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NOTE

Functional Analysis of a *Hansenula polymorpha MNN2-2* Homologue Encoding a Putative UDP-*N*-acetylglucosamine Transporter Localized in the Endoplasmic Reticulum

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The *Kluyveromyces lactis* UDP-GlcNAc transporter (KlMnn2-2p) is responsible for the biosynthesis of *N*-glycans containing *N*-acetylglucosamine. A putative gene of *Hansenula polymorpha* encoding a KlMnn2-2p homologue, Hp*MNN2-2*, was identified and investigated for its function. The deletion mutant strain of Hp*MNN2-2* (Hp*mnn2-2Δ*) showed increased sensitivity to geneticin, hygromycin B, and tunicamycin. However, the Hp*mnn2-2*Δ strain exhibited increased resistance to Calcofluor white, an inhibitor of chitin biosynthesis, along with a reduced chitin content. The localization of HpMnn2-2p at the endoplasmic reticulum-enriched membrane, different from the Golgi localization of a *K. lactis* homologue, further supports the involvement of HpMnn2-2p in cell wall chitin biosynthesis.

***Keywords*:** *Hansenula polymorpha*, UDP-GlcNAc transporter, endoplasmic reticulum, chitin

The methylotrophic yeast *Hansenula polymorpha* has been developed as a potential host for production of therapeutic proteins with human-compatible *N*-glycans. This thermotolerant yeast has been successfully engineered to produce glycoproteins homogeneously carrying a high mannose type ($Man_5GlcNAc_2$) or a trimannosyl core ($Man_3GlcNAc_2$) glycans (Kim *et al.*, 2006; Oh *et al.*, 2008). To produce glycoprotein with human-complex type *N*-glycans, the engineered *H. polymorpha* strains are further manipulated by heterologous expression of functional genes required for the addition of *N*-acetylglucosamine (GlcNAc), galactose (Gal), and *N*-acetylneuraminic acid (NANA) at the Golgi apparatus. Monosaccharides such as GlcNAc, Gal, and NANA are activated as a nucleotide-sugar form in the cytoplasm and in the nucleus, and then transported via specific nucleotide-sugar transporters into the luminal side of organelles (Gerardy-Schahn *et al.*, 2001). Whereas most yeast species, including *Saccharomyces cerevisiae* and *Pichia pastoris*, generally produce *N*-glycans composed of only mannose residues (Gemmill and Trimble, 1999), *Kluyveromyces lactis* can add GlcNAc on the outer chain part of its *N*-glycans through the action of *N*-acetylglucosaminyltransferase (KlGnT1) (Abeijon *et al.*, 1996). The transport of GlcNAc to be incorporated into the *K. lactis N*-glycans is specifically mediated via a UDP-GlcNAc transporter (KlMnn2-2p) localized at the Golgi apparatus. More intriguingly, the genes for KlGnT1 and KlMnn2-2p are contiguous in the genome of *K. lactis* (Guillen *et al.*, 1999). It has been recently reported that the heterologously expressed KlMnn2-2p could supply efficient UDP-GlcNAc as a substrate of human GnT1 into the Golgi lumen in the glycoengineered *P. pastoris* (Choi *et al.*, 2003). Although *S. cerevisiae* was also found to contain a homologous gene for UDP-GlcNAc transporter (Sc*YEA4*), the ScYea4p was shown to be mainly related to chitin biosynthesis (Roy *et al.*, 2000).

Our previous study on the structure of *H. polymorpha* glycans indicated the absence of GlcNAc in the outer chain portion of *N*-glycans in *H. polymorpha* (Kim *et al.*, 2004b). Interestingly, however, two open reading frames (ORFs) with high homology to the *K. lactis* genes coding for a UDP-GlcNAc transporter (Kl*MNN2-2*) and a *N*-acetylglucosaminyltransferase (Kl*GNT1*), respectively, were identified from the *H. polymorpha* whole genome database (Ramezani-Rad *et al.*, 2003). Considering *H. polymorpha* cells lack terminal GlcNAc residues in their sugar chains, the presence of such genes in *H. polymorpha* is unexpected. In the present study, we investigated the cellular function of an *H. polymorpha* homologue encoding a putative UDP-GlcNAc transporter, Hp*MNN2-2*, particularly in *N*-glycan modification and cell wall chitin synthesis.

