Identification and characterization of two \(\alpha\)-1,6-mannosyltransferases, Anl1p and Och1p, in the yeast \textit{Yarrowia lipolytica}

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In this study, the identification and characterization of the \textit{Yarrowia lipolytica} homologues of \textit{Saccharomyces cerevisiae} \(\alpha\)-1,6-mannosyltransferases Anp1p and Och1p, designated YlAnl1p and YlOch1p, are described. In order to confirm the function of the \textit{Y. lipolytica} proteins, including the previously isolated YLMnn9p, in the \(N\)-glycosylation pathway, a phenotypic analysis of the disrupted strains \(\Delta YLMnn9\), \(\Delta Ylan1\), \(\Delta Ylch1\), \(\Delta Ylan1\Delta Ylmnn9\) and \(\Delta Ylmnn9\Delta Ylch1\) was performed. Disruption of the \(YLMNN9\), \(YIANL1\) and \(YIOCH1\) genes caused an increased sensitivity to SDS, compatible with a glycosylation defect, and to Calcofluor White, characteristic of cell-wall defects. Moreover, Western-blot analysis of a heterologous glycosylated protein confirmed a direct role of \(YLMnn9p\) and \(Ylan1p\) in the \(N\)-glycosylation process. These mutant strains, \(\Delta Ylmnn9\), \(\Delta Ylan1\), \(\Delta Ylch1\), \(\Delta Ylan1\Delta Ylmnn9\) and \(\Delta Ylmnn9\Delta Ylch1\) may thus be used to establish a model for the \textit{Y. lipolytica} \(N\)-linked glycosylation pathway.

INTRODUCTION

In eukaryotic cells, transmembrane and secreted proteins undergo several modifications during their maturation. \(N\)-Glycosylation is one of these modifications, and contributes to the functional conformation and the final destination of these proteins (Parodi, 2000). The process begins in the endoplasmic reticulum (ER) lumen: a core oligosaccharide composed of 14-residue Glc3Man9GlcNAc2 is transferred to the asparagine of the consensus sequence Asn–X–Ser/Thr by the M-Pol II complex. The M-Pol II complex consists of five proteins, of which two \(\alpha\)-1,6-mannosyltransferases, Anp1p and Och1p, designated \(YIANL1\) and \(YIOCH1\), are described. In order to confirm the function of the \textit{Y. lipolytica} proteins, including the previously isolated YLMnn9p, in the \(N\)-glycosylation pathway, a phenotypic analysis of the disrupted strains \(\Delta YLMnn9\), \(\Delta Ylan1\), \(\Delta Ylch1\), \(\Delta Ylan1\Delta Ylmnn9\) and \(\Delta Ylmnn9\Delta Ylch1\) was performed. Disruption of the \(YLMNN9\), \(YIANL1\) and \(YIOCH1\) genes caused an increased sensitivity to SDS, compatible with a glycosylation defect, and to Calcofluor White, characteristic of cell-wall defects. Moreover, Western-blot analysis of a heterologous glycosylated protein confirmed a direct role of \(YLMnn9p\) and \(Ylan1p\) in the \(N\)-glycosylation process. These mutant strains, \(\Delta Ylmnn9\), \(\Delta Ylan1\), \(\Delta Ylch1\), \(\Delta Ylan1\Delta Ylmnn9\) and \(\Delta Ylmnn9\Delta Ylch1\) may thus be used to establish a model for the \textit{Y. lipolytica} \(N\)-linked glycosylation pathway.

Indeed, in the Golgi, the extension of the oligosaccharide chain involves many specific glycosyltransferases. This specificity generates the observed diversity of glycan structures between different species and cell types (Drickamer & Taylor, 1998). In the yeast \textit{Saccharomyces cerevisiae}, the glycosyltransferases are mannosyltransferases which lead to the formation of two main types of mannan outer chain. Many proteins of the cell wall and periplasm receive a large mannan structure that contains a long \(\alpha\)-1,6-linked backbone of about 50 mannoses with short \(\alpha\)-1,2 and \(\alpha\)-1,3 side chains. In contrast, the proteins of the internal organelles display a smaller core-type structure, with only a few mannoses (Munro, 2001).

