

Cas3p Belongs to a Seven-Member Family of Capsule Structure Designer Proteins

Frédérique Moyrand,¹ Yun C. Chang,² Uwe Himmelreich,³
Kyung J. Kwon-Chung,² and Guilhem Janbon^{1*}

Unité de Mycologie Moléculaire, Institut Pasteur, Paris, France¹; Max-Planck-Institute for Neurological Research, Cologne, Germany³; and Molecular Microbiology Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland²

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The polysaccharide capsule is the main virulence factor of the basidiomycetous yeast *Cryptococcus neoformans*. Four genes (*CAP10*, *CAP59*, *CAP60*, and *CAP64*) essential for capsule formation have been previously identified, although their roles in the biosynthetic pathway remain unclear. A genetic and bioinformatics approach allowed the identification of six *CAP64*-homologous genes, named *CAS3*, *CAS31*, *CAS32*, *CAS33*, *CAS34*, and *CAS35*, in the *C. neoformans* genome. This gene family is apparently specific in a subclass of the basidiomycete fungi. Single as well as double deletions of these genes in all possible combinations demonstrated that none of the *CAP64*-homologous genes were essential for capsule formation, although the *cas35Δ* strains displayed a hypocapsular phenotype. The chemical structure of the glucuronomannan (GXM) produced by the *CAS* family deletants revealed that these genes determined the position and the linkage of the xylose and/or *O*-acetyl residues on the mannose backbone. Hence, these genes are all involved in assembly of the GXM structure in *C. neoformans*.

Cryptococcus neoformans is an encapsulated basidiomycetous yeast and is the most common cause of fungal meningoencephalitis, which is lethal if untreated (6). *C. neoformans* is a heterogeneous species with four serotypes that can be classified into three varieties. *C. neoformans* var. *grubii* (serotype A) is the most common, representing 90% of the clinical isolates around the world. *C. neoformans* var. *neoformans* corresponds to serotype D, and *C. neoformans* var. *gatti* includes both serotypes B and C (20, 33). The capsule of this yeast is the major virulence factor, along with the production of melanin (5). Acapsular strains are avirulent, and the capsule components modulate the host immune response (8, 49). A major component of *C. neoformans* capsule is glucuronoxylomannan (GXM), a large polymer of (1→3)- α -mannose with xylose, glucuronic acid, and *O*-acetyl branches. The serotypic specificity is determined by both the number and position of the *O*-acetyl and xylose residues (12). The minor components of the capsule include galactoxylomannan and mannoproteins (reviewed in reference 4). Cherniak and colleagues defined six elementary structures called structure reporter groups (SRGs), designated M1 to M6, which defined GXM's constitutive elements (12). The GXM structure of each strain is composed of a major SRG specific for each serotype and one or several minor SRGs. Eight chemotypes were thus defined according to the presence of one or the other minor SRG. In the same study, the authors demonstrated that the structure of GXM is very heterogeneous even within each serotype, although the main SRG is generally specific for some serotypes.

To date, only a few genes involved in capsule biosynthesis have been identified. In the 1990s, four genes necessary for

capsule production were cloned by functional complementation of acapsular mutant strains (8–11). The functions of these genes, however, have not been clearly assigned, and since the homologs of these genes have been identified in several non-encapsulated fungi, their roles might not be restricted to capsule production (4). More recently, a number of elements in the cyclic AMP signal transduction pathway have been shown to play an important role in environmental regulation of capsule size (4, 18). However, many environmental factors, such as pH, serum, carbon and nitrogen sources, iron deprivation, CO₂ concentration, and osmolarity, influence the size and perhaps even the structure of the capsule (reviewed in reference 26). A number of other unidentified signaling pathways are also likely to be involved in the regulation of capsule production (4, 53). Even though most of the genes involved in the biosynthesis of capsule polysaccharide precursors have been identified (2, 37, 44, 52), only two genes potentially involved in the capsule polymerization have been studied to date. Genes involved in capsule secretion or precursor transport have yet to be identified, even though a role of Cap59p in capsule secretion has been invoked (22, 27, 44).