The Hp*MNN2-2* gene product (HpMnn2-2p) showed 36.7% and 34.9% sequence identities to KlMnn2-2p and ScYea4p, respectively (Fig. 1A). The HpMnn2-2p was predicted to contain an N-terminal 18-amino acid signal peptide (http://www.cbs.dtu.dk/services/SignalP/) and six transmembrane helix domains (http://www.cbs.dtu.dk/services/TMHMM/), as generally observed in nucleotide sugar transporters (Kawakita *et al.*, 1998). Interestingly, HpMnn2-2p possesses the C-terminal endoplasmic reticulum (ER) retention signal (KKXX)-like motif (LSKM, http://psort.hgc.jp/form2.html). The nucleotide sequence of the Hp*MNN2-2* gene derived from *H. polymorpha*

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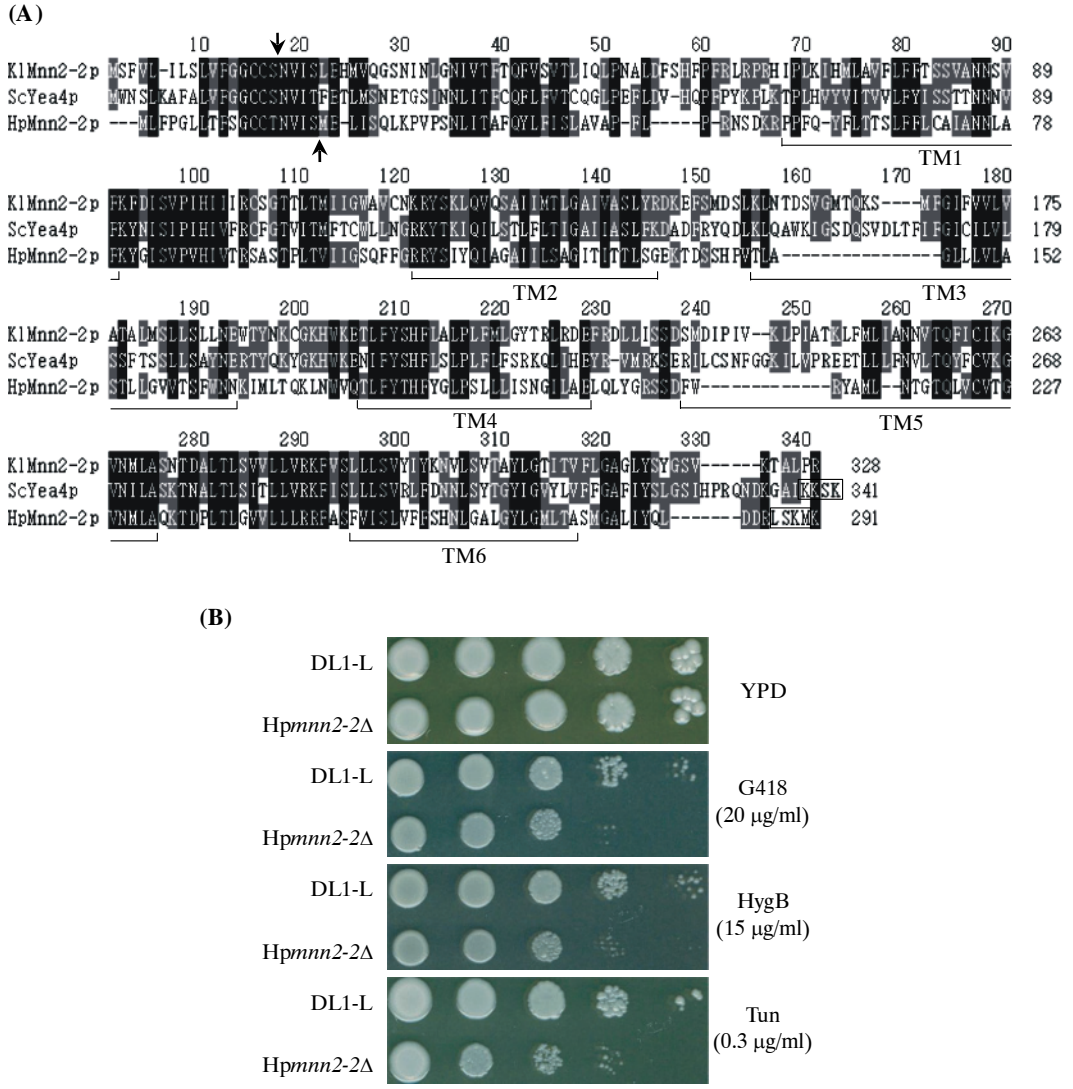