The structure of the mannan outer chain has been investigated by the study of \(mnn\) (mannan defective) mutants isolated by Ballou and co-workers (Ballou, 1982, 1990). The analysis of the partial \(N\)-glycan structures in these mutants has allowed the ordering of the steps of mannan synthesis. Studies of other glycosylation mutants (\(van\), \(och\), \(vrg\)) have shown that mutants severely affected in mannan outer-chain extension have additional phenotypes: enhanced hygromycin B sensitivity and sodium orthovanadate resistance (Ballou \textit{et al.}, 1991; Kanik-Ennulat \textit{et al.}, 1995). The degree of sensitivity or resistance is correlated with the severity of the glycosylation defect in these mutants. Underglycosylated cells are not affected in protein secretion.

The recent work of Munro suggests a model for the complete \(N\)-glycosylation pathway in \textit{S. cerevisiae} (Munro, 2001; Stolz & Munro, 2002). In this model, the formation of the mannan outer chain is initiated by the Och1p protein, a type II membrane \(\alpha\)-1,6-mannosyltransferase that defines an early Golgi compartment (Nakanishi-Shindo \textit{et al.}, 1993; Romero \textit{et al.}, 1994; Nakayama \textit{et al.}, 1997). Upon arrival in the Golgi, Och1p adds a single \(\alpha\)-1,6-mannose to all \(N\)-glycan cores. The formation of the long \(\alpha\)-1,6-linked backbone is generated by two enzyme complexes. The M-Pol I complex contains two \(\alpha\)-1,6-mannosyltransferases, Mnn9p and Van1p. It is responsible for the first committed step in the generation of the mannan structure by adding about ten mannoses to the nascent \(\alpha\)-1,6-linked chain. The M-Pol II complex consists of five proteins, of which two
$\alpha$-1,6-mannosyltransferases, Mnn9p and Anp1p, are involved in the last step of the extension of the long $\alpha$-1,6-linked backbone by adding a large number of mannoses (about 50). The side chains are then completed by the action of two $\alpha$-1,2-mannosyltransferases, Mnn2p and Mnn5p, and a single $\alpha$-1,3-mannosyltransferase, Mnn1p. In contrast, formation of the small core-type N-glycan involves only Och1p, one unknown $\alpha$-2-mannosyltransferase and Mnn1p. MNN9 (Yip et al., 1994), ANP1 (Chapman & Munro, 1994) and VAN1 (Kanik-Ennulat et al., 1995) are three members of a gene family. They encode $\alpha$-1,6-mannosyltransferases co-localized within the cis Golgi compartment (Jungmann & Munro, 1998). Null alleles of the three genes are viable, and the strains mutated in mnn9 show the most severe underglycosylation and osmotic fragility. The anp1 mutants are the least severely affected.

We wished to investigate this specific part of the secretory pathway in the yeast \textit{Yarrowia lipolytica}. The non-conventional yeast \textit{Y. lipolytica} is a good alternative model organism for fundamental and applied studies (Barth & Gaillardin, 1996, 1997). It has a secretion process closer to that of mammals than does \textit{S. cerevisiae} (Beckerich et al., 1998; Boisramé et al., 1998) and it is a better host for the expression of heterologous secreted proteins in an active form (Madzak et al., 2000). At the beginning of this project, only one \textit{Y. lipolytica} N-glycosylation pathway gene sequence, a homologue of the \textit{S. cerevisiae} MNN9 gene, was available in the public databases. Additionally, the N-glycan structure of \textit{Y. lipolytica} was not as yet determined. To perform a functional analysis of the Golgi glycosylation pathway in \textit{Y. lipolytica}, we characterized gene sequences encoding homologues of proteins known to be involved in this pathway in \textit{S. cerevisiae}, such as mannosyltransferases. The objective of the work presented in this paper was to confirm the function of the encoded proteins in \textit{Y. lipolytica} by the construction and analysis of the glycosylation mutants. Our aim is (i) to establish a model for the glycosylation pathway, and (ii) to be able to modify it in order to permit production and secretion of mammalian-type proteins in \textit{Y. lipolytica}.