We previously isolated a group of mutant strains that synthesize capsule with a modified structure in comparison with the original strain. We used these mutants to isolate two genes (*CAS1* and *UXS1*), which are necessary for *O* acetylation and xylosylation of the capsule, respectively (27, 37). Using isogenic sets of strains and purified polysaccharide, we demonstrated the influence of the capsule structure on the pathophysiology of cryptococcal infection in a murine model (31). In this study, we report the isolation of a new gene (*CAS3*) which is necessary to complete *O* acetylation of the capsule. We demonstrated that Cas3p belongs to a seven-member protein family that includes Cap64p, an essential component of capsule production (11). Furthermore, all of the genes from this family

* Corresponding author. Mailing address: Unité de Mycologie Moléculaire, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: (33) 1 45688356. Fax: (33) 1 45688420. E-mail: janbon@pasteur.fr.

TABLE 1. Strains used in this study

Strain	Genotype	Reference
H99	<i>MAT</i> α	39
JEC21	<i>MAT</i> α	50
JEC43	<i>MAT</i> <i>oura5</i>	50
JEC155	<i>MAT</i> <i>oura5 ade2</i>	50
JEC156	<i>MAT</i> <i>a ura5 ade2</i>	50
JEC33	<i>MAT</i> <i>alys2</i>	50
JEC32	<i>MAT</i> <i>a lys2</i>	50
TYCC77	<i>MAT</i> α <i>cap64</i> Δ :: <i>ADE2 ura3</i>	11
NE159	<i>MAT</i> α <i>cap64</i> Δ :: <i>ADE2</i>	This study
NE81	<i>MAT</i> α <i>cas3-1 ura5</i>	37
NE84	<i>cas3</i> mutant	This study
NE68	<i>MAT</i> <i>acas3-2 ura5</i>	This study
NE87	<i>MAT</i> <i>acas3-4 ura5</i>	This study
NE113	<i>MAT</i> <i>acas3-5 ura5</i>	This study
NE53	<i>MAT</i> <i>acas3</i> Δ :: <i>ADE2 ura5</i>	This study
NE190	<i>MAT</i> <i>acas3</i> Δ :: <i>ADE2</i>	This study
NE195	<i>MAT</i> <i>acas31</i> Δ :: <i>URA5 ade2</i>	This study
NE197	<i>MAT</i> <i>acas32</i> Δ :: <i>URA5 ade2</i>	This study
NE152	<i>MAT</i> <i>acas33</i> Δ :: <i>URA5 ade2</i>	This study
NE156	<i>MAT</i> <i>acas34</i> Δ :: <i>URA5 ade2</i>	This study
NE234	<i>MAT</i> <i>acas35</i> Δ :: <i>ADE2 ura5</i>	This study
NE228	<i>MAT</i> <i>acas31</i> Δ :: <i>URA5</i>	This study
NE230	<i>MAT</i> <i>acas32</i> Δ :: <i>URA5</i>	This study
NE238	<i>MAT</i> <i>acas33</i> Δ :: <i>URA5</i>	This study
NE232	<i>MAT</i> <i>acas34</i> Δ :: <i>URA5</i>	This study
NE244	<i>MAT</i> <i>acas35</i> Δ :: <i>ADE2</i>	This study
NE223	<i>MAT</i> <i>acas31</i> Δ :: <i>URA5 cas3</i> Δ :: <i>ADE2</i>	This study
NE224	<i>MAT</i> <i>acas32</i> Δ :: <i>URA5 cas3</i> Δ :: <i>ADE2</i>	This study
NE213	<i>MAT</i> <i>acas33</i> Δ :: <i>URA5 cas3</i> Δ :: <i>ADE2</i>	This study
NE218	<i>MAT</i> <i>acas34</i> Δ :: <i>URA5 cas3</i> Δ :: <i>ADE2</i>	This study
NE245	<i>MAT</i> <i>acas31</i> Δ :: <i>URA5 cas3</i> Δ :: <i>ADE2</i>	This study
NE253	<i>MAT</i> <i>acas32</i> Δ :: <i>URA5 cas35</i> Δ :: <i>ADE2</i>	This study
NE249	<i>MAT</i> <i>acas33</i> Δ :: <i>URA5 cas35</i> Δ :: <i>ADE2</i>	This study
NE257	<i>MAT</i> <i>acas34</i> Δ :: <i>URA5 cas35</i> Δ :: <i>ADE2</i>	This study

were systematically deleted, and mutants deficient in one or more of these genes were characterized. Study of the GXM structure of the deletion strains demonstrated that the proteins encoded by these genes determine the position and the linkage of the xylose and *O*-acetyl residues on the mannose backbone.

