staining of invertase. Cells grown to an optical density at 600 nm of 6 in 3 ml of YPD medium were transferred onto YPsuc medium (2% yeast extract, 2% Bacto peptone, and 1% sucrose) to derepress invertase expression and were then incubated at 25°C for 3 h. Lanes 1, 3, and 5 contain cell lysates of W303-1A, *Scoch1*Δ, and TOY137 strains transformed with a control vector, respectively. Lanes 2, 4, and 6 contain cell lysates of W303-1A, *Scoch1*Δ, and TOY137 strains transformed with pYEp352GAPII-YIOCH1, respectively.

formed with a Shodex Asahipak NH2P-50 column (0.46 by 25 cm; Showa Denko K. K., Japan) at a rate of 1.0 ml/min. The column was equilibrated with a solution comprising 80% solvent A (200 mM acetic acid-triethylamine [pH 7.3]-acetonitrile, 1:9) and 20% solvent B (200 mM acetic acid-triethylamine [pH 7.3]-

acetonitrile, 9:1). After sample injection, the proportion of solvent B was increased linearly up to 95% for 52 min. PA oligosaccharides were detected by fluorescence (excitation λ, 320 nm, and emission λ, 400 nm) with a Waters 2475 fluorescence detector.

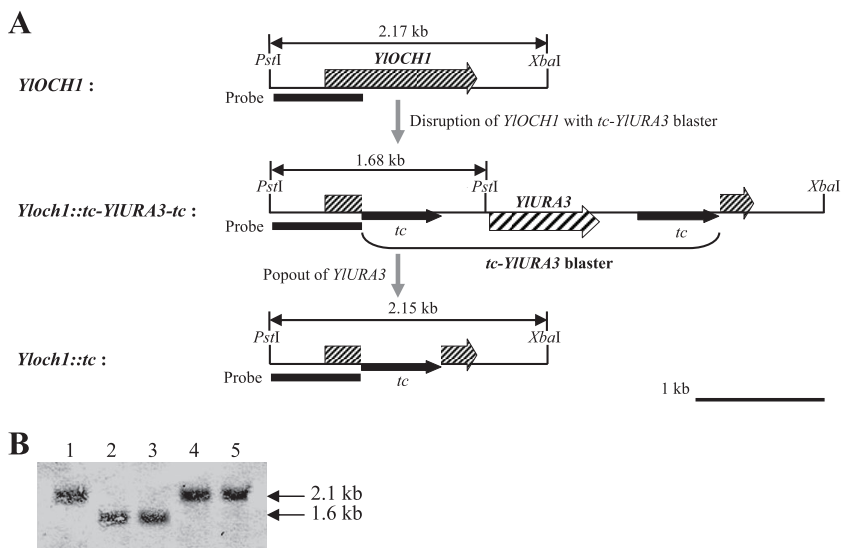


FIG. 2. Disruption of *YIOCH1*. (A) Schematic representation of various genomic fragments containing *YIOCH1* or mutated alleles. The 1.2-kb fragment of *YIOCH1* was replaced with the *tc-YIURA3-tc* cassettes flanked by regions homologous to *YIOCH1* by in vivo DNA recombination. (B) Southern blot analysis of the *YIOCH1* disruption. Lane 1, SMS397A (wild type); lanes 2 and 3, *Yloch1*Δ (*URA3*) strain (*Yloch1::tc-YIURA3-tc*); lanes 4 and 5, *Yloch1*Δ strain (*ura3*) (*Yloch1::tc*). Each genomic DNA strand was digested with PstI and XbaI and hybridized with the digoxigenin-labeled 690-bp DNA fragment.

In vitro α -1,6-mannosyltransferase activity assay. Membrane fractions were obtained as described previously (14). Cells grown in YPD medium were harvested, washed with 1% KCl, and resuspended in 5 ml of PMS buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MnCl_2$, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, and 2 μ g of each protease inhibitor [antipain, chymostatin, leupeptin, and pepstatin A]/ml). Glass beads (425 to 600 μ m in diameter) were added to half of the cell suspension volume, and the mixture was homogenized four times for 1 min at 4°C. Homogenates were centrifuged at $10,000 \times g$ for 20 min, and the supernatant obtained was further centrifuged at $100,000 \times g$ for 1 h. High-speed pellets were collected and resuspended in PMS buffer, and protein concentrations were determined using a protein assay agent (Bio-Rad). The α -1,6-mannosyltransferase activity assay was performed as described by Nakajima and Ballou (16). One hundred micrograms of high-speed pellet proteins was incubated in 100 μ l of 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM $MnCl_2$, 1 mM GDP-mannose, 0.5 mM 1-deoxymannojirimycin, and 100 pmol of the $Man_8GlcNAc_2$ -PA acceptor at 30°C for 2 h. The reaction was terminated by boiling at 99°C for 5 min, and the reaction mixture was filtered through an Ultrafree-MC membrane (10,000-Da cutoff; Millipore) and analyzed by HPLC.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis. N-linked oligosaccharides released from 100 μ g of the purified recombinant EGI were isolated by using a porous graphitized carbon column (Alltech). To elute neutral and acidic glycans, 900 μ l of a solution consisting of 25% (vol/vol) acetonitrile (Applied Biosystems) and 0.05% (vol/vol) trifluoroacetic acid (Aldrich) was added onto the column. The effluent was collected, lyophilized on a freeze-dry system (Ilshin, Korea), and dissolved in 10 μ l of HPLC-grade water. The glycan sample (0.7 μ l) was mixed with 0.7 μ l of a mixture of 6-aza-2-thiothymine and 2,5-dihydroxybenzoic acid (vol/vol, 1:1) as a matrix, and the mixture was loaded onto a ground steel MSP 96 target (microScout target; Bruker Daltonics, Germany). The mass spectrum was analyzed with a microflex mass spectrometer (Bruker Daltonics, Germany) in the positive reflector mode (for the detection of neutral sugars) or in the negative linear mode (for the detection of acidic sugars).