**METHODS**

**Strains, plasmids and media.** \textit{Escherichia coli} strain DH5x was used for transformation, plasmid amplification and preparation. It was grown on Luria–Bertani (LB) medium, supplemented with 50 $\mu$g ampicillin ml$^{-1}$ when necessary, at 37 °C.

All disrupted strains of \textit{Y. lipolytica} presented in this paper were constructed in the strain INAG136463 (MatB, scrI::ADE1, SCR2, his-1, leu-2, ura3). Yeast strains were grown on rich YPD medium, with 0.5 M sorbitol when necessary, or on minimal YNB medium supplemented with the appropriate amino acids plus proline as nitrogen source, at 28 °C. When required, 1-25 mg ml$^{-1}$ 5'-fluoroorotic acid was added to solid media for selection of \textit{ura} strains.

The pGAA plasmid containing the \textit{Arxula adeninivorans} glucosamylase (GAA) coding sequence under the control of the \textit{hp4d} hybrid promoter (Swennen et al., 2002) was used to transform wild-type and mutant haploid strains. To express GAA, the \textit{Y. lipolytica} cells were cultivated on rich YPD in 50 mM phosphate buffer, pH 6-8, at 28 °C.

**Transformation of strains, DNA isolation and sequencing.** Basic DNA manipulation and transformation in \textit{E. coli} were performed according to standard methods. Yeast transformation was carried out by the lithium acetate method (Xuan et al., 1988). Plasmid DNA from \textit{E. coli} was prepared using the Qiaprep Kit (Qiagen) and DNA fragments were purified from agarose gels using the Gel Extraction Kit (also from Qiagen). Sequencing was performed by Eurogentec (Belgium). The oligonucleotides used for sequencing are shown in Table 1.

**Gene deletion.** Disruption was performed using the two-step ‘pop-in/pop-out’ method (Rothstein, 1983). This method uses the \textit{URA3} marker and a disrupted copy of the gene in an integrative vector. The deleted copy of the gene was obtained by ligation of two amplified products (Table 1), one corresponding to the upstream region of the gene and the other to the downstream region. Both digested fragments were cloned in the pINA300 vector. The resulting plasmids named pINA300-'\textit{Yip}mnn9', pINA300-'\textit{Yip}loch1' and pINA300-'\textit{Yip}lan1' linearized by \textit{NheI} or \textit{BstEII} were transformed into the \textit{Y. lipolytica} 136463 wild-type haploid strain.

**Two-hybrid experiment.** The \textit{S. cerevisiae} strain P694x MATa, trp1-901, leo2-3,112, ura3-52, his3-200, gpdA, gal80A, lys2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ (James et al., 1996) was used for the two-hybrid experiment.

The \textit{YIAN1L} and \textit{YIMNN9} ORFs were amplified with two primer pairs: ANLhyb5'-Nco and ANLhyb5'-Bgl or MNNhyb5'-Nco and MNNhyb5'-Bam (Table 1) in order to clone them in-frame with either the DNA-binding domain of Gal4p in pAS2A or the activating domain in pACT2. The first primer of each couple contains a \textit{NcoI} restriction site and, a \textit{BglII} or a \textit{BamHI} restriction site is present in the second one. After restriction of the two amplified fragments, the digested products, of 1250 and 920 bp, respectively, were ligated with the pAS2A or pACT2 vector cut with \textit{NcoI} and \textit{BamHI}.

**Immunofluorescence.** Cells grown in rich YPD medium to an OD$_{600}$ of 1–2 were fixed by addition of 5% formaldehyde to the culture. After centrifugation, cells were incubated for 2 h at room temperature without shaking in 50 mM potassium phosphate buffer, pH 6-5, 0.5 mM MgCl$_2$, 5% formaldehyde. Cells were then resuspended in 10 ml 0.1 M potassium phosphate buffer, pH 7-5, containing 25 mM $\beta$-mercaptoethanol and 1:2 M sorbitol, and permeabilized using 10 mg Zymolyase 20T (Seikagaku) and 20 mg Cytochalasin (Sigma) for 40 min at 37 °C. Cells were washed in PBS, and 10 $\mu$L of the cell suspension in PBS was transferred to wells of immunofluorescence slides pretreated with polylysine. Cells were treated with 10 $\mu$L PBS, 0.5% BSA and 0.05% Nonidet P-40 for 15 min and washed before the addition of 10 $\mu$L 1:300-diluted anti-Hap (Santa Cruz Biotechnology) or anti-cmycp antibodies (Upstate Biotechnology). After 1 h of incubation and washes in PBS, bound primary antibodies were reacted with 1:300-diluted goat anti-rabbit Texas red-conjugated IgG (Jackson Immunoresearch). Slides were treated with 300 $\mu$g ml$^{-1}$ 4,6-diamidino-2-phenylindole (DAPI) and mounted in one drop of mounting medium (1:10 PBS, 9:10 glycerol, 1 mg p-phenylenediamine ml$^{-1}$).