MATERIALS AND METHODS

Strains and culture media. The *C. neoformans* strains used in this study are listed in Table 1. The strains were routinely cultured on yeast-peptone-dextrose medium at 30°C (42). Minimal medium (SD), induction medium, and 5-fluoroorotic acid medium were prepared as previously described (19, 27). *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.) was used for the propagation of all plasmids.

MAbs. The anticapsular monoclonal antibodies (MAbs) E1 (17); CRND-8 (kindly provided by T. Shinoda, Tokyo, Japan) (25); and 4H3, 2H1, and 5E4 (kindly provided by A. Casadevall, Albert Einstein College of Medicine, Bronx, N.Y.) (7) were used for immunoblotting and immunofluorescence experiments as previously described (16, 27). They were selected because they have different specificities (3).

Capsule measurements. To determine the capsule thickness, cells were grown overnight at 30°C in induction medium. Yeast cells were suspended in India ink, the distance from the cell wall to the outer border of the capsule for 20 cells was measured under a magnification of $\times 100$ by using a grid with a resolution of 0.10 μm , and the mean thickness was determined for each strain.

Preparation and analysis of nucleic acid. Purification of genomic DNA was carried out as previously described (21). The RNeasy Mini and Oligotex kits (Qiagen S.A., Courtabouef, France) were used to purify total RNA and mRNA, respectively, from *C. neoformans* cells. Southern blotting and colony hybridization were carried out by using standard protocols (40). Probe labeling, hybridization, washing, and detection of hybridized bands were performed with the digoxigenin nonradioactive nucleic acid labeling and detection system (Roche

Diagnostic, Meylan, France) according to the manufacturer's instructions. DNA was sequenced by ESGS (Evry, France) with synthetic primers. The University of Wisconsin Genetics Computer Group programs were used for analysis of nucleic acid sequences (15). Restriction endonuclease digestions and ligations were carried out by standard methods, as recommended by the suppliers.

Cloning of *CAS3* from *C. neoformans* var. *neoformans*. Strain NE81 was transformed by electroporation (19) with a genomic library constructed in plasmid pCnTEL-1, kindly provided by B. Wickes (San Antonio, Tex.). By using a procedure described previously (27, 37), one plasmid able to complement the mutation of strain NE81 was isolated. Following subcloning, a 2.7-kb EcoRI-EcoRI DNA fragment was sequenced.

3' and 5' RACE analysis. The C and N termini of Cas3p, Cas31p, Cas32p, Cas33p, and Cas34p were determined using the Smart RACE (rapid amplification of cDNA ends) system kit from Clontech (Palo Alto, Calif.) as previously described (27). The primers CAS3XF and CAS3XR used for the 5'- and 3'-end amplifications are listed at <http://www.pasteur.fr/recherche/unites/Mymol/>. The cDNA fragments were then cloned into the pGEM-T Easy vector (Promega, Madison, Wis.) and sequenced.

Chromosome separation by pulsed-field gel electrophoresis. *C. neoformans* chromosome plugs for pulsed-field gel electrophoresis were prepared as previously described (51), and the chromosomes were separated on a 0.9% agarose gel ($1\times$ Tris-acetate-EDTA) by using a CHEF DR II apparatus (Bio-Rad) with the following parameters: 12°C, 16-h pulse from 100 to 200 s followed by 52-h pulse from 200 to 450 s, 2 V cm^{-1} .

Disruption of the genes. (i) *CAS31*. Two 1-kb DNA fragments specific for the *CAS31* upstream and downstream regions were amplified by PCR with the HFPCR kit from Clontech and the primer pairs CAS31-5'5'-CAS31-5'3 and CAS31-3'5'-CAS31-3'3, respectively. Each amplified fragment was then cloned into the pGEM-t Easy cloning vector (Promega), resulting in the plasmids pNE195 and pNE170, respectively. The SpeI-SmaI fragment of pNE195 was then cloned into the SpeI-SmaI sites of the pNE170 plasmid to construct plasmid pNE196. Finally, plasmid pNE203 was obtained by inserting the XhoI-BamHI fragment of the pCIP3 plasmid (8), which contains the *URA3* gene at the SmaI site of pNE196, after filling in of the cohesive ends.