RESULTS

YIOCH1 is a functional homolog of ScOCH1 encoding an initiating α -1,6-mannosyltransferase. Since the role of YIOCH1 in outer-chain biosynthesis has not yet been completely defined despite the previous study by Barnay-Verdier et al. (3), we wanted to investigate whether YIOCH1 is a functional homolog of ScOCH1. We first examined the effect of YIOCH1 expression on the growth phenotypes of *S. cerevisiae och1* mutant strains, such as sensitivity to hygromycin B and resistance to orthovanadate, which are general characteristics observed in yeast mutant strains defective in N glycosylation (2, 7, 18). A complementation vector, pYEp352GAPII-YIOCH1, which expresses YIOCH1 under the control of the *S. cerevisiae* GAPDH gene promoter, was constructed and introduced into the *S. cerevisiae* wild-type and *och1* Δ mutant strains. In contrast to the strains transformed with a control plasmid, pYEp352GAPII, the *S. cerevisiae och1* mutant strains transformed with pYEp352GAPII-YIOCH1 recovered the phenotypes of the wild-type strain, including the normal growth rate, resistance to hygromycin B, and sensitivity to sodium orthovanadate (Fig. 1A). Next, we performed an invertase gel electrophoretic mobility assay in order to determine whether YIOCH1 can eliminate the glycosylation defect in the *Scoch1* Δ mutant strains. The invertases secreted from the *Scoch1* Δ mutant cells migrated faster than those secreted from the wild-type cells (Fig. 1B), which indicates the lack of heavy glycosylation in the invertases from the *Scoch1* Δ mutants. It is evident, however, that the introduction of the YIOCH1 gene restored the ability of the *Scoch1* Δ mutant strains to secrete fully glycosylated invertase. Along with the recovery of the normal growth phenotype, the elimination of the glycosylation defect in the *S. cerevisiae och1* mutant strains by the expression of

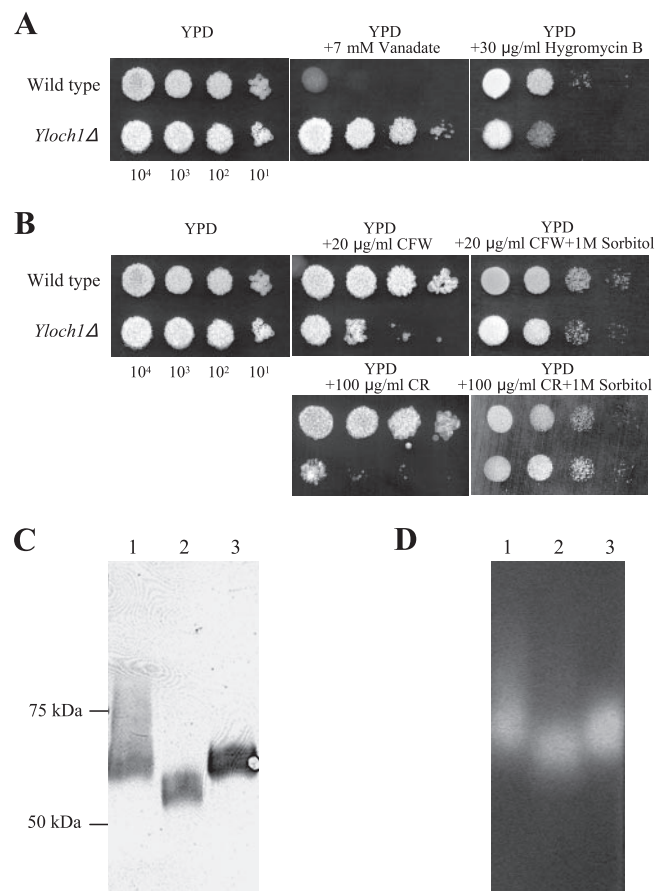


FIG. 3. Phenotypic analysis of the *Yloch1* mutant strain. (A) Vanadate resistance and hygromycin B sensitivity. (B) Calcofluor white and Congo red sensitivity. The wild-type and *Yloch1* mutant cells were grown in YPD, and 10- μ l serial (1/10) dilutions of each strain were spotted onto YPD plates containing 7 mM vanadate, 30 μ g of hygromycin B/ml, 20 μ g of calcofluor white (CFW)/ml, or 100 μ g of Congo red (CR)/ml. The plates were incubated for 2 days at 28°C. (C) Western blot analysis of *T. reesei* EGI secreted from the wild-type and *Yloch1* Δ cells. The secreted EGI from the wild-type and *Yloch1* Δ cells was purified, separated by 10% SDS-PAGE, and detected with anti-His antibody. Lane 1, EGI secreted from the wild type; lane 2, EGI treated with endo H; lane 3, EGI secreted from *Yloch1* Δ cells. (D) The supernatants (16 μ l) from cell cultures grown in 3 ml of YPD medium for 30 h were subjected to 10% PAGE without SDS. The substrate gel containing 1% carboxyl methyl cellulose was overlaid with the PAGE gel. Both gels were wrapped and incubated at 30°C for 3 h. The substrate gel was stained with 1% Congo red solution for 10 min. Lane 1, EGI secreted from the wild type; lane 2, EGI treated with endo H; lane 3, EGI secreted from *Yloch1* Δ cells.

YIOCH1 demonstrates that YIOCH1 is a functional homolog of *S. cerevisiae* OCH1.

Effect of YIOCH1 deletion on cell growth and N glycosylation. To study the in vivo function of the YIOCH1 gene in *Y. lipolytica*, the *Yloch1* null mutant (*Yloch1* Δ) strain was constructed and confirmed by Southern analysis (Fig. 2). Interestingly, unlike the *S. cerevisiae och1* mutant, the *Yloch1* Δ mutant grew as well as the wild-type strain both under normal growth conditions and at a high temperature, 34°C (data not shown). To test whether the *Yloch1* Δ mutant has the phenotypes of defective glycosylation, we grew the *Yloch1* Δ mutant on me-

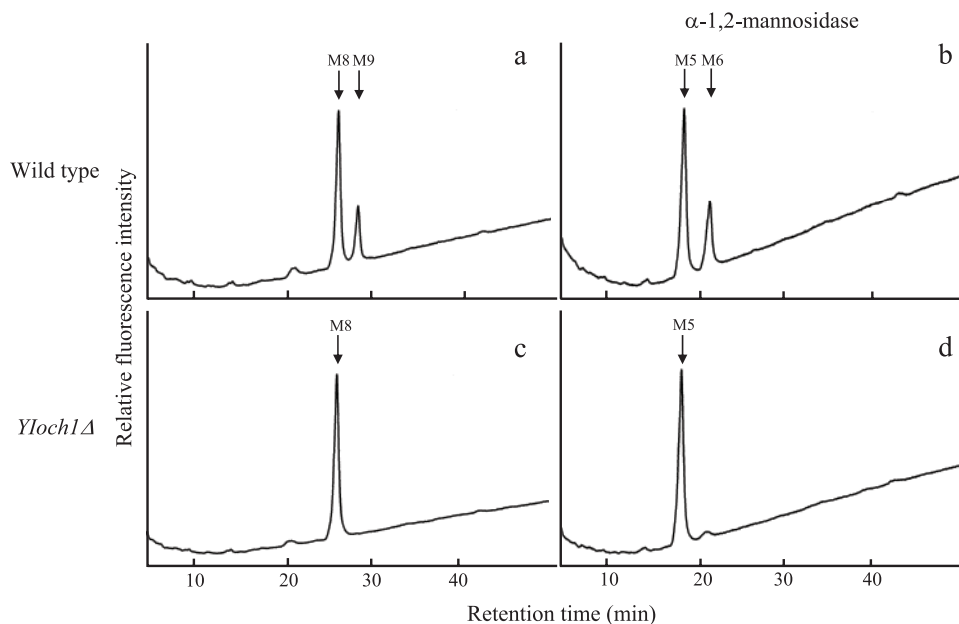


FIG. 4. In vitro activity analysis of *Y. lipolytica* *OCH1* gene product. Solubilized membrane fractions from the *Y. lipolytica* wild type (a and b) and *Yloch1Δ* (c and d) strains were prepared as enzyme sources, and $\text{Man}_8\text{GlcNAc}_2\text{-PA}$ was used as an acceptor. The reaction products were analyzed by HPLC. The reaction products treated with α -1,2-mannosidase were also analyzed by HPLC. M5, $\text{Man}_5\text{GlcNAc}_2\text{-PA}$; M6, $\text{Man}_6\text{GlcNAc}_2\text{-PA}$; M8, $\text{Man}_8\text{GlcNAc}_2\text{-PA}$; M9, $\text{Man}_9\text{GlcNAc}_2\text{-PA}$.

dium containing vanadate or hygromycin B. The *Yloch1Δ* mutant was resistant to vanadate and sensitive to hygromycin B compared to the wild-type strain (Fig. 3A), indicating that the *Yloch1Δ* mutant was defective in terms of glycosylation. The *Yloch1Δ* mutant also showed poor growth on medium containing calcofluor white or Congo red. However, the addition of an osmotic stabilizer, 1 M sorbitol, to the medium restored growth to the level of wild-type growth (Fig. 3B), although supplementation with 0.5 M sorbitol was shown to be insufficient to support the growth of the *Yloch1Δ* mutant (3). This result suggested that the *Yloch1Δ* mutant has alterations in its cell wall composition, probably in mannoproteins (7, 22). The phenotypes of the glycosylation defect and the alteration of cell wall composition and structure in the *Yloch1Δ* mutant imply that the function of *YIOCH1* may be important for the N-glycosylation process in *Y. lipolytica*.

To further examine protein N glycosylation in the *Yloch1Δ* mutant, *T. reesei* EGI, which has nine potential N-linked glycosylation sites (9), was expressed in *Y. lipolytica*. The *Yloch1Δ* mutant produced relatively homogeneously sized EGI proteins compared to the wild-type strain, which secreted rather heterogeneous forms of EGI, as judged by the smeared pattern on Western blots; this smearing was eliminated upon treatment with endo H, which removes oligomannosidic N-glycans (Fig. 3C and D). This result led us to believe that the *Yloch1Δ* mutant certainly lacked hyperglycosylation activity and that *YIOch1p* might be involved in the outer-chain elongation of N-linked oligosaccharides in *Y. lipolytica*.