**Calcofluor White staining.** Cells grown in rich YPD medium to 5×10$^8$ cells ml$^{-1}$ were fixed by addition of 4% formaldehyde to the culture at room temperature for 10 min. After centrifugation, cells were incubated for 1 h at room temperature without shaking in PBS containing 4% formaldehyde. Cells were then pelleted, washed and resuspended in 500 $\mu$L PBS. A 100 $\mu$L sample of cells was treated with 10 $\mu$L of 1 mg Calcofluor White ml$^{-1}$ in the dark for 1 h, and mounted in one drop of mounting solution (1 part PBS, 9 parts...
Table 1. Catalogue of the primers used in this study

| Oligonucleotide  | Sequence (5’–3’)
|------------------|---------------------
| ANL5’seq1        | GCA GGA GAA GGG AAC ACC GAA |
| ANL3’seq2        | CAA GAC GGT GCC CTA CCC G |
| ANL3’seq3        | GGC GTC GCT CTC GAT CGA |
| ANL3’cons1       | AGC TCA ATG CTA GCC CGT GG |
| ANL5’cons2       | CGG ACG TCC TCT CCA TCG C |
| OCHseq5’         | ATT CCT GTC GAC TTT TTT CAT C |
| OCHseq3’         | TCC TCC TGA TAT ACG CCA TT |

Pop-in/pop-out strategy: position compared to ATG +1

| Oligonucleotide  | Sequence (5’–3’)
|------------------|---------------------
| ANLu-640         | GCA TGG TAG CGG CAG TAC TGT |
| ANLu-55          | GGA GAG GAC GTC GGT GCC TA |
| ANLD + 1285Xba   | GCT CTA GAG GCA GGA GAA GGG AAC ACC GA |
| ANLD + 1825Bam   | CGG GAT CCC GAA CAT CGC CCA CC |
| OCHu + 510Eco    | CGG AAT TCC GAT TCC TGT CGA CTT TTT GCA TC |
| OCHu + 10        | ATG AGA GCC ATG GTG TGA CG |
| OCHd + 890Nco    | CAT GCC ATG GCA TGA TGG CAG CGA GAT CAT GGA CT |
| OCHd + 1572Bam   | CGG GAT CCC GTC CTC CTG ATA TAC GGC ATT |
| MNNu - 95Eco     | CGG AAT TCC GTC GAG AGT GAA TTT CAG GC |
| MNNu + 365Asc    | TTG GCG GGC CAA CTT GTC CTT GGC CTT CTG |
| MNNd + 740Asc    | TTG GCG GGC CTC GAG GCC TAC GGA GAA ATG |
| MNNd + 1115Bam   | CGG GAT CCG TCA ATC ATA CGA ATG TTG TAC CAG C |

Two-hybrid system

| Oligonucleotide  | Sequence (5’–3’)
|------------------|---------------------
| ANLHyb5’-Nco     | CAT GCC ATG GCA TGT GGC TGT GAC GTT GAT TGT GTC |
| ANLHyb3’-Bgl     | GGA AGA TCT TCC TCT ATA TTA GTC TAA TCA AGG C |
| MNNHyb5’-Nco     | CAT GCC ATG GCA TGT GGC TCG AAA CAT GGC CGG |
| MNNHyb3’-Bam     | CGG GAT CCC GAT CGC TCT ACT CAT TGT AAT AGG |
| Ochip tag        | CCA CAG CTT GGG TCG GCC TTC CAT GAA CCG G |