(ii) *CAS32*. By using the same strategy as for *CAS31*, two 1-kb DNA fragments specific for the *CAS32* upstream and downstream regions were amplified by PCR with the HFPCR kit from Clontech and the primer pairs CAS325'5'-CAS32-5'3 and CAS32-3'5'-CAS32-3'3, respectively. Each amplified fragment was then cloned in the pGEM-t Easy cloning vector (Promega) to construct plasmids pNE169 and pNE168, respectively. The SpeI-BamHI fragment of pNE169 was then cloned into the SpeI-BamHI sites of the pNE168 plasmid to construct pNE176. Finally, plasmid pNE190 was obtained by inserting the XhoI-BamHI fragment of the pCIP3 plasmid (8), which contains the *URA3* gene at the XhoI-BamHI sites of pNE176.

(iii) *CAS3*, *CAS33*, *CAS34*, and *CAS35*. The disruption cassettes for *CAS3*, *CAS33*, *CAS34*, and *CAS35* were constructed by PCR fusion by using a strategy similar to that described by Kuwayama and colleagues for *Clostridium difficile* (32). Briefly, upstream and downstream gene fragments and the selection marker genes (*ADE2* or *URA5*) were PCR amplified from plasmids pRCD28 (46) and pCIP3 (8), respectively, by using the HFPCR kit from Clontech. The primers used for these amplifications are listed at <http://www.pasteur.fr/recherche/unites/Mymol/> (see Fig. 3 for their positions). CAS3X-5'3 and CAS3X-3'5 contain sequences recognized by the M13R and M13F primers, respectively. Similarly, the MKRCAS3Xf and MKRCAS3XR primers were designed to anneal to both M13R and M13F, as well as CAS3X-5'3, CAS3X-3'5, respectively. Consequently, the CAS3X-3'5 and CAS3X-5'3 sequences contain the reverse complements of MKRCAS3Xf and MKRCAS3XR, respectively. In addition, 5 ng of each of the three amplified and gel-purified fragments was utilized as the templates for PCR fusion with the primers CAS3X-5'5 and CAS3X-3'3 according to the following program: 95°C for 5 min, followed by 35 cycles of 94°C for 30 s and 68°C 5 min. The final PCR-amplified fragment represented the *cas3* Δ ::*URA5*, *cas34* Δ ::*URA5*, *cas35* Δ ::*ADE2*, and *cas3* Δ ::*ADE2* alleles.

The NotI-linearized plasmids pNE190 and pNE203 for *CAS32* and *CAS31*, respectively, or the PCR-amplified fragments for the four other genes were used to transform strains JEC155 and JEC156 by biolistic DNA delivery. Transformants were selected on minimal medium with 1 M sorbitol but lacking uracil or adenine, depending on the markers used (48).

Polysaccharide analysis. Each strain was grown for 3 days in induction medium, and GXM was prepared as previously reported (37). Isolated GXMs were sonicated and lyophilized three times before being dissolved in 0.5 ml of 99.96% D₂O (Sigma, St. Louis, Mo.) for nuclear magnetic resonance (NMR) analysis. NMR spectra were acquired with a Bruker Avance 600 NMR spectrometer,