Membrane fraction of *Yloch1Δ* mutant lacks an initiating α -1,6-mannosyltransferase activity. To determine whether the *Yloch1Δ* mutant lacks an initiating α -1,6-mannosyltransferase that uses the core oligosaccharide as a substrate, solubilized membrane fractions were prepared from the wild-type and

Yloch1Δ mutant strains and used as enzyme sources for an in vitro α -1,6-mannosyltransferase assay. Whereas, as expected, the peak corresponding to $\text{Man}_9\text{GlcNAc}_2\text{-PA}$ was detected as a reaction product in the membrane fraction of the wild-type strain, the acceptor, $\text{Man}_8\text{GlcNAc}_2\text{-PA}$, was not converted into $\text{Man}_9\text{GlcNAc}_2\text{-PA}$ by the membrane fraction of the *Yloch1Δ* mutant (Fig. 4). Furthermore, the $\text{Man}_9\text{GlcNAc}_2\text{-PA}$ product was converted into $\text{Man}_6\text{GlcNAc}_2\text{-PA}$ and the $\text{Man}_8\text{GlcNAc}_2\text{-PA}$ core oligosaccharide was converted into $\text{Man}_5\text{GlcNAc}_2\text{-PA}$ when the reaction products were treated with α -1,2-mannosidase from *A. saitoi*, which specifically removes α -1,2-linked mannose residues at the nonreducing ends of manno-oligosaccharides (Fig. 4). These results strongly indicate that *YIOCH1* encodes an initiation-specific α -1,6-mannosyltransferase acting on the core oligosaccharide.

Structural analysis of N-linked oligosaccharides released from EGI and lipase in the *Yloch1Δ* mutant. To obtain detailed information on the in vivo function of *YIOch1p* in N glycosylation, we analyzed the structures of N-glycans assembled on EGI secreted from the *Y. lipolytica* wild-type and *Yloch1Δ* strains. In the case of the wild-type strain, two major peaks corresponding to $\text{Man}_8\text{GlcNAc}_2$ and $\text{Man}_9\text{GlcNAc}_2$ in the chromatogram of the oligosaccharides released from EGI were observed; some other minor peaks also appeared (Fig. 5A). In the case of the *Yloch1Δ* strain, however, we found a single major peak for $\text{Man}_8\text{GlcNAc}_2$, indicating that the *Yloch1Δ* mutant was defective in its ability to synthesize oligosaccharides larger than $\text{Man}_8\text{GlcNAc}_2$ (Fig. 5A, panels a and c). To further investigate the structures of N-linked oligosaccharides synthesized from the wild-type and *Yloch1Δ* mutant strains, we analyzed the oligosaccharides by treating them with α -1,2- and α -1,6-mannosidases. The $\text{Man}_8\text{GlcNAc}_2$ and $\text{Man}_9\text{GlcNAc}_2$ oligosaccharides generated from the wild-type

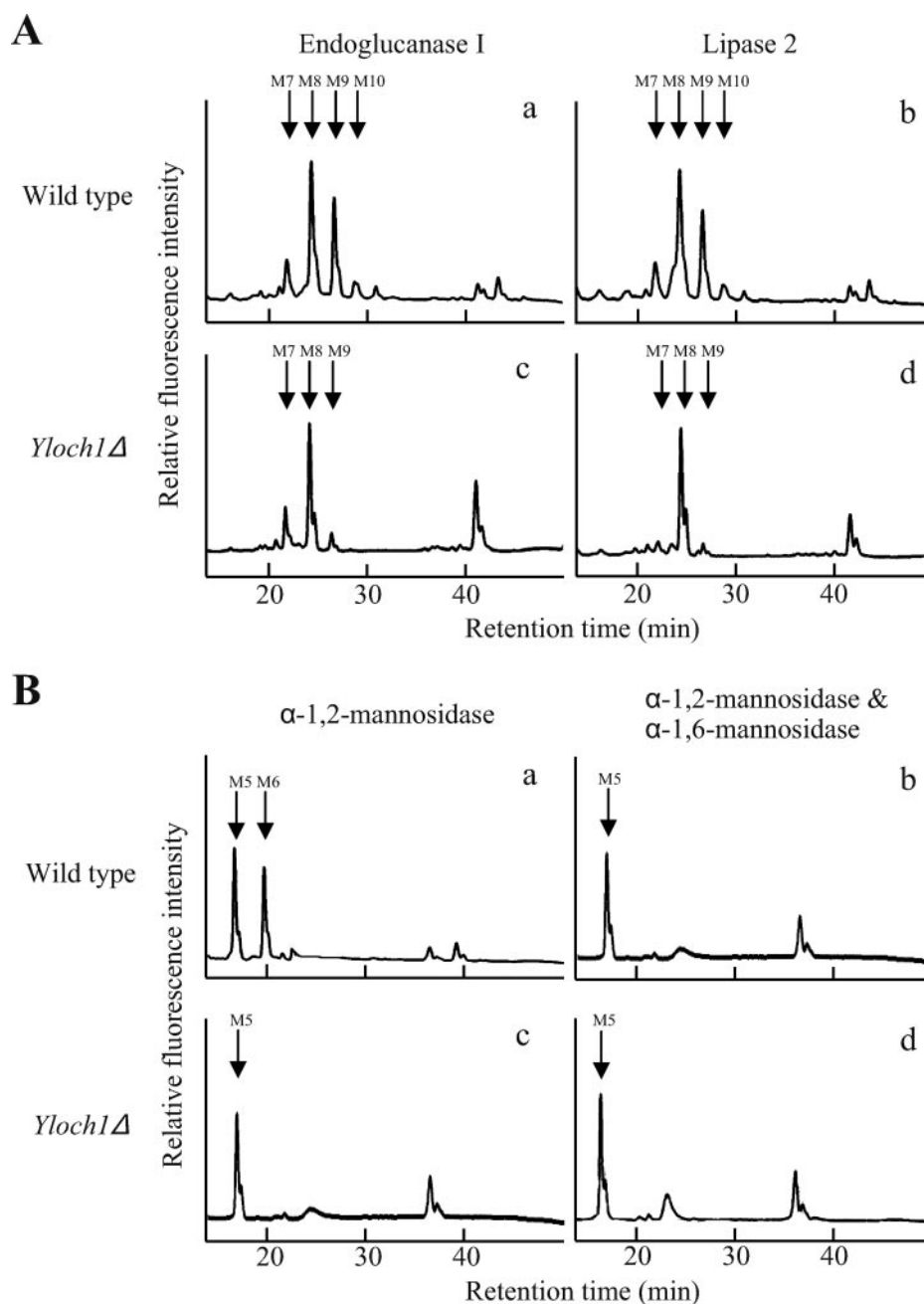
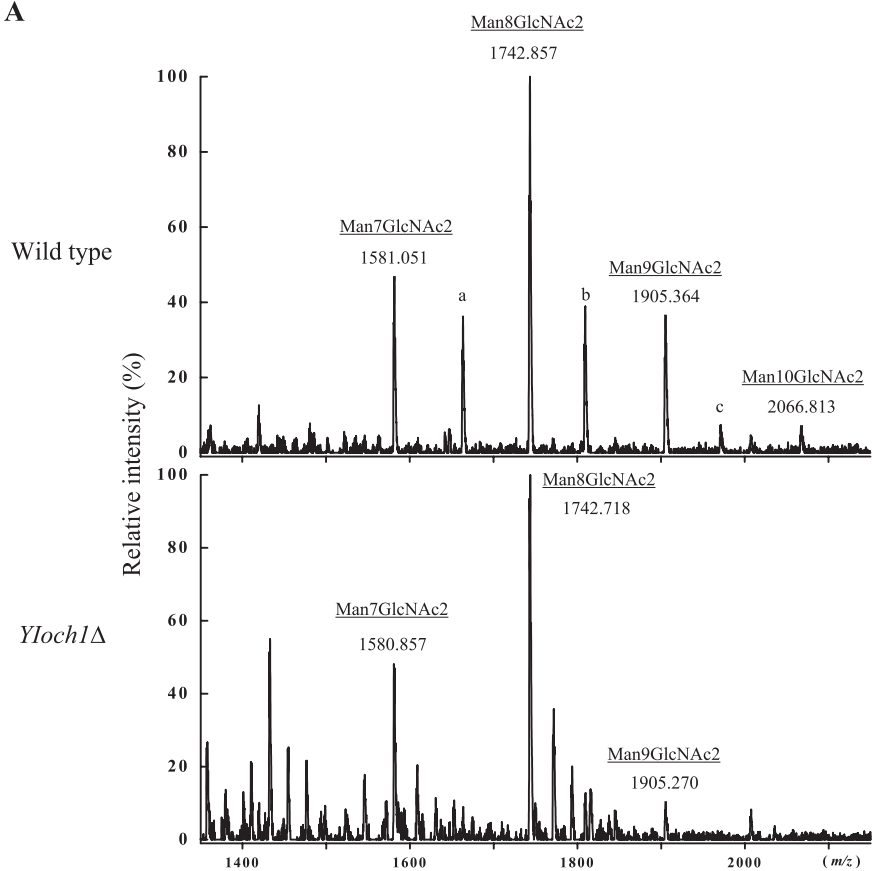
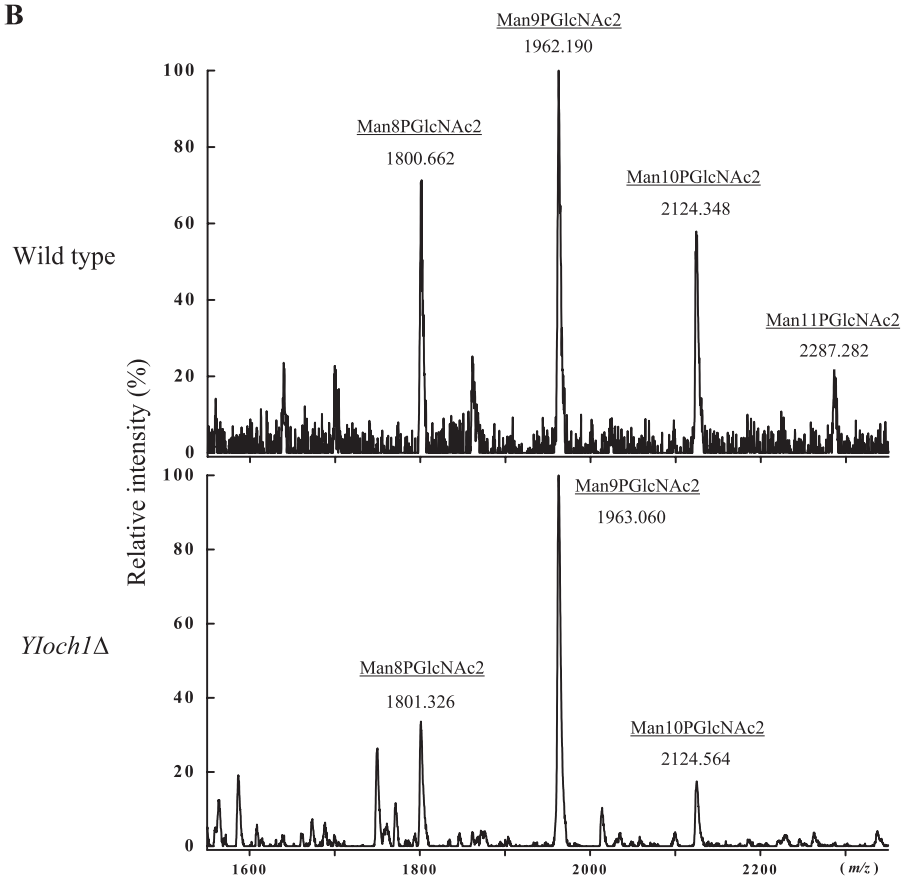


FIG. 5. HPLC analysis of N-linked oligosaccharides assembled on glycoproteins. (A) Chromatograms of the N-linked oligosaccharides released from EGI (a and c) and lipase (b and d) from the wild-type and *Yloch1Δ* strains. (B) Chromatograms of the N-linked oligosaccharides released from EGI secreted by the wild-type (a and b) and *Yloch1Δ* strains (c and d) after treatment with α -1,2-mannosidase and α -1,6-mannosidase. The elution times of authentic PA-sugar chains are indicated by arrows. M5, $\text{Man}_5\text{GlcNAc}_2\text{-PA}$; M6, $\text{Man}_6\text{GlcNAc}_2\text{-PA}$; M7, $\text{Man}_7\text{GlcNAc}_2\text{-PA}$; M8, $\text{Man}_8\text{GlcNAc}_2\text{-PA}$; M9, $\text{Man}_9\text{GlcNAc}_2\text{-PA}$; M10, $\text{Man}_{10}\text{GlcNAc}_2\text{-PA}$.

and *Yloch1Δ* strains were treated with α -1,2-mannosidase from *A. saitoi*. $\text{Man}_8\text{GlcNAc}_2$ was converted completely into $\text{Man}_5\text{GlcNAc}_2$, and $\text{Man}_9\text{GlcNAc}_2$ was converted completely into $\text{Man}_6\text{GlcNAc}_2$ (Fig. 5B, panels a and c). The $\text{Man}_5\text{GlcNAc}_2$ and $\text{Man}_6\text{GlcNAc}_2$ oligosaccharides were subsequently digested with α -1,6-mannosidase from *X. manihotis*, a highly specific exoglycosidase that removes unbranched α -1,6-linked D-mannopyranosyl residues from oligosaccharides. The $\text{Man}_6\text{GlcNAc}_2$ oligosaccharides were converted into

$\text{Man}_5\text{GlcNAc}_2$ (Fig. 5B, panels b and d), which indicates that the $\text{Man}_6\text{GlcNAc}_2$ species has a single unbranched α -1,6-linked mannose. These results demonstrate that the *Yloch1Δ* mutant strain has a defect in terms of the addition of the first α -1,6-mannose residue onto the core oligosaccharide, $\text{Man}_8\text{GlcNAc}_2$.

In order to demonstrate that the function of *YIOCHI* is generally required for the addition of the first α -1,6-mannose residue onto the core oligosaccharide, $\text{Man}_8\text{GlcNAc}_2$, in *Y.*

A**B**

lipolytica, we analyzed the N-linked oligosaccharide structure of an endogenous glycoprotein, lipase (YLip2p), which is a secreted protein with two potential N-linked glycosylation sites (20). The HPLC profiles of the oligosaccharides assembled on the lipases secreted from the wild-type and *Yloch1Δ* strains were the same as those of the oligosaccharides assembled on EGI (Fig. 5A, panels b and d). Therefore, it is clear that *YIOCHI* encodes an enzyme that initiates the outer-chain elongation by adding the first α -1,6-linked mannose to the core oligosaccharide in *Y. lipolytica*.

There were two minor peaks appearing much later than the major peaks in the HPLC profiles of the oligosaccharides from the wild-type strain (Fig. 5). Judging from a comparison of the HPLC profiles with the data from the study by Wang et al. (25), in which the mannosylphosphate transferase activity of *S. cerevisiae* Mnn6p was assayed, we suspected that the two minor peaks might represent phosphate-containing oligosaccharides. To determine whether N-linked oligosaccharides in *Y. lipolytica* are phosphorylated, we analyzed N-glycans derived from the recombinant EGI by using MALDI-TOF mass spectrometry. The glycans were analyzed in the positive reflector mode for the detection of neutral sugars (Fig. 6A) and in the negative linear mode for the detection of acidic sugars (Fig. 6B). The major peaks detected in the negative mode corresponded to molecular masses higher than those of the neutral sugars, which nearly equaled the molecular masses of mannosylphosphorylated $\text{Man}_7\text{GlcNAc}_2$ to $\text{Man}_9\text{GlcNAc}_2$ ($\text{Man}_8\text{PGlcNAc}_2$ to $\text{Man}_{10}\text{PGlcNAc}_2$) species. These results indicate that N-linked oligosaccharides assembled on glycoproteins in *Y. lipolytica* are modified by mannosylphosphate. In addition, the observation that the overall glycan mass spectra are consistent with the HPLC data (Fig. 5A and 6A) confirms that the *Yloch1Δ* mutant is defective in synthesizing oligosaccharides larger than $\text{Man}_8\text{PGlcNAc}_2$.

DISCUSSION

The Och1 protein has been proven to be an initiating α -1,6-mannosyltransferase that plays a key role in the addition of the first mannose to the core oligosaccharide in several yeast species, including *S. cerevisiae*, *P. pastoris*, *Schizosaccharomyces pombe*, and *H. polymorpha* (14, 15, 17, 19, 28). However, *Y. lipolytica* Och1p was suspected to have a minor role in the outer-chain elongation of N glycosylation or to be important only for specific proteins (3). In this study, we reexamined the *Y. lipolytica* *OCHI* gene and analyzed the structure of the N-linked oligosaccharide from the *Yloch1Δ* mutant based on our observation that the recombinant glycoprotein EGI secreted from the *Yloch1Δ* mutant appeared to be less heavily glycosylated than the recombinant EGI from the wild-type strain. Here, we present several lines of strong evidence indicating that *YIOCHI* encodes an α -1,6-mannosyltransferase

that plays an important role in the addition of the first mannose to the core oligosaccharide in *Y. lipolytica*, as in other yeasts. First, the ability of the *YIOCHI* gene to eliminate the defects of the *S. cerevisiae* *och1* mutant, i.e., retarded growth rate and a reduction in the sizes of N-linked oligosaccharides, demonstrated that *YIOCHI* is a functional homolog of *ScOCHI*. Second, the membrane fraction of the *Yloch1Δ* mutant lacked an initiation-specific α -1,6-mannosyltransferase activity. Third, the N-linked oligosaccharides attached to the recombinant EGI and the endogenous lipase secreted from the *Yloch1Δ* mutant cells were composed of a single predominating species, $\text{Man}_8\text{GlcNAc}_2$, and some minor ones, which is consistent with the rapid mobility of the glycoproteins from the *Yloch1Δ* mutant cells. Last, the structural analysis of N-glycans revealed that the N-linked oligosaccharides from *Yloch1Δ* did not contain additional α -1,6-linked mannoses. Taken together, these data provide clear evidence that the major function of *YIOch1p* is to initiate α -1,6-linked mannose elongation of the core oligosaccharide, $\text{Man}_8\text{GlcNAc}_2$. However, we cannot exclude the possible presence of another α -1,6-mannosyltransferase that may play a minor role in the outer-chain elongation of certain glycoproteins in *Y. lipolytica*.

In *S. cerevisiae*, N-linked oligosaccharides are capped with immunogenic α -1,3-linked mannose residues (16, 27), and α -1,2-linked mannoses of N-linked oligosaccharides are not digested with α -1,2-mannosidase if they are attached to α -1,3-linked mannose. However, in *Y. lipolytica*, $\text{Man}_8\text{GlcNAc}_2$ was converted completely into $\text{Man}_5\text{GlcNAc}_2$ upon treatment with α -1,2-mannosidase. This result, along with the finding that no open reading frame that is homologous to *S. cerevisiae* *MNN1* appears in the *Y. lipolytica* genome database (<http://cbi.labri.fr/Genolevures/>), strongly suggests that the core oligosaccharides from *Y. lipolytica* may not be terminally capped by α -1,3-linked mannose residues.

Analysis using MALDI-TOF mass spectrometry suggests that N-linked glycans assembled on secreted glycoproteins from *Y. lipolytica* contain phosphates and that the two minor peaks appearing in the HPLC profiles of the oligosaccharides from the wild-type strain (Fig. 5) may represent mannosylphosphorylated forms of $\text{Man}_7\text{GlcNAc}_2$ to $\text{Man}_9\text{GlcNAc}_2$ (Fig. 6). The absence of the latter of the two minor peaks in the chromatograms corresponding to the oligosaccharides from the *Yloch1Δ* mutant strain and the α -1,6-mannosidase-treated oligosaccharides from the wild-type strain (Fig. 5B) indicates that the difference between the two peaks comes from an additional α -1,6-linked mannose at the latter peak. Therefore, we think that mannosylphosphates are transferred to the core forms of N-glycans in *Y. lipolytica*. Further studies are presently in progress to identify genes involved in the mannosylphosphorylation of N-linked oligosaccharides in *Y. lipolytica*.

Recently, many studies have reported the engineering of

FIG. 6. MALDI-TOF mass spectrometry analysis of N-linked oligosaccharides assembled on EGI. (A) Mass spectra analyzed in the positive reflector mode for the detection of neutral sugars released from the recombinant EGI secreted from the wild-type (top panels) and *Yloch1Δ* (bottom panels) strains. The intermediate peaks for the wild type, designated a, b, and c, are assumed to represent the mannosylphosphorylated forms of $\text{Man}_7\text{GlcNAc}_2$, $\text{Man}_8\text{GlcNAc}_2$, and $\text{Man}_9\text{GlcNAc}_2$, based on their m/z of 1,662.792, 1,808.617, and 1,970.866, respectively. (B) Mass spectra analyzed in the negative reflector mode for the detection of acidic sugars released from the recombinant EGI secreted from the wild-type (top panel) and *Yloch1Δ* (bottom panel) strains. The analyzed glycan samples are free, nonreduced forms without any labeling.

novel yeast strains secreting therapeutic glycoproteins with human-compatible types of N-linked oligosaccharides (10, 14, 24, 26). *Y. lipolytica* is considered to be one of the most suitable strains for heterologous protein expression (12) and can be developed for the production of glycoproteins. In this study, we found that the *Yloch1Δ* mutant strain, despite the complete block of the outer-chain elongation of N-linked oligosaccharides, could grow as well as the wild-type strain under normal growth conditions. This growth property of the *Yloch1Δ* mutant strain will be very useful for the further development of *Y. lipolytica* as a host for the production of glycoproteins. Along with the possibility of a lack of the immunogenic terminal α -1,3-mannose linkages in *Y. lipolytica*, the *Yloch1Δ* mutant strain can be exploited as a platform strain for developing another potential yeast system that can produce recombinant glycoproteins with human-compatible oligosaccharides.

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REFERENCES

- Ballou, C. E. 1990. Isolation, characterization, and properties of *Saccharomyces cerevisiae mnn* mutants with nonconditional protein glycosylation defects. *Methods Enzymol.* **185**:440–470.
- 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. USA* **88**:3209–3212.
- Barnay-Verdier, S., A. Boisrame, and J. M. Beckerich. 2004. Identification and characterization of two α -1,6-mannosyltransferases, An1p and Och1p, in the yeast *Yarrowia lipolytica*. *Microbiology* **150**:2185–2195.
- Barth, G., and C. Gaillardin. 1997. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. *FEMS Microbiol. Rev.* **19**:219–237.
- Cohen, R. E., L. Ballou, and C. E. Ballou. 1980. *Saccharomyces cerevisiae* mannosyltransferase mutants. Isolation of the *mnn5* mutant and comparison with the *mnn3* strain. *J. Biol. Chem.* **255**:7700–7707.
- Dean, N. 1999. Asparagine-linked glycosylation in the yeast Golgi. *Biochim. Biophys. Acta* **1426**:309–322.
- Dean, N. 1995. Yeast glycosylation mutants are sensitive to aminoglycosides. *Proc. Natl. Acad. Sci. USA* **92**:1287–1291.
- Devlin, C., and C. E. Ballou. 1990. Identification and characterization of a gene and protein required for glycosylation in the yeast Golgi. *Mol. Microbiol.* **4**:1993–2001.
- Garcia, R., J. A. Cremata, O. Quintero, R. Montesino, K. Benkestock, and J. Stahlberg. 2001. Characterization of protein glycoforms with N-linked neutral and phosphorylated oligosaccharides: studies on the glycosylation of endoglucanase 1 (Cel7B) from *Trichoderma reesei*. *Biotechnol. Appl. Biochem.* **33**:141–152.
- Gerngross, T. U. 2004. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nat. Biotechnol.* **22**:1409–1414.
- Hwang, J. S., D. H. Seo, and J.-Y. Kim. 2006. Soluble forms of YICrh1p and YICrh2p, cell wall proteins of *Yarrowia lipolytica*, have β -1,3-glycosidase activity. *Yeast* **23**:803–812.
- Juretzek, T., M. Le Dall, S. Mauersberger, C. Gaillardin, G. Barth, and J. Nicaud. 2001. Vectors for gene expression and amplification in the yeast *Yarrowia lipolytica*. *Yeast* **18**:97–113.
- Kim, J. W., T. J. Park, D. D. Ryu, and J.-Y. Kim. 2000. High cell density culture of *Yarrowia lipolytica* using a one-step feeding process. *Biotechnol. Prog.* **16**:657–660.
- Kim, M. W., E. J. Kim, J.-Y. Kim, J. S. Park, D. B. Oh, Y. Shimma, Y. Chiba, Y. Jigami, S. K. Rhee, and H. A. Kang. 2006. Functional characterization of the *Hansenula polymorpha HOC1*, *OCH1*, and *OCR1* genes as members of the yeast *OCH1* mannosyltransferase family involved in protein glycosylation. *J. Biol. Chem.* **281**:6261–6272.
- Murakami, K., and N. Sugio. January 1997. Production of glycoprotein. Japanese patent JP 1997000261-A1.
- Nakajima, T., and C. E. Ballou. 1975. Yeast manno-protein biosynthesis: solubilization and selective assay of four mannosyltransferases. *Proc. Natl. Acad. Sci. USA* **72**:3912–3916.
- Nakanishi-Shindo, Y., K. Nakayama, A. Tanaka, Y. Toda, and Y. Jigami. 1993. Structure of the N-linked oligosaccharides that show the complete loss of α -1,6-polymannose outer chain from *och1*, *och1 mnn1*, and *och1 mnn1 alg3* mutants of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**:26338–26345.
- Nakayama, K., T. Nagasu, Y. Shimma, J. Kuromitsu, and Y. Jigami. 1992. *OCH1* encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides. *EMBO J.* **11**:2511–2519.
- Nakayama, K., Y. Nakanishi-Shindo, A. Tanaka, Y. Haga-Toda, and Y. Jigami. 1997. Substrate specificity of α -1,6-mannosyltransferase that initiates N-linked mannose outer chain elongation in *Saccharomyces cerevisiae*. *FEBS Lett.* **412**:547–550.
- Pignede, G., H. Wang, F. Fudalej, C. Gaillardin, M. Seman, and J. M. Nicaud. 2000. Characterization of an extracellular lipase encoded by *LIP2* in *Yarrowia lipolytica*. *J. Bacteriol.* **182**:2802–2810.
- Pignede, G., H. J. Wang, F. Fudalej, M. Seman, C. Gaillardin, and J. M. Nicaud. 2000. Autocloning and amplification of *LIP2* in *Yarrowia lipolytica*. *Appl. Environ. Microbiol.* **66**:3283–3289.
- 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.
- Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Vervecken, W., V. Kaigorodov, N. Callewaert, S. Geysens, K. De Vusser, and R. Contreras. 2004. In vivo synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*. *Appl. Environ. Microbiol.* **70**:2639–2646.
- 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.
- Wildt, S., and T. U. Gerngross. 2005. The humanization of N-glycosylation pathways in yeast. *Nat. Rev. Microbiol.* **3**:119–128.
- Yip, C. L., S. K. Welch, F. Klebl, T. Gilbert, P. Seidel, F. J. Grant, P. J. O'Hara, and V. L. MacKay. 1994. Cloning and analysis of the *Saccharomyces cerevisiae MNN9* and *MNN1* genes required for complex glycosylation of secreted proteins. *Proc. Natl. Acad. Sci. USA* **91**:2723–2727.
- Yoko-o, T., K. Tsukahara, T. Watanabe, N. Hata-Sugi, K. Yoshimatsu, T. Nagasu, and Y. Jigami. 2001. *Schizosaccharomyces pombe och1(+)* encodes α -1,6-mannosyltransferase that is involved in outer chain elongation of N-linked oligosaccharides. *FEBS Lett.* **489**:75–80.