**RESULTS**

Isolation and sequencing of the *Y. lipolytica ANL1* gene

The *S. cerevisiae* MNN9, ANP1 and VAN1 genes are three members of a gene family. They encode **z-1,6-mannosyltransferases**, are co-localized in the cis Golgi compartment, and are involved in the extension and elongation of the *z-1,6-linked mannan outer chain*. In order to isolate the *Y. lipolytica* homologues, we performed a BLAST analysis of the *S. cerevisiae* gene sequences against the *Y. lipolytica* RST 'Génolevures' database (http://cbi.labri.fr/Genolevures/index.php). The Génolevures *Y. lipolytica* genomic DNA library (Casaregola et al., 2000) covers approximately 25% of the genome, and was prepared by generating fragments ranging in size from 3 to 5 kb. A 1 kb portion at both extremities of each insert was sequenced, so that each individual insert was represented in the library by two random sequence tags (RST). By this method, a clone which contained the RST AW0AA030C10 was identified. This RST displayed a sequence similarity with a part of the *S. cerevisiae* ANP1, VAN1 and MNN9 genes. We completed the sequence of the clone by PCR amplifications with the primers shown in Table 1. The generated sequences were compared with *S. cerevisiae* genes, to confirm the sequence similarity, and with the Génolevures DNA library. Sequence data from 5’-end amplification allowed the identification of a new RST (AW0AA005B04) that overlapped the first RST and extended into the 5’ region, as shown in Fig. 1. This sequence contained a putative initiator methionine.

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residue and the promoter region of the gene. The complete sequence was assembled using the Staden software (Staden et al., 1998). It contains a 1355 bp ORF, predicted to encode a protein of 451 amino acids, with a putative transmembrane domain from amino acids 49 to 62. The deduced amino-acid sequence showed overall similarities of 69% and 67% with the Anp1p of Schizosaccharomyces pombe and S. cerevisiae, respectively; thus we named it YlAnl1p, for Anp1p-like protein. An alignment of these three Anp1p and ScVan1p proteins is presented in Fig. 2.

Characterization of the YlAnl1p and YlMnn9p interaction

In order to confirm a functional role for YlAnl1p in the Golgi glycosylation pathway and to establish a model for the glycosylation pathway in Y. lipolytica, we decided to investigate the existence of M-Pol complexes. The two M-Pol complexes described in S. cerevisiae contain Mnn9p, which is associated either with Anp1p or Van1p, as demonstrated by co-immunoprecipitation (Jungmann & Munro, 1998). To address the question of whether YlMnn9p (Jaafar et al., 2003) and YlAnl1p interact directly in vivo, we used the S. cerevisiae two-hybrid system (Fields & Song, 1989). The YlAN11 and YlMNN9 ORFs, amplified with two pairs of primers described in Table 1, were cloned in-frame with the DNA-binding domain of Gal4p in pAS2 and with the activating domain of Gal4p in pACT2, respectively. These two plasmids, pAS2ΔA-Anl1p and pACT2-Mnn9p, were used to co-transform the S. cerevisiae PJ694 strain (James et al., 1996). Results are presented in Fig. 3. While all controls were negative (sectors 2 and 3), co-expression of GAL4-BD-Anl1p and GAL4-AD-Mnn9p (sector 1) allowed transformants to grow on medium devoid of adenine and histidine (Fig. 3B). This demonstrates that YlAnl1p and YlMnn9p interact physically.

Identification of the Y. lipolytica OCH1 gene

Previous studies of S. cerevisiae have demonstrated that the formation of the mannan outer chain is initiated by Och1p, a type II membrane α-1,6-mannosyltransferase protein (Nakanishi-Shindo et al., 1993; Romero et al., 1994; Nakayama et al., 1997). To study the glycosylation pathway in Y. lipolytica, we searched this yeast for sequences homologous to the Och1p protein. When the complete Y. lipolytica DNA genomic sequencing project initiated by the Gênolevures research group had been completed, we compared the S. cerevisiae OCH1 coding sequence with the Y. lipolytica sequences and identified a putative ORF. The primer pair OCHseq5’/OCHseq3’ (Table 1) was used to amplify a 1983 bp product from Y. lipolytica 136463 genomic DNA. It contained a 1195 bp ORF predicted to encode a 397 amino acid protein with a putative transmembrane domain from amino acids 10 to 23. The deduced amino acid sequence displayed an overall identity of 47% with ScOch1p, strongly suggesting that the gene isolated was indeed Y. lipolytica OCH1; hence we named it YIOCH1. Fig. 4 shows an alignment of Och1p from Y. lipolytica, S. cerevisiae, Candida albicans and Sch. pombe.

Immuno localization of YIOch1p

α-1,6-Mannosyltransferases, on account of their specific role in the N-glycosylation pathway, are localized into the Golgi apparatus. Previous work with S. cerevisiae has shown that Och1p belongs to the cis Golgi compartment (Nakayama et al., 1992; Gaynor et al., 1994). In order to determine the intracellular localization of YIOch1p, we tagged this protein. A 1561 bp fragment of the YIOCH1 gene, amplified using the OCHu-510Eco/OCH3’-Hind primers (Table 1), was cloned into an integrative plNA300’ recombinant vector which contained 13 copies of the cmyc epitope. The resulting construction was transformed into the Y. lipolytica 136463 wild-type strain. Transformants were expected to carry a complete tagged and a truncated untagged copy of the YIOCH1 gene. We studied the localization of the YIOch1p–cmyc protein by immunofluorescence. Since, at the time of the study, no Golgi marker protein was available for Y. lipolytica, we had to use the S. cerevisiae Kex2 protease Golgi marker protein (Redding et al., 1991) as control. The pattern observed for YIOch1p–cmyc was similar to those observed for Kex2p, as shown in Fig. 5. This assay demonstrates the Golgi localization of Y. lipolytica Och1p.
**Fig. 2.** Alignment of ANP family amino-acid sequences of *Y. lipolytica*, *S. cerevisiae* and *Sch. pombe*. The alignment was performed using CLUSTALX 1.81. Shading indicates the percentage of identity between the sequences. Amino acids conserved between all four sequences are shaded black. Dark-grey shading corresponds to amino acids conserved between three of the four sequences, and light grey to amino acids conserved between two of the four sequences. ANL1 is the sequence from *Y. lipolytica*, ANP1-SP is the homologue from *Sch. pombe* (AC: spac4f10.10c). ANP1-SC (AC: P32629) and VAN1-SC (AC: P23642) are sequences from *S. cerevisiae*.
Deletion of **YIMNN9**, **YIANL1** and **YIOCH1** genes

We performed a complete deletion of **YIMNN9**, **YIANL1** and **YIOCH1** by using the two-step ‘pop-in/pop-out’ strategy (Rothstein, 1983). ΔYlmnn9, Δylanl, Δyloch1, Δylanl1Δyimnn9 and Δylmnn9Δyloch1 mutant strains were constructed using this method. Growth of strains containing the Δylmnn9 deletion was dependent on the addition of 0.5 M sorbitol. This indicates that the Δylmnn9 mutant displays a severe osmotic fragility, as previously described for the **S. cerevisiae** Δmnn9 mutant.

Effect of **YIMNN9**, **YIANL1** and **YIOCH1** deletion on sensitivity to hygromycin B and SDS

Previous work with yeasts has shown that mutants affected specifically in Golgi N-glycosylation processing display characteristic phenotypes, such as enhanced hygromycin B sensitivity, SDS sensitivity and sodium orthovanadate resistance (Ballou et al., 1991; Kanik-Ennulat et al., 1995; Dean, 1995). The degree of sensitivity or resistance is correlated with the severity of the glycosylation defect in these mutants. In order to show that the **Y. lipolytica** proteins Mnn9p, Anl1p and Och1p operate in the glycosylation pathway, we performed phenotypic analysis. To determine the sensitivity of the parental 136463 and mutant strains, 5 μl aliquots of serial dilutions of overnight cultures of the strains were plated on YPD plates containing various amounts of SDS. All the mutant strains exhibited a higher sensitivity to SDS than the parental 136463 strain (Fig. 6a). Δylmnn9 was the most affected mutant: its growth was severely inhibited from 0-015% SDS upwards. The Δylanl1 strain was the least severely affected mutant, with SDS sensitivity at a concentration of 0-05%. It is interesting to note that the double-mutant strains, Δylmnn9Δylanl and Δylmnn9Δyloch1, did not display a higher sensitivity to SDS than the Δylmnn9 mutant. These results suggest that the three **Y. lipolytica** α-1,6-mannosyltransferases YlMnn9p, YlAnl1p and YlOch1p are actually involved in the N-glycosylation pathway. Moreover, the deletion of the **YIMNN9** gene induces similar glycosylation defects in the **S. cerevisiae** (Ballou, 1990; Yip et al., 1994) and **C. albicans** Δmnn9 strains (Southard et al., 1999). The fact that the double-mutant strains are no more severely affected than the single-mutant Δylmnn9 strain proves that Mnn9p has a major function in N-glycosylation in **Y. lipolytica**, as postulated by Munro for **S. cerevisiae** (Munro, 2001; Stolz & Munro, 2002).

Effect of **YIMNN9**, **YIANL1** and **YIOCH1** deletion on cell-wall structure

A modification of the N-glycosylation process, and more particularly undermannosylation, induces defects in cell-wall integrity in yeast. Calcofluor White, a compound that interferes with the synthesis or assembly of cell-wall components, can be used as a parameter to investigate these changes (Ram et al., 1994; Van der Vaart et al., 1995; Moukadiri et al., 1997). In experiments performed as above, all mutant strains were more sensitive to Calcofluor White than the parental strain; Δylmnn9 was the most sensitive strain. Its growth was severely inhibited at a concentration of 5 μg ml⁻¹, and completely inhibited at a concentration of 10 μg ml⁻¹, whereas none of the other mutants or the parental 136463 strain was affected at this concentration. As for the SDS sensitivity test, the Δylanl1 strain was the least-affected mutant: it displayed Calcofluor-White sensitivity at a concentration of 15 μg ml⁻¹. The double-mutant strains Δylmnn9Δylanl1 and Δylmnn9Δyloch1 had a similar Calcofluor-White sensitivity to that of the Δylmnn9 strain (Fig. 6b).

To confirm that the cell-wall structure is affected in these mutant strains, we performed Calcofluor-White staining, which allows a qualitative estimation of the accumulation of chitin in the cell wall. In a wild-type context, this accumulation occurs only in damaged or fragile regions of the cell wall, such as in the bud scar, but in an underglycosylation context a high and continuous chitin accumulation is visible all around the cell wall. This allows the cell to maintain cell-wall integrity. As the cell wall is mainly composed of mannan proteins which are hypermannosylated, underglycosylation induces a general cell-wall fragility that can be highlighted by this specific chitin accumulation. Wild-type and defective cells of Δylmnn9, Δylanl1 and Δyloch1 strains were treated with 10 μl 1 mg Calcofluor White ml⁻¹ and mounted in a drop of mounting solution. As expected, in the parental 136463 strain, chitin

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**Fig. 3.** Analysis of YIAN1p and YIMNN9p interaction using the two-hybrid system. The PJ69-4x strain was co-transformed with the following plasmid combinations: 1, pAS2ΔAn1p and pACT2-Mnn9p; 2, pAS2ΔAn1p and pACT2; 3, pAS2ΔΔ and pACT2-Mnn9p, and plated on minimal medium lacking leucine and tryptophan (A), and minimal medium lacking leucine, tryptophan, adenine and histidine (B).
accumulation was observed only in the bud scar; conversely, the mutant strains displayed chitin accumulation specific to the underglycosylation context, as shown in Fig. 7. Taken together, these results indicate that the cell-wall structure of the mutant strains is characteristic of an underglycosylated cell wall.

**Fig. 4.** Alignment of OCH1 amino-acid sequences of *Y. lipolytica*, *S. cerevisiae*, *C. albicans* and *S. pombe*. The alignment was performed using CLUSTALX 1.81. Shading indicates the percentage of identity between the sequences. Amino acids conserved between all four sequences are shaded black. Dark-grey shading corresponds to amino acids conserved between three of the four sequences, and light grey to amino acids conserved between two of the four sequences. OCH1 is the sequence from *Y. lipolytica*, OCH1-SC (AC: P31755) is the sequence from *S. cerevisiae*, OCH1-CA is the homologue from *C. albicans* (AC: CA5690) and OCH1-SP is the homologue from *S. pombe* (AC: spac1006.05c).
Comparison of glucoamylase (Gaap) detection between the mutants

In order to obtain direct evidence of the function of the Y. lipolytica MNN9, ANL1 and OCH1 genes in the N-glycosylation pathway, we compared the mobility of the A. adeninivorans Gaap secreted glycoprotein in wild-type and ΔYlmnn9, ΔYlanl1 and ΔYloch1 strains by Western blot. In accordance with the results observed for A. adeninivorans (Bui et al., 1996), the native Gaap secreted in a wild-type Y. lipolytica background revealed a smear of diffuse bands between 80 kDa and 120 kDa that corresponded to heterogeneous glycosylation events of its nine potential N-glycosylation sites. In contrast, endo H-deglycosylated...
protein migrated as a discrete band (Fig. 8). To appreciate and compare the glycosylation defect in the mutant strains, the electrophoretic patterns of endo H-digested or -non-digested supernatants were analysed. In contrast to the parental strain, the Gaap secreted by ΔYlmnn9 and ΔYlanl1 strains revealed a discrete band, observed at a higher size than that of the endo H-deglycosylated protein (Fig. 8). This shift is consistent with glycosylation events restricted to the addition of core oligosaccharides in the ER. From the study of the ER-restricted glycosylated intermediate of the alkaline extracellular protease, the size of this core oligosaccharide in Y. lipolytica is estimated to be about 2 kDa (He et al., 1992). Moreover, the Gaap secreted by the ΔYlanl1 strain had a higher molecular mass than that of the ΔYlmnn9 strain. This may indicate that the degree of the glycosylation defect induced by the deletion of YLMNN9 or YLANL1 is different and correlates with the role of the gene in the N-glycosylation pathway. No direct glycosylation defect could be observed for the ΔYloch1 strain.

**DISCUSSION**

Here we report the identification and the characterization of two *Y. lipolytica* α,1,6-mannosyltransferases, Anl1p and Och1p.

Following a sequence-dependent approach, we found one ORF (YLANL1) encoding a 451 amino acid protein that has a high degree of similarity with *Sch. pombe* and *S. cerevisiae* Anp1p proteins. Similarly to these Anp1ps, a predicted transmembrane domain is present (amino acids 42 to 63). In order to characterize the *Y. lipolytica* YlAnl1p, we performed a two-hybrid system analysis of the YlAnl1p and Mnn9p interaction. Using this system, the *Y. lipolytica* Anl1p and Mnn9p were shown to interact directly, as has been demonstrated (by co-immunoprecipitation) for *S. cerevisiae* (Jungmann & Munro, 1998). A recent analysis of the phylogenetic tree of the identified members of the ANP family in *Y. lipolytica*, *S. cerevisiae*, *C. albicans* and *Sch. pombe* revealed an interesting point. In contrast to
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*S. cerevisiae*. *Y. lipolytica* possess two Anp1p-like proteins, An1p and An2p, but no Van1p-like protein (Fig. 9). These data demonstrate the particularity of the *Y. lipolytica* ANP family and raise the possibility of a specific role for its protein members. In order to confirm this hypothesis, a two-hybrid screening study, using YLMnn9p and YLAN1p as baits, will elucidate the multi-protein complexes involved in the Golgi N-glycosylation pathway in this yeast.

By screening the genome database of *Y. lipolytica*, we found a second ORF (*YIOCH1*), which predicts a protein with a putative transmembrane domain and has an overall identity of 47% with *S. cerevisiae* Och1p. By indirect immunofluorescence we concluded that YIOch1p is localized to the Golgi apparatus.

The ΔYLMnn9, ΔYLAN1, ΔYIOCH1, ΔYLAN1ΔYLMnn9 and ΔYLMnn9ΔYIOCH1 mutant strains displayed sensitivity to SDS. This is a characteristic phenotype expected for mutants defective in some aspect of protein glycosylation. Moreover, ΔYLAN1 or ΔYIOCH1 deletions induce some alteration in the cell-wall structure, as shown by Calcofluor-White sensitivity and chitin accumulation for the ΔYLMnn9 deletion. These results provide indirect evidence that YLAN1p and YIOCH1p are involved in the specific Golgi N-glycosylation process.


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