Fig. 1. Sequence alignment of yeast UDP-GlcNAc transporters and growth analysis of a Hp*mnn2-2*Δ mutant strain. (A) The yeast UDP-GlcNAc transporters. KlMnn2-2p, *K. lactis* Mnn2-2p; ScYea4p, *S. cerevisiae* Yea4p; and HpMnn2-2p, *H. polymorpha* Mnn2-2p. Arrows indicate cleavage sites on secretion signal peptides of the proteins, and solid boxes indicate putative ER-retention signal-like sequences. Transmembrane domains (TM1-6) of HpMnn2-2p were described as a bundle. The amino acids were aligned and figured out by using two web-based programs, Multiple Sequence Alignment by CLUSTALW (http://align.genome.jp/) and BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). (B) Drug sensitivity test of the Hp*mnn2-2*Δ strain. The yeast cells were cultured in YPD media at 37°C overnight, transferred into fresh YPD media at initial A_{600}=0.1, and grown up to A_{600}=1. After washing with water, the cells were spotted with 10-fold dilution on each YPD plate containing geneticin (G418), hygromycin B (Hyg B), or tunicamycin (Tun), respectively, and incubated at 37°C for 36 h.

DL1-L strain was submitted to GenBank under accession number FJ226758.

To examine a function of the Hp*MNN2-2* gene, the null mutant strain with the Hp*MNN2-2* gene deletion was constructed in the background of *H. polymorpha* DL1-LdU strain (Table 1) using fusion polymerase chain reaction (PCR) with specific primer sets and *in vivo* recombination, as described previously (Kim *et al.*, 2006). All oligonucleotides used in this study (Table 1) were synthesized by Bioneer Co. (Korea). After cultivation of yeast cells to A_{600}=1, growth phenotypes of the parental and Hp*mnn2-2Δ* strains were compared by spotting on YPD (1% yeast extract, 2% peptone, 2% glucose) media containing geneticin (G418, 20 μg/ml), hygromycin B (15 μg/ml) or tunicamycin (0.3 μg/ml). All of the chemicals were purchased from Sigma-Aldrich (USA). As shown in Fig. 1B, the Hp*mnn2-2*Δ strain became more sensitive to G418, hygromycin B, and tunicamycin compared to the parental strain. It was suggested that the increased sensitivity to aminoglycoside antibiotics such as G418 and hygromycin B was indicative of a change of cell wall permeability, which could be generated by *N*-glycosylation defect of cell wall mannoproteins (Dean, 1995). Also, the increased sensitivity to tunicamycin, an inhibitor of an early step in *N*-glycosylation, might reflect the defect of Hp*mnn2-2Δ* in *N*-glycosylation

Table 1. Strains and oligonucleotides used in this study

Strain	Genotype	Reference
DL1-L	*leu2*	(Kim *et al.*, 2006)
DL1-LdU	*leu2 ura3*	(Kim *et al.*, 2006)
A16	*leu2*	(Kim *et al.*, 2004a)
NCYC495	*leu1-1*	(Park *et al.*, 2007)
Hp*mnn2-2*Δ	*leu2 ura3 mnn2-2::lacZ-URA3-lacZ*	This study
Hp*ocr1*Δ	*leu2 ura3 ocr1::lacZ-URA3-lacZ*	(Kim *et al.*, 2006)
Name	**Sequence**	**Reference**
HpMNN2-2_NF	ATGCTTTTTCCGGGGCTACTC	This study
HpMNN2-2_NR	ACGGCCAGTCACAGTCAACGGTGTCGATGC	This study
HpMNN2-2_ CF	CTTTCGCCACTGCTAATTAGCAACGGCATAC	This study
HpMNN2-2_CR	TTATTTCATTTTACTTAGTCTACGTC	This study
HpMNN2-2 NC_F	CGCCTCGGGCACAGCTTTCAGGAG	This study
HpMNN2-2_NC_R	CTTCGGAGAGTGTGGTTATAGTTA	This study
HpMNN2-2_CC_F	TGTTGTCACCTCGTTTTGGAATAAC	This study
HpMNN2-2_CC_R	GGGCGAAAGCGGGTTTGGCAGAGA	This study
HpURA3_NF	GCATCGACACCGTTGACTGTGACTGGCCGTCGTTTTACAACGT	This study
HpURA3_NR	TCTCTCTGTGCAATAAATCCAATG	This study
HpURA3_CF	GCACATGGAGTGACTGGCGCA	This study
HpURA3_CR	GTATGCCGTTGCTAATTAGCAGTGGCGAAAGGGGGAGGCTGCA	This study
HpMNN2-2_F	TGAC**AGATCTAAGCTT**ATGCTTTTTCCGGGGCTACT	This study
HpMNN2-2(ΔTAA)_R	TGAC**AGATCT**TTTCATTTTACTTAGTCTATCGT	This study
3x HA_F	ATGC**AGATCT**TACCCATACGATGTTCCTG	This study
3x HA_R	ATGC**AAGCTT**TTAGGCGGCCGGAGCGTAAT	This study
yEGFP(ΔATG)_F	GATC**AGATCT**TCTAAAGGTGAAGAATTATTCA	This study
yEGFP_R	GATC**AAGCTT**TTATTTGTACAATTCATCCATAC	This study
KlMNN2-2_F	TGAC**AGATCTAAGCTT**ATGAGTTTTGTATTGATTTTGTCGTT	This study
KlMNN2-2(ΔTGA)_R	TGAC**AGATCT**GCGAGGCAGTGCAGTTTTGACCG	This study