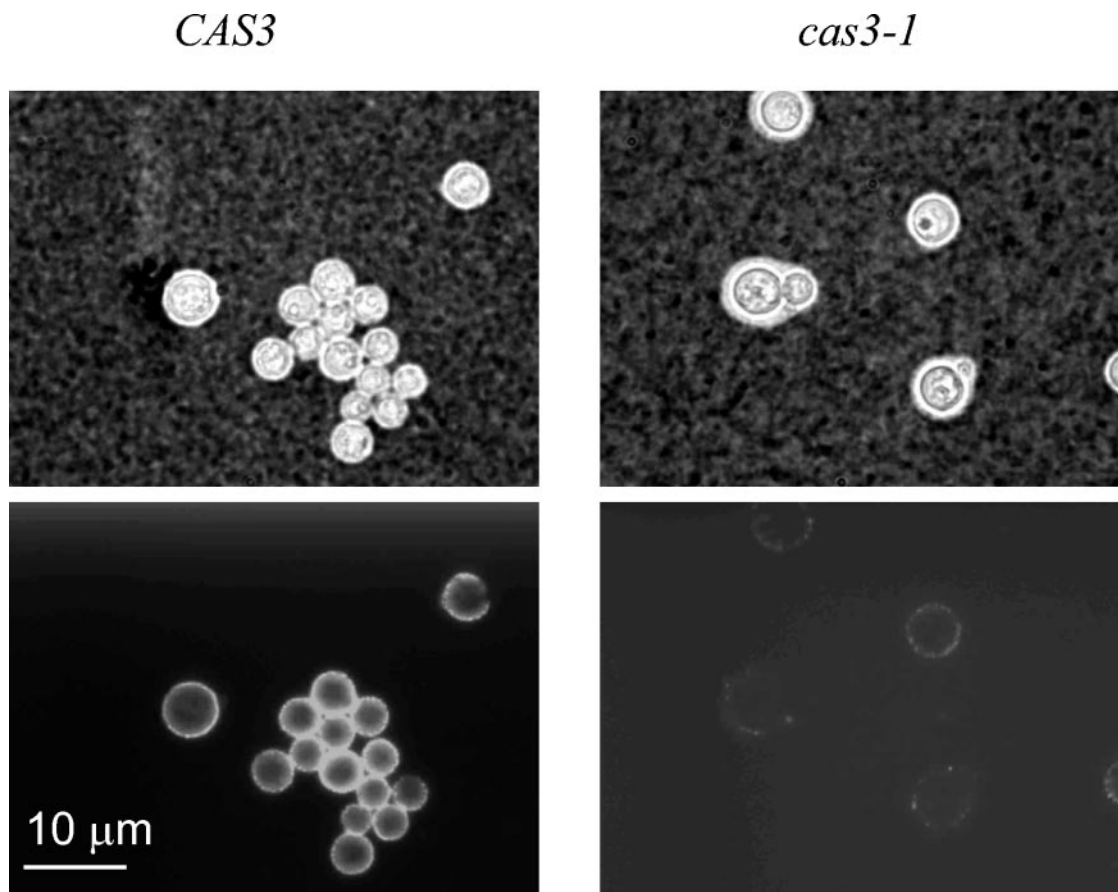


FIG. 1. Comparison of the *cas3-1* (NE81) and *CAS3* (JEC43) strains by using India ink negative staining (top panels) and immunofluorescence labeling with the MAb CRND-8 (bottom panels). Cells were grown to stationary phase in capsule-inducing medium at 30°C (see Materials and Methods), washed with sterile water, and stained with India ink or labeled with the MAb.

using a 5-mm (^1H , ^{13}C) inverse-detection dual-frequency probe, operating at 600.13 and 150.913 MHz, respectively. The temperature was maintained at 70°C during the NMR measurements. ^1H NMR spectra were acquired with a spectral width of 3,000 Hz, a time domain of 32 K, 128 acquisitions, and a repetition time of 6.5 s. Two-dimensional NMR spectra were acquired by using standard Bruker pulse sequences (XWINNMR 3.5). Double-quantum-filtered (^1H , ^1H) COSY, TOCSY, and NOESY spectra were obtained in the phase-sensitive mode. The spectral width was 3,000 Hz, and 256 increments of 16 acquisitions were acquired, each containing 2,000 data points. The acquisition time was 340 ms, and the relaxation delay was 1.5 s. Squared sine-bell functions shifted by $\pi/2$ were applied for data processing in t_1 and t_2 . The mixing time for the TOCSY spectra was either 40 or 150 ms. (^1H , ^{13}C) single-bond shift correlation (HSQC) spectra were obtained in the ^1H detection mode. Acquisition parameters were as follows: spectral widths in t_1 and in t_2 , 12,000 and 3,000 Hz, respectively; optimization for $^1\text{J}_{\text{C,H}}$ coupling constants, 140 Hz; acquisition of 256 increments in t_1 , each consisting of 32 to 64 acquisitions of 2,000 data points; acquisition time, 170 ms; relaxation delay, 1 s. A sine-bell function shifted by $\pi/2$ was applied in t_2 and a Gaussian-Lorentzian function was applied in t_1 . Cross-peak volumes were determined for comparison of relative intensity ratios of carbohydrate residues. (^1H , ^{13}C) multiple-bond heteronuclear correlation (HMBC) spectra were acquired similarly to the HSQC spectra. The spectral width in t_1 was 15,000 Hz. Experiments were optimized for $^n\text{J}_{\text{C,H}}$ long-range coupling constants of approximately 2 Hz to determine the linkage between carbohydrate moieties. The number of acquisitions per increment was 64.

Nucleotide sequence accession numbers. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY421722, AY421723, AY421724, AY421725, AY421726, AY428736, and AY063511.

RESULTS

Cloning of *CAS3*. As reported previously, the phenotype of *cas3* mutant strains differed from that of *cas1* Δ or *uxs1* Δ strains (27, 37). As shown in Fig. 1, binding of the anti-GXM-specific MAb CRND-8 to cells of strain NE81 was significantly weaker than that to cells of the original strain JEC43. This phenotype was not complemented by any of the previously identified *CAS* genes, suggesting that the gene mutated in strain NE81 was not one of the previously identified *CAS* genes. We then used functional complementation to clone the *CAS3* gene as previously described for the *CAS1* and *UXS1* genes (27, 37). Following subcloning and complementation analysis, the sequence of a 2,663-bp EcoRI-EcoRI DNA fragment capable of complementing the mutation in NE81 was identified. The complete cDNA sequence of the *CAS3* gene was obtained after 3' and 5' RACE amplifications. The *CAS3* gene encodes a protein of 57 kDa with 34% similarity to the capsule protein Cap64p (11). We sequenced the 5' ends of five independent cDNA clones and determined that the transcription start site was located between 76 and 80 nucleotides upstream of the AUG site. Sequencing of the 3' end showed that the polyadenylation site was located 154 bp downstream of the stop codon. Alignment of the cDNA and genomic sequences revealed the presence of

TABLE 2. Similarities between the different members of the Cas3p/Cap64p family

Protein	% Similarity to:					
	Cas3p	Cas31p	Cas32p	Cas33p	Cas34p	Cas35p
Cas3p						
Cas31p	77					
Cas32p	44	44				
Cas33p	36	36	35			
Cas34p	34	35	29	26		
Cas35p	31	30	31	36	23	
Cas64p	34	35	34	42	25	62

12 introns having an average length of 55.25 nucleotides, with the 12th intron being located downstream of the stop codon. BLAST searches in the general databases did not reveal any other homologous sequence except for the *CAP64* homologs in the Homobasidiomycete fungi *Pleurotus ostreatus* (GenBank accession no. AJ318523) and *Phanerochaete chrysosporium* (<http://www.jgi.doe.gov/programs/whiterot.htm>). However, no homolog was present in the genome of the Heterobasidiomycete *Ustilago maydis* or any other eukaryotic or prokaryotic genome.

Identification and cloning of *CAS3* homologs in the *C. neoformans* genome. A search for homologous sequences in the genome of *C. neoformans* strain JEC21 at the Institute for Genomic Research website (<http://www.tigr.org/tdb/e2k1/cna1/>) revealed the presence of five other putative proteins sharing homologies with Cas3p and Cap64p. Specific primers were chosen, and 5' and 3' RACE amplification allowed the elucidation of the entire cDNA sequence. The proteins encoded by these sequences were named Cas31p, Cas32p, Cas33p, Cas34p, and Cas35p. Cas31p corresponds to the Cap64p homolog that has been shown to copurify with the α -1,3-mannosyl transferase protein Cmt1p (44). Using a specific probe, we located *CAS3* on one of the largest chromosomes in serotype D (JEC20 and JEC21) and serotype A (H99) genomes (data not shown). This result is consistent with the last assembly of the strain JEC21 genome, where *CAS3* is located in chromosome scaffold 2. In this assembly, *CAS31*, *CAS32*, and *CAS35* are located in chromosome scaffold 1, *CAP64* and *CAS34* are located in chromosome scaffold 3, and *CAS33* is located in scaffold 11. However, the genes located on the same chromosome are separated by large regions, and a simple explanation for the organization of these genes is not readily apparent.

The proteins encoded by these genes are comparable in size (506, 481, 551, 603, 674, and 628 amino acids for Cas3p, Cas31p, Cas32p, Cas33p, Cas34p, and Cas35p, respectively) and have a putative N-terminal hydrophobic domain similar to that of Cap64p. Table 2 illustrates the degree of homology between the different sequences, and the sequence alignment is presented in Fig. 2. A search for sequences homologous to Cas31p, Cas32p, Cas33p, Cas34p, and Cas35p did not reveal any homologs in addition to those previously identified with Cas3p. However, analysis of the Cas33p sequence showed the presence of a domain between amino acids 191 and 353 (COG2755.1, TesA) that is conserved in different esterases and lysophospholipases (Fig. 2). Interestingly, this domain was present in different esterases or transferases involved in polysaccharide synthesis or degradation in microorganisms, such as the acetyl xylan esterase in the fungus *Neocallimastix patrici-*

rum (13), the acylneuraminate cytidyltransferase in *Streptococcus agalactiae* (24), and the rhamnogalacturonan acetyltransferase in *Xanthomonas campestris* (14).

***CAS3* sequence modifications.** In our initial screen for *cas* mutant strains, we isolated five strains displaying the same alteration of anticapsular MAb binding pattern, defined as the Cas3 phenotype, including strain NE81, which was used to clone *CAS3* (37). Transformation of the remaining four mutant strains with an autonomously replicating plasmid containing the wild-type *CAS3* gene restored binding of the antibodies to wild-type levels in each strain (data not shown). The *CAS3* gene was amplified from each mutant, and two independent PCR products were sequenced. With the exception of strain NE84, mutations were identified within the *CAS3* gene, suggesting the importance of the corresponding residues for *CAS3* function. As shown in Fig. 2, the *cas3-1* allele from strain NE81 encodes a tryptophan residue in place of the conserved glycine residue at position 457. The *cas3-2* allele from strain NE68 revealed a mutation corresponding to position 356, where a conserved proline residue was replaced with a leucine residue. The *cas3-4* allele from strain NE87 contained two mutations, encoding Y195H and N222K, located within a highly conserved region encompassed by the putative TesA domain. Finally, the protein encoded by the *cas3-5* allele in strain NE113 was truncated due to the presence of an ochre mutation at position 460. In strain NE84, a mutation in the *CAS3* gene was not found. However, a mutation might be in a regulatory region of *CAS3* outside the amplified fragment.

The comparison of the *CAS* gene sequences revealed the presence of a partially conserved DXD motif essential for the activity of different glycosyltransferases. We used site-directed mutagenesis to replace the first two amino acids of this motif with two alanine residues but these substitutions had no detectable effect on the function of Cas3p (data not shown). In contrast, tagging the C-terminal region of Cas3p with His₆ completely abolished the Cas3p function (data not shown). Thus, the C-terminal region is essential for the function of Cas3p.

Gene disruption. We disrupted every gene of the *CAS3* family with either the *ADE2* or *URA5* selection marker. Correct integration of the cassette was determined by PCR with a primer that annealed to a region outside the disruption cassette (*CAS3Xex*) and a primer that annealed to sequences within the marker (*URA5F*, *URAR*, or *ADE2a*) (Fig. 3). We screened 20 clones resulting from each transformation and identified two putative homologous integrants each for *CAS3*, *CAS33*, and *CAS34* and four each for *CAS31*, *CAS32*, and *CAS35*. A second pair of primers was used to determine the correct integration of the cassette (Fig. 3). PCR amplifications and restrictions of the *CAS3Xex-CAS3Xex2* region were used to verify the disruption of the wild-type gene in each putative deletion strain. In addition, Southern analysis was used to confirm replacement of the genes with the corresponding marker (data not shown). To construct isogenic sets of strains, genetic crosses were performed. Strain NE53 (*MAT α cas3 Δ ::ADE2 ura5*) was crossed with strain JEC33 (*MAT α lys2*), and the prototrophic progeny strains of this cross were selected on SD medium. Strains containing disrupted or wild-type genes were identified by PCR, and one strain with each possible genotype (*MAT α cas3 Δ ::ADE2*, *MAT α cas3 Δ ::ADE2*, *MAT α CAS3*, and *MAT α CAS3*) was then stored for further studies.

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Cas3 : -----MLVNSSSMLVTRSK-----
Cas31 : -----MLLASRGS-----
Cas32 : -----MPLGSPRRSH-----
Cas33 : MPDDHELDSYGIQASAEAGSSSSRPLLSRDPSPPPSPRRGRSHPVASHG-----
Cas34 : -----MFSPLSPYRLP-----
Cas35 : -----MLRPRNHANVPSAAEPDTPMSHYPSGHVHAHPDAAGATVGAHVAEYFPDADHEAGAGDTPRRAS
Cap64 : -----MLREAKVGLDTPSSAP-----RAF

Cas3 : -----VRIYLCISVMIINIVLITLFLPTDKAHGLMGDEAWKELAKYGL
Cas31 : -----VKLFLNLSLALNIVFFAAFFPSKKAYKL-----
Cas32 : -----ILLAALLSLVLFVFLHTHHPQSHFLSGPISKLLKVFGLYSSS
Cas33 : -----YPSIHSMPFRLLKRPVILSIFPFLVFLYSLVHPVKGPPLPKISITSGGA
Cas34 : -----KPIFVIVCLLIIIGLLFLSFASHDESRGYID-----
Cas35 : AGTAAGVIGEKRNVNERRKGLAGRRKFGFRLKGLMGRKKGSKFGLTQKGMIVTAVVGLIFLFLKLVFSSNGSDGHHQTYDTAHLIPRDY
Cap64 : S-----GVSRRITLWLVAVVVGFTFSRLLFPAHPD-----SSLGVLLTSKDY

Cas3 : TRWSDDGAFLTVPARGCSMCEVDPVLCET--EDNKRSLAFSGTNRRLKRVLAKLRRGETINVGAIGGSVTKGYELN-----
Cas31 : -----DAFAVKQDSNCGMCQFDPCLCEDLG--EDNQRALAYVGTNARLRRVLAQLRRGERFTVGVVGGSVSKGFLD-----
Cas32 : DGLETKDKGIRKRIDKIRGYCEAEDAFEREYGRTNRLSRAYEGSHBRMRLLOKIMRGEPLTISAIGGSVTKG-----
Cas33 : VSQP--YEEKIVQDCICGTTDEGKRLCSLYHEEGRLNSRLIECTGARMRKVLQKARNGEKIKIIVLGGSVSACHVHPS
Cas34 : -----IFKKAGANVGSASRLSGNAEEDAMREWEFRRALQHEGTSARVQAFIDKARSKAFTVASIGGSVSKGRSLTPPKSSQLVHNTF
Cas35 : LN-----HSLTDPAPFVEFCPVFGDAIAAKRGQFELRSLRLHTGTGARVQRILOKAMSGAPITISILGGSVSACTAG-----
Cap64 : LN-----ASATDPAPFVEFCPVFGDDEVAARRGQWGLRSLRLHMGSGARVQRVVKAMAGLPVTSVILGGSVSACHAG-----

Cas3 : -----RYNEPYYDTEPNLHRIIFDHLVSLYPAFNGVKTDSDGRKEGK-----H
Cas31 : -----KDVG-YFDTPTNLNRIVFDRLNLFPAFNGVKVGENGREENM-----N
Cas32 : -----HQVWQNETWEHKEFEWELTEFAGEGVEIK-----
Cas33 : -----DRFPQGDREAGGCGYTSLLMEWEKETFDPADHE-----
Cas34 : RRSSEGDSVRPSDVPDDSEQDNIDLTEVTPSSAQELEFTGSAIENQGKNVSHPIAPLVHLGASTLYSHENLHVQIFEWLNETFPNPDN
Cas35 : -----DDETHEKCYPHRVFDWNTVFFHPANE-----
Cap64 : -----NDEVSAGCYPAQFFDWNNSVFPHPSE-----

          H in cas3-4          DDX          K in cas3-4
Cas3 : GYINGGGGATGTYFYSYCEWEHVADLDLIFLEQAINDELLLRNIDSYELLVRSLLDLPTSFAIVNHLVFAIMFNSTIT--LGGD-LHQSI
Cas31 : SYINGVGGVGDYFYSYCEWEHLEDDMDLVFIELAINDEVLTRNINSYELLVRSLLDLPLNGPAVMNFQIIFALKFNTVT--NGGD-MHHGT
Cas32 : -QVNGAAPATGSDYFYSYCEWELHPSDSDLVLELAVNDEGPEHVENMENLLRGLLDLPLNKPAVMLTEAMAFSNGMG--GGGRMHLBV
Cas33 : -ETNGAIGGMDSSYFYSYCEWELHPSDSDLVLELAVNDEGPEHVENMENLLRGLLDLPLNKPAVMLTEAMAFSNGMG--GGGRMHLBV
Cas34 : REINGVGGVGAAYFGWCEKEHTEPADLVLELGINDLDLEFDVFSQTEHLVRLGLELENKPAVINHET--TLFVPSL--SSSS-FHNDV
Cas35 : -LTNGATPKTDSAYYAYCNSHHLBDKTDLVILEFDASDPNDPEWLQHFELLVRSVLRPEMPAVINLGHESLQVQAQNGFAGPELLHNVD
Cap64 : -LTNGASRRRTDSAYYAYCNSHHLBDKTDLVILEFDASDPNDPEWLQHFELLVRSVLRPEMPAVINLGHESLQVQAQNGFAGPELLHNVD

Cas3 : AQFYDLPVLSLRNALLDMLKNESEISEYFFVHPE-----GDI DLRHISRKGHNVMGRIGAAVMDSQICEMDKYEQGP-
Cas31 : AQFYDLPVLSLRNAAYNFIQNESLIPEYFIHGