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

Stéphanie Barnay-Verdier, Anita Boisramé and Jean-Marie Beckerich

Laboratoire de Microbiologie et Génétique Moléculaire, CNRS-Institut National Agronomique Paris-Grignon-INRA, 78850 Thiverval-Grignon, France

In this study, the identification and characterization of the *Yarrowia lipolytica* homologues of *Saccharomyces cerevisiae* \(\alpha\)-1,6-mannosyltransferases Anp1p and Och1p, designated YlAnl1p and YlOch1p, are described. In order to confirm the function of the *Y. lipolytica* proteins, including the previously isolated YlMnn9p, in the \(N\)-glycosylation pathway, a phenotypic analysis of the disrupted strains \(\Delta YlMnn9\), \(\Delta YlAnl1\), \(\Delta YlOch1\), \(\Delta YlAnl1\Delta YlMnn9\) and \(\Delta YlMnn9\Delta YlOch1\) was performed. Disruption of the *YlMNN9*, *YLAN1* 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 YlAnl1p in the \(N\)-glycosylation process. These mutant strains, \(\Delta YlMnn9\), \(\Delta YlAnl1\), \(\Delta YlOch1\), \(\Delta YlAnl1\Delta YlMnn9\) and \(\Delta YlMnn9\Delta YlOch1\) may thus be used to establish a model for the *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. \(N\)-Glycosylation is a step/glycosylation process. These mutant proteins, including *Anp1* and *Och1*, have shown that mutants severely affected in mannose outer-chain extension have additional phenotypes: enhanced hygromycin B sensitivity and sodiumorthovanadate resistance (Ballou et al., 1991; Kanik-Ennulat 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 *S. cerevisiae* (Munro, 2001; Stolz & Munro, 2002). In this model, the formation of the mannose outer chain is initiated by the Och1p protein, a type II membrane \(\alpha\)-1,6-mannosyltransferase that defines an early Golgi compartment (Nakanishi-Shindo et al., 1993; Romero et al., 1994; Nakayama 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 mannose 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 display a smaller core-type structure, with only a few mannoses (Munro, 2001).

The structure of the mannose outer chain has been investigated by the study of *mmn* (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 mannose synthesis. Studies of other glycosylation mutants (*van*, *och*, *vrg*) have shown that mutants severely affected in mannose outer-chain extension have additional phenotypes: enhanced hygromycin B sensitivity and sodium orthovanadate resistance (Ballou et al., 1991; Kanik-Ennulat 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.

In *Yarrowia lipolytica*, the identification and characterization of the *YlMnn9* protein, in the \(N\)-glycosylation pathway, is a step/glycosylation process. These mutant proteins, including *Anp1* and *Och1*, have shown that mutants severely affected in mannose outer-chain extension have additional phenotypes: enhanced hygromycin B sensitivity and sodium orthovanadate resistance (Ballou et al., 1991; Kanik-Ennulat 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.

Studies of other glycosylation mutants (*van*, *och*, *vrg*) have shown that mutants severely affected in mannose outer-chain extension have additional phenotypes: enhanced hygromycin B sensitivity and sodium orthovanadate resistance (Ballou et al., 1991; Kanik-Ennulat 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.

In this study, the identification and characterization of the *Yarrowia lipolytica* homologues of *Saccharomyces cerevisiae* \(\alpha\)-1,6-mannosyltransferases Anp1p and Och1p, designated YlAnl1p and YlOch1p, are described. In order to confirm the function of the *Y. lipolytica* proteins, including the previously isolated YlMnn9p, in the \(N\)-glycosylation pathway, a phenotypic analysis of the disrupted strains \(\Delta YlMnn9\), \(\Delta YlAnl1\), \(\Delta YlOch1\), \(\Delta YlAnl1\Delta YlMnn9\) and \(\Delta YlMnn9\Delta YlOch1\) was performed. Disruption of the *YlMNN9*, *YLAN1* 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 YlAnl1p in the \(N\)-glycosylation process. These mutant strains, \(\Delta YlMnn9\), \(\Delta YlAnl1\), \(\Delta YlOch1\), \(\Delta YlAnl1\Delta YlMnn9\) and \(\Delta YlMnn9\Delta YlOch1\) may thus be used to establish a model for the *Y. lipolytica* \(N\)-linked glycosylation pathway.
z-1,6-mannosyltransferases, Mnn9p and Anp1p, are involved in the last step of the extension of the long z-1,6-linked backbone by adding a large number of mannoses (about 50). The side chains are then completed by the action of two z-1,2-mannosyltransferases, Mnn2p and Mnn5p, and a single z-1,3-mannosyltransferase, Mnn1p. In contrast, formation of the small core-type glycan involves only Och1p, one unknown z-1,3-mannosyltransferase and Mnn1p. MNN9 (Yip et al., 1994), ANP1 (Chapman & Munro, 1994) and VAN1 (Canik-Ennulat et al., 1995) are three members of a gene family. They encode z-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 Yarrowia lipolytica. The non-conventional yeast 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 S. cerevisiae (Beckerich et al., 1996, 1997). It has a secretion process closer to that of mammals than does S. cerevisiae (Beckerich et al., 1996, 1997). It has a secretion process closer to that of mammals than does S. cerevisiae (Beckerich et al., 1996, 1997). It has a secretion process closer to that of mammals than does S. cerevisiae (Beckerich et al., 1996, 1997). It has a secretion process closer to that of mammals than does S. cerevisiae (Beckerich et al., 1996, 1997).

The objective of the work presented in this paper was to confirm the function of the encoded proteins in this pathway in S. cerevisiae, such as mannosyltransferases. The objective of the work presented in this paper was to confirm the function of the encoded proteins in this pathway in S. cerevisiae, such as mannosyltransferases. The objective of the work presented in this paper was to confirm the function of the encoded proteins in this pathway in S. cerevisiae, such as mannosyltransferases. The objective of the work presented in this paper was to confirm the function of the encoded proteins in this pathway in S. cerevisiae, such as mannosyltransferases.

METHODS

Strains, plasmids and media. Escherichia coli strain DH5α was used for transformation, plasmid amplification and preparation. It was grown on Luria–Bertani (LB) medium, supplemented with 25 mM (pH 6.0) glucose, 100 μg ml⁻¹ ampicillin ml⁻¹ and 10 μg ml⁻¹ tetracycline ml⁻¹ when necessary, at 37 °C.

All disrupted strains of Y. lipolytica presented in this paper were constructed in the strain INAG13646 (MATb, scr1::ADE1, SCR2, his-1, leu-2, ura3). Yeast strains were grown on rich YPD medium, with 5% of 50 mM phosphate buffer, pH 6.8, at 28 °C.

Transformation of strains, DNA isolation and sequencing. Basic DNA manipulation and transformation in E. coli were performed according to standard methods. Yeast transformation was performed by the lithium acetate method (Xuan et al., 1988). Plasmid DNA from 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 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-YLMMN9, pINA300-YLloch1 and pINA300-Ylan1 linearized by Nhel or BstEII were transformed into the Y. lipolytica 13646 wild-type haploid strain.

Two-hybrid experiment. The S. cerevisiae strain P694α MATα, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ (James et al., 1996) was used for the two-hybrid experiment.

The YIANN1 and YIMNN9 ORFs were amplified with two primer pairs: ANLhyb5’-Nco and ANLhyb3’-Bgl or MNNhyb5’-Nco and MNNhyb3’-Bam (Table 1) in order to clone them in-frame with either the DNA-binding domain of Gal4p in βASΔΔ or the activating domain in βCT2. The first primer of each couple contains a Nco restriction site, and a BglII or a 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 βASΔΔ or βCT2 vector cut with NcoI and BamHI.

Immunofluorescence. Cells grown in rich YPD medium to an OD500 of 1–2 were fixed by addition of 5% formaldehyde to the culture at room temperature for 10 min. After centrifugation, cells were resuspended in 0.5 ml PBS, pH 7.2, resuspended in 1 molar phosphate buffer, pH 7.5, containing 25 mM β-mercaptoethanol and 1 M sorbitol, and permeabilized using 10 μg ml⁻¹ Zymolyase 20T (Seikagaku) and 20 μg ml⁻¹ Calcofluor White (Sigma) for 40 min at 37 °C. Cells were washed in PBS, and 10 μl of the cell suspension in PBS was transferred to wells of immunofluorescence slides pretreated with polylysine. Cells were treated with 10 μl PBS, 0.5% BSA and 0.05% Nonidet P-40 for 15 min and washed before the addition of 10 μl 1:300-diluted anti-Hap (Santa Cruz Biotechnology) or anti-cnmcp antibody (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 μg ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI) and mounted in one drop of mounting medium (1:10 PBS, 9:10 glycerol, 1 mg ml⁻¹ p-phenylenediamine ml⁻¹).

Calcofluor White staining. Cells grown in rich YPD medium to 5×10⁶ 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 50 mM phosphate buffer, pH 6.8, at 28 °C.
Table 1. Catalogue of the primers used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequencing and gene walking</strong></td>
<td></td>
</tr>
<tr>
<td>ANLN5’seq1</td>
<td>GCA GGA GAA GGG AAC ACC GAA</td>
</tr>
<tr>
<td>ANLN3’seq2</td>
<td>CAA GAC GGT GCC CTA CCC G</td>
</tr>
<tr>
<td>ANLN3’seq3</td>
<td>GGC GTC GCT CTC GAT CGA</td>
</tr>
<tr>
<td>ANLN3’cons1</td>
<td>AGC TCA ATG CTA GCC CGT GG</td>
</tr>
<tr>
<td>ANLN5’cons2</td>
<td>CCG ACG TCC TCT CCA TCG C</td>
</tr>
<tr>
<td>OCHseq5’</td>
<td>ATT CCT GTC GAC TTT TTG CAT C</td>
</tr>
<tr>
<td>OCHseq3’</td>
<td>TCC TCC TGA TAT ACG CCA TT</td>
</tr>
</tbody>
</table>

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

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

**Two-hybrid system**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANLHyb5’-Nco</td>
<td>CAT GCC ATG GCA TGT GCC TGT GAC GTT GAT TGT TTCC</td>
</tr>
<tr>
<td>ANLHyb3’-Bgl</td>
<td>GGA AGA TCT TCC TCT ATA TTA GTC TAA TCA AGG C</td>
</tr>
<tr>
<td>MNNHyb5’-Nco</td>
<td>CAT GCC ATG GCA TGT GCC TCG AAA CAT GGC CGG</td>
</tr>
<tr>
<td>MNNHyb3’-Bam</td>
<td>CGG GAT CCC GAT CCG TCT ACT CAT TGT AAT GG</td>
</tr>
<tr>
<td>Ochi p tag</td>
<td></td>
</tr>
<tr>
<td>OCH3’-Hind</td>
<td>CCC AAG CTT GGG TCG GCC TTC CAT GAA CCG G</td>
</tr>
</tbody>
</table>

glycerol, 1 mg p-phenylenediamine ml⁻¹, 50 ng DAPI ml⁻¹, pH 8, adjusted with 0-5 M sodium carbonate).

**Western blot analysis.** Aliquots of supernatant from yeast cultures were digested by 1 μl endo H (Biolabs) overnight at 37 °C. Digested and undigested aliquots of supernatant were mixed with sample buffer (100 mM Tris/HCl, pH 6.8, 2 % β-mercaptoethanol, 20 % glycerol, 4 % SDS, 0-02 % Bromophenol Blue), heated for 10 minutes at 65 °C and loaded on a 10 % polyacrylamide denaturing gel. After migration, separated proteins were transferred onto a nitrocellulose membrane. Rabbit anti-Gaap antibodies, kindly provided by Dr D. Swennen (INRA UMR 1238, France), were used as primary antibodies, peroxidase-conjugated anti-IgG antibodies as secondary ones, and detection was done by the ECL method (Amersham).

**Nucleotide sequence accession numbers.** We used the YIMNN9 sequence, isolated and characterized by Zueco and co-workers (Jaafar et al., 2003), available from EMBL/GenBank/DDBJ under accession no. AF44127. The accession numbers for the YIAN1 and YIOCHI sequences reported in this work are given in the footnote.

**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 \(x\)-1,6-mannosyltransferases, are co-localized in the cis Golgi compartment, and are involved in the extension and elongation of the \(x\)-1,6-linked mannann 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 AW0A030BC10 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 sequences, 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.
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 YIANl1p and YILMMn9n9p interaction

In order to confirm a functional role for YIANl1p 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 YLMn9n9p (Jaafar et al., 2003) and YIANl1p interact directly in vivo, we used the S. cerevisiae two-hybrid system (Fields & Song, 1989). The YIANL1 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, pAS2ΔΔ-Anl1p and pACT2-Mnn9n9p, were used to co-transform the S. cerevisiae PJ694x 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-Mnn9n9p (sector 1) allowed transformants to grow on medium devoid of adenine and histidine (Fig. 3B). This demonstrates that YIANl1p 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 Génolevures 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.

Immunolocalization 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. 1. Schematic representation of the position of the RSTs and primers used for YIANL1 gene walking. Maps of the pAW0AA030C10 and pAW0AA005B04 plasmids which encoded ‘Génolevures’ RST are positioned with regard to part of YIANL1. The primers used for sequencing and gene walking are represented in light (positive strand) and dark (reverse) grey, respectively.
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*. 

http://mic.sgmjournals.org 2189
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, ΔYlanl1, ΔYoch1, ΔYlanl1ΔYlmnn9 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 amount 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, ΔYlmnn9ΔYlanl1 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 YMnn9p, YAnl1p and YOch1p 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 under-glycosylation 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...
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 *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. 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 *Sch. pombe* (AC: spac1006.05c).

---

**Fig. 7.** Taken together, these results indicate that the cell-wall structure of the mutant strains is characteristic of an underglycosylated cell wall.
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

Fig. 5. Immunolocalization of the YIOch1p tagged protein. YIOch1-cmycp was localized in cells expressing a chromosomal YIOch1-cmyc gene. Cells were grown overnight in rich medium, prepared for immunofluorescence, and treated with DAPI to visualize nuclear DNA (a) and with rabbit anti-cmycp antibodies followed by Texas red-conjugated anti-rabbit IgG to localize YIOch1-cmycp (b). The YIOch1-cmycp signal was absent in the non-tagged parental strain (data not shown). The pattern of the YIOch1-cmycp signal was comparable to that observed for the S. cerevisiae Golgi marker Kex2 protease (Redding et al., 1991) (c). Bar, 2 μm.

Fig. 6. Sensitivity to SDS and Calcofluor White. Cells of the parental 136463 or the ΔYlanl1, ΔYlmnn9, ΔYloch1, ΔYlanl1ΔYlmnn9 and ΔYlmnn9ΔYloch1 strains were grown in YPD (supplemented with 0-5 M sorbitol), and 5 μl droplets of serial 1 : 10 dilutions of each strain were inoculated on the surface of YPD plates containing 0-015 %, 0-025 %, 0-05 % SDS (a) or 5 μg ml⁻¹, 10 μg ml⁻¹, 15 μg ml⁻¹ Calcofluor White (b). CW, Calcofluor White; WT, wild-type.
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 ΔYlan1 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 ΔYlan1 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 YLAN1 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 (YLAN1) 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 Ylan1p, we performed a two-hybrid system analysis of the Ylan1p and Mnn9p interaction. Using this system, the Y. lipolytica An1p 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
The Golgi apparatus. S. cerevisiae putative transmembrane domain and has an overall identity of 47% with S. cerevisiae ANP1 (AC: Q00314) and C. albicans (Sp) ANP1 (AC: spac4f10.10c) and MNN9 (AC: P39107) and VAN1 (AC: P23642); and (Ca) MNN9 (AC: P53697) and VAN1 (AC: Q00314) are represented.

White sensitivity and chitin accumulation for the mutants defective in some aspect of protein glycosylation. SDS. This is a characteristic phenotype expected for N-glycosylation process. Isolation, characterization and properties of Saccharomyces cerevisiae mnn mutants with non-conditional glycosylation defects. Methods Enzymol 185, 440–470.

**References**


S. cerevisiae, Y. lipolytica possesses two Anp1p-like proteins, Anl1p and Anl2p, 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, ΔYlan1l, ΔYloch1, ΔYlan1lΔYlmnn9 and ΔYlmnn9ΔYloch1 mutant strains displayed sensitivity to SDS. This is a characteristic phenotype expected for mutants defective in some aspect of protein glycosylation. Moreover, ΔYlan1l or ΔYloch1 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 Ylan1lp and YIOch1p are involved in the specific Golgi N-glycosylation process.

To explore more directly the role of these α-1,6-mannosyltransferases in this process, we examined the secreted N-glycosylated protein Gaap from A. adeninivorans in wild-type and mutant strains. This experiment demonstrated that Ylmnn9p and Ylan1lp have a direct effect on N-glycosylation of the Gaap secreted protein. Moreover, the most severe glycosylation defect was observed in the ΔYlmnn9 mutant. Taken together, all presented results suggest a major role for Ylmnn9p and a probable secondary role for Ylan1lp in the Golgi N-glycosylation pathway, as postulated in S. cerevisiae.

However, no effect on Gaap glycosylation has been shown in the ΔYloch1 mutant. Thus, our current data suggest a minor role for Yloch1p in Golgi N-glycosylation. We cannot exclude, however, the possibility that YIOCH1 has a severe effect on the glycosylation of a specific protein that we have not assayed. In S. cerevisiae, two tightly related α-1,6-mannosyltransferase homologues, Och1p and Hoc1p, have been identified and characterized. Och1p has been described as a critical protein in the extension of N-linked oligosaccharide chains, and catalyses the addition of the first α-1,6-linked mannose to the core oligosaccharide (Nakanishi-Shindo et al., 1993; Romero et al., 1994; Nakayama et al., 1997). In contrast, Hoc1p has been defined as a regulator protein belonging to the M-Pol II complex, and is involved in the last step of the long α-1,6-linked backbone extension (Neiman et al., 1997; Munro, 2001; Stolz & Munro, 2002). However, in the complete Y. lipolytica genome sequence we found only one sequence homologue to these two genes. This specific characteristic of Y. lipolytica allows us to propose that YIOCH1 might have a different role in the N-glycosylation pathway from those described for the S. cerevisiae proteins. To define the functional role of YIOch1p we will determine its membership of the M-Pol II type complex by the two-hybrid system.

In order to establish a model for the Y. lipolytica Golgi N-glycosylation pathway, future work will consist of further investigating the modification of the native Y. lipolytica glycosylated chain in all mutant strains by analysis of the oligosaccharidic chains of secreted proteins using mass spectrometry.

**Acknowledgements**

This work was supported by a PhD grant from the Institut National de la Recherche Agronomique (France) and NESTEC (Switzerland).