Bold characters represent recognition site(s) for restriction enzyme (AAGCTT for HindIII, AGATCT for BglII).
Underlines represent complementary sequences for fusion-PCR primer binding.

(Herscovics and Orlean, 1993). However, the *N*-glycosylation profiles of total cell wall mannoproteins from the wild type and Hp*mnn2-2*Δ strains were almost identical (data not shown), indicating that the function of Hp*MNN2-2* was not involved in *N*-glycosylation of cell wall components.

It has been shown that, whereas a UDP-GlcNAc transporter associated with *N*-glycosylation, such as KlMnn2-2p, is located on the Golgi membrane, a UDP-GlcNAc transporter involved in chitin biosynthesis, such as ScYea4p, is located on the ER membranes (Abeijon *et al.*, 1996; Roy *et al.*, 2000). To identify intracellular localization of HpMnn2-2p in *H. polymorpha*, the Hp*MNN2-2* gene tagged with triple hemagglutinin (HA_3) of influenza virus or fused with the *yEGFP* gene was expressed under the control of the methanol-induced *MOX* promoter and analyzed by western blotting or fluorescence microscopy. The DNA fragments for HA_3 or Hp*MNN2-2* without a stop codon (ΔTAA) were amplified using primer sets 3xHA_F/3xHA_R or HpMNN2-2_F/HpMNN2-2(ΔTAA)_R (Table 1) and cloned into pDrive PCR cloning vector (QIAGEN, Germany), generating pDrive-HA_3 or pDrive-HpMNN2-2(ΔTAA), respectively. Subsequently, the BglII-digested Hp*MNN2-2*(ΔTAA) was inserted into the BglII site in pDrive-HA_3, resulting in pDrive-HpMNN2-2-HA_3. Then, the HindIII-digested HpMNN2-2-HA_3 was inserted into the HindIII site of the pMOX-GFP1 (Park *et al.*, 2007) to replace the *yEGFP* gene fragment, generating pHpMNN2-2-HA_3. To make the chimeric Hp*MNN2-2* gene fused with the *yEGFP* gene at its 3'-end, a DNA fragment of *yEGFP* without a start codon was amplified by the primer pair (yEGFP(ΔATG)_F/yEGFP_R) (Table 1) using pMOX-GFP1 as a template and subcloned into pDrive, resulting in pDGFP. The BglII-digested Hp*MNN2-2* (ΔTAA) was inserted into the BglII site of pDGFP, making pDHMGFP. Finally, the HindIII-digested chimeric gene from pDHMGFP was ligated with the HindIII-digested pMOX-GFP1, resulting in pHpMNN2-2_yEGFP. The Kl*MNN2-2* gene was amplified by the KlMNN2-2_F/KlMNN2-2(ΔTGA)_R primer set (Table 1) from the genomic DNA of *K. lactis* KCTC7153 strain (KCTC, Korea). The pKlMNN2-2-HA_3 expression plasmid was constructed with the same procedure described in construction of the pHpMNN2-2-HA_3. Recombinant strains harboring each expression plasmid were pre-cultured in YPD medium and transferred into YPM (1% yeast extract, 2% peptone, and 2% methanol) medium. Protein samples prepared from the ER and Golgi-enriched membrane fractions, as described in the legend of Fig. 2, were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then subjected to immunoblot analysis using rat anti-hemagglutinin (HA) antibody (1:500, Sigma) or rabbit anti-green fluorescent protein (GFP) antibody (1:1,000, Invitrogen, USA).

As shown in Fig. 2A, the HpMnn2-2-HA_3 or HpMnn2-2-yEGFP protein was mainly detected in the ER-enriched fraction (P2) like HpDer1p, a *H. polymorpha* homologue to the

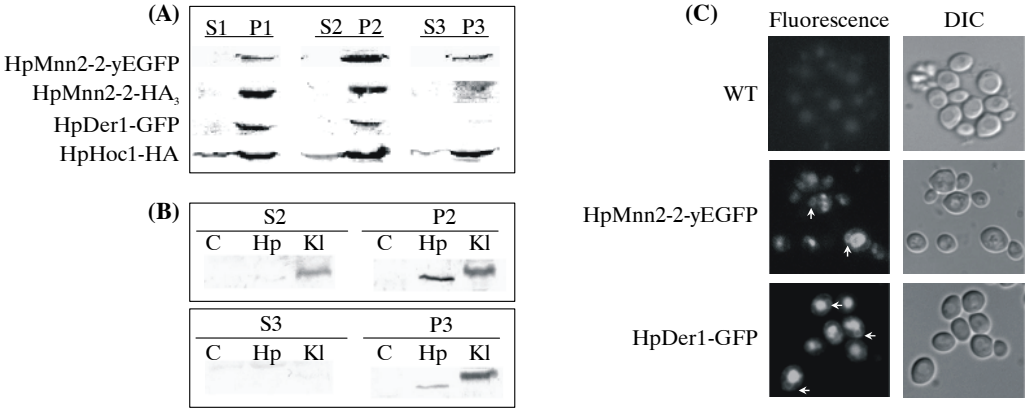

Fig. 2. Cellular localization of HpMnn2-2p. (A) Western blotting with anti-HA or anti-GFP antibodies. S and P stand for the soluble and pellet fractions including organelle membranes. HpDer1-GFP and HpHoc1-HA are marker proteins for ER and Golgi membranes, respectively. To fractionate ER- and Golgi-enriched membranes, cells grown in YPM media for 36-48 h were suspended with solution A containing 50 mM potassium phosphate (pH 7.5), 1.4 M sorbitol, 10 mM NaN_3, Zymolyase® T-100 (*Arthrobacter luteus,* 1 mg/g wet cell weight, AMS Biotechnology, UK), and 40 mM β-mercaptoethanol. After incubation at 37°C for 40 min, the spheroplasts were collected, resuspended in lysis solution, composed of 0.8 M sorbitol, 10 mM triethanolamine (pH 7.2), protease inhibitor cocktail (2 μl/ml, Sigma), and lysed by Dounce homogenizer (Wheaton Scientific, USA). Crude protein extracts (S1) of yeasts were prepared by centrifugation at 3,000×*g* for 10 min from lytic mixture of cells. The ER-enriched membrane fraction (P2) was prepared by centrifugation of S1 for 15 min at 10,000×*g* (Optima™ TLX, Beckman Coulter, USA), and the supernatant (S2) was centrifuged at 100,000×*g* for 60 min, yielding the Golgi-enriched membrane fraction (P3). (B) Western blotting of two yeast UDP-GlcNAc transporters with anti-HA antibodies. P2 and P3 stand for the ER- and Golgi-enriched membrane fractions, respectively. C, fractions from HpDL1-L; Hp, fractions from the recombinant strain expressing the HpMnn2-2p-HA_3; Kl, fractions from the recombinant strain expressing the KlMnn2-2p-HA_3. (C) Fluorescent micrograph of *H. polymorpha* cells. Cells were cultured in YPM media for 36 h and then analyzed by an Olympus BX-FLA fluorescence microscope (Olympus, Japan). The images were captured with a MicroMAX CCD camera system (Roper, USA).

well-known ER marker ScDer1p (Knop *et al.*, 1996). In contrast, the KlMnn2-2-HA_3 protein heterologously expressed in *H. polymorpha* was localized in the Golgi membrane fraction (Fig. 2B). In addition, the fluorescence image of the HpMnn2-2-yEGFP fusion protein showed a similar pattern to that of the ER-membrane protein HpDer1p (Fig. 2C), although some portion of HpMnn2-2-yEGFP protein was mislocalized into a vacuole due to overexpression by the strong *MOX* promoter. Therefore, different from KlMnn2-2p in *K. lactis*, it appears that HpMnn2-2p is localized in the ER-membrane in *H. polymorpha*, which is consistent with the presence of ER retention motif at its C-terminal.

It was reported that the Sc*yea4Δ* strain exhibited an increased resistance to Calcofluor white (CFW) and had a reduced content of chitin (~65%) compared to the wild type (Roy *et al.*, 2000). Chitin is a polymer consisting of β-1,4-linked *N*-acetylglucosamines and composed of a minor portion (1-2%) of cell wall in *S. cerevisiae*, but it is important for cell survival (Klis *et al.*, 2002). The lower inhibitory effect of CFW was attributed to the lower amount of chitin in the Sc*yea4*Δ mutant. As a result, we examined the sensitivity to CFW (Fluorescent Brightener 28, 200 μg/ml) and the chitin level of the Hp*mnn2-2*Δ strain. The *H. polymorpha* mutant strain exhibited more resistance to growth inhibition by CFW than the parental strain, as observed in the Sc*yea4*Δ strain (Fig. 3A). Furthermore, the chitin level of the Hp*mnn2-2*Δ strain was partially decreased (~10%), as expected from the increased resistance to CFW (Fig 3B). For chitin measurement, yeast cells at mid-log phase (200-300 mg wet cell weight) were incubated in 2 ml 6% potassium hydroxide (KOH) with occasional mixing for 90 min at 80°C to remove the mannan layer of the cell wall. The insoluble pellets were obtained by mixing with 100 μl glacial acetic acid, and resuspended in 1 ml 50 mM sodium phosphate buffer (pH 6.0). After addition of 10 μl *Streptomyces griseus* chitinase (10 mU/μl, Sigma) to remove chitobiose units from chitin, the reactant was incubated with mixing for 6 h at 37°C and then subsequently was treated with jack bean β-*N*-acetylglucosaminidase (0.2 UN, Sigma) overnight at 25°C, which cleaves chitobiose to its monomer *N*-acetylglucosamine. The amounts of chitin (as *N*-acetylglucosamine equivalents) were determined by a colorimetric method (Reissig *et al.*, 1955), with slight modification as described in the legend of Fig. 3B.

Several *H. polymorpha* strains, such as A16, NCYC495, and Hp*ocr1Δ* strains (Table 1), were also tested to validate the relationship between the sensitivity to CFW and the level of chitins. The Hp*ocr1*Δ strain is a null mutant strain with deletion of the Hp*OCR1* gene, which is involved in both *N*- and *O*-glycosylation of secretory proteins (Kim *et al.*, 2006). The defect in glycosylation would generate a defect of cell wall integrity, which would trigger a compensation response to increase the level of chitins by activation of chitinase synthase III (Bulik *et al.*, 2003). As expected, the Hp*ocr1Δ* strain displaying the most sensitivity to CFW among the tested strains showed the highest level of chitin, while the *H. polymorpha* NCYC495 strain exhibiting the most resistance to CFW showed the lowest level of chitin, supporting the idea that the increased resistance of the Hp*mnn2-2*Δ strain to CFW might result from loss of transporter function due to the decreased level of chitin, probably due to reduction of UDP-GlcNAc

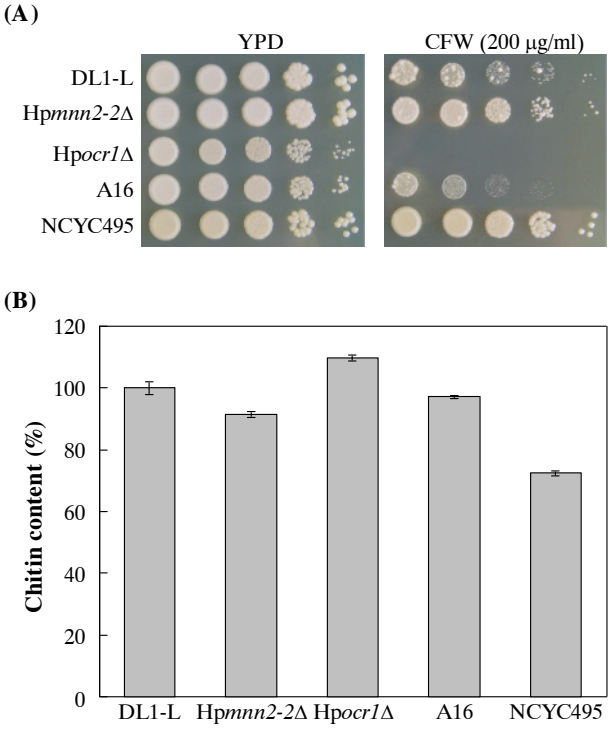

Fig. 3. Involvement of Hp*MNN2-2* in chitin biosynthesis. (A) Sensitivity test of several *H. polymorpha* strains to CFW. The cells were cultured in YPD media at initial A_{600}=0.1 and grown until they reached A_{600}=1. After washing with water, the cells were spotted with 10-fold dilution on each plate and incubated at 37°C for 40 h. (B) Comparison of chitin contents from several *H. polymorpha* strains. The amounts of chitin (as *N*-acetylglucosamine equivalents) were determined by a slightly modified colorimetric method (Reissig *et al.*, 1955). After chitinase and β-*N*-acetylglucosaminidase treatment, the cell wall sample was fractioned by centrifugation at 12,000 rpm for 2 min, and 20 μl of the supernatant was mixed with 10 μl solution A (0.8 M potassium tetraborate, pH 9.2). The mixture was boiled for 3 min, and 170 μl solution B [0.5% (w/v) *p*-dimetylaminobenzaldehyde (DMAB), 1.3% (v/v) concentrated HCl, and 100% (v/v) glacial acetic acid] was added. After incubation for 20 min at 37°C, the mixture was measured by VersaMax^{TM} tunable microplate reader (Molecular Devices, USA) at A_{585}. The levels of chitins were normalized to the wet weight of cells, and the standard deviation is depicted as an error bar.

supply as a substrate. The decrease of chitin content in the Hp*mnn2-2*Δ strain was relatively lower than that of the Sc*yea4*Δ strain, which might reflect the intrinsic low level of chitins (0.20 μg/mg wet cell weight) in *H. polymorpha* wild type stains compared to the chitin content (0.72-1.3 μg/mg wet cells) in *S. cerevisiae* wild type (Bulawa, 1992; Roy *et al.*, 2000).

N-Acetylglucosamines can be used as a building block for biosynthesis of *N*-glycan, cell wall chitin, and a glycosylphosphatidylinositol anchor in yeast and fungal species (Milewski *et al.*, 2006). Our data in the present study suggest the involvement of the UDP-GlcNAc transporter encoded by Hp*MNN2-2* in cell wall chitin biosynthesis. However, until now, there has been no evidence of a direct relationship between ER-localized UDP-GlcNAc transporter and cell wall chitin biosynthesis, because chitin biosynthesis is mediated by a chitin synthase complex using cytoplasmic UDP-GlcNAc as a substrate in fungal plasma membrane (Bowman and Free, 2006). Castro *et al.* (1999) suggested that the ER-localized UDP-glucose transporter may be involved in cell wall β-1,6-glucan synthesis in *S. cerevisiae*, and Roy *et al.* (2000) discussed a possibility that ScYea4p may interact with other chitin-related component(s). Recently, Sesma *et al.* (2009) reported that Sc*YEA4* mutant strain showed a reduced release of extracellular UDP-GlcNAc, suggesting that the cytosolic UDP-GlcNAc transports into the ER lumen by ScYea4p and luminal UDP-GlcNAc releases through a secretory pathway. Our recent microarray data of transcriptome analysis of treatment with dithiothreitol, a cell wall stress-induced reagent, showed upregulation (~2.8 fold) of the Hp*MNN2-2* transcript (unpublished data), proposing the possibility that the function of HpMnn2-2p is also involved in the transport of UDP-GlcNAc to the plasma membrane through a secretory pathway to provide a substrate for chitin synthase(s) in *H. polymorpha*, as suggested in *S. cerevisiae*. Further biochemical study is in progress to provide more direct evidence supporting the proposed function of HpMnn2-2p as a transporter for chitin biosynthesis.

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References

Abeijon, C., P.W. Robbins, and C.B. Hirschberg. 1996. Molecular cloning of the Golgi apparatus uridine diphosphate-*N*-acetylglucosamine transporter from *Kluyveromyces lactis*. *Proc. Natl. Acad. Sci. USA* 93, 5963-5968.

Bowman, S.M. and S.J. Free. 2006. The structure and synthesis of the fungal cell wall. *BioEssays* 28, 799-808.

Bulawa, C.E. 1992. *CSD2*, *CSD3*, and *CSD4*, genes required for chitin synthesis in *Saccharomyces cerevisiae*: the *CSD2* gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. *Mol. Cell. Biol.* 12, 1764-1776.

Bulik, D.A., M. Olczak, H.A. Lucero, B.C. Osmond, P.W. Robbins, and C.A. Specht. 2003. Chitin synthesis in *Saccharomyces cerevisiae* in response to supplementation of growth medium with glucosamine and cell wall stress. *Eukaryot. Cell* 2, 886-900.

Castro, O., L.Y. Chen, A.J. Parodi, and C. Abeijón. 1999. Uridine diphosphate-glucose transport into the endoplasmic reticulum of *Saccharomyces cerevisiae:* in vivo and in vitro evidence. *Mol. Biol. Cell* 10, 1019-1030.

Choi, B.-K., P. Bobrowicz, R.C. Davidson, S.R. Hamilton, D.H. Kung, H. Li, R.G. Miele, J.H. Nett, S. Wildt, and T.U. Gerngress. 2003. Use of combinatorial genetic libraries to humanize *N*-linked glycosylation in the yeast *Pichia pastoris*. *Proc. Natl. Acad. Sci. USA* 100, 5022-5027.

Dean, N. 1995. Yeast glycosylation mutants are sensitive to aminoglycosides. *Proc. Natl. Acad. Sci. USA* 92, 1287-1291.

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.

Gerardy-Schahn, R., S. Oelmann, and H. Bakker. 2001. Nucleotide sugar transporters: biological and functional aspects. *Biochimie* 83, 775-782.

Guillen, E., C. Abeijon, and C.B. Hirschberg. 1999. The genes for the Golgi apparatus *N*-acetylglucosaminyltransferase and the UDP-*N*-acetylglucosamine transporter are contiguous in *Kluyveromyces*

lactis. *J. Biol. Chem.* 274, 6641-6646.

Herscovics, A. and P. Orlean. 1993. Glycoprotein biosynthesis in yeast. *FASEB J.* 7, 540-550.

Kawakita, M., N. Ishida, N. Miura, G.-H. Sun-Wada, and S. Yoshioka. 1998. Nucleotide sugar transporters: elucidation of their molecular identity and its implication for future studies. *J. Biochem.* 123, 777-785.

Kim, Y.-H., K.Y. Han, K. Lee, J.-H. Heo, H.A. Kang, and J. Lee. 2004a. Comparative proteome analysis of *Hansenula polymorpha* DL1 and A16. *Proteomics* 4, 2005-2013.

Kim, M.W., E.J. Kim, J.-Y. Kim, J.-S. Park, D.-B. Oh, Y.-I. 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.

Kim, M.W., S.K. Rhee, J.-Y. Kim, Y. Shimma, Y. Chiba, Y. Jigami, and H.A. Kang. 2004b. 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.

Klis, F.M., P. Mol, K. Hellingwerf, and S. Brul. 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 26, 239-256.

Knop, M., A. Finger, T. Braun, K. Hellmuth, and D.H. Wolf. 1996. Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *EMBO J.* 15, 753-763.

Milewski, S., I. Gabriel, and J. Olchowy. 2006. Enzymes of UDP-GlcNAc biosynthesis in yeast. *Yeast* 23, 1-14.

Oh, D.-B., J.-S. Park, M.W. Kim, S.A. Cheon, E.J. Kim, H.Y. Moon, O. Kwon, S.K. Rhee, and H.A. Kang. 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.

Park, J.-N., M.J. Sohn, D.-B. Oh, O. Kwon, S.K. Rhee, C.-G. Hur, S.Y. Lee, G. Gellissen, and H.A. Kang. 2007. Identification from transcriptome analysis and application to whole-cell heavy metal detection systems of the cadmium-inducible *Hansenula polymorpha SEO1* gene promoter. *Appl. Environ. Microbiol.* 73, 5990-6000.

Ramezani-Rad, M., C.P. Hollenberg, J. Lauber, H. Wedler, E. Griess, C. Wanger, K. Albermann, J. Hani, M. Piontek, U. Dahlems, and *et al*. 2003. The *Hansenula polymorpha* (strain CBS4732) genome sequencing and analysis. *FEMS Yeast Res.* 4, 207-215.

Reissig, J.L., J.L. Strominger, and L.F. Leloir. 1955. A modified colorimetric method for the estimation of *N*-acetylamino sugars. *J. Biol. Chem.* 217, 959-966.

Roy, S.K., Y. Chiba, M. Takeuchi, and Y. Jigami. 2000. Characterization of yeast Yea4p, a uridine diphosphate-*N*-acetylglucosamine transporter localized in the endoplasmic reticulum and required for chitin synthesis. *J. Biol. Chem.* 275, 13580-13587.

Sesma, J.I., C.R. Esther, Jr., S.M. Kreda, L. Jones, W. O'Neal, S. Nishihara, R.A. Nicholas, and E.R. Lazarowski. 2009. Endoplasmic reticulum/Golgi nucleotide sugar transporters contribute to the cellular release of UDP-sugar signaling molecules. *J. Biol. Chem.* 284, 12572-12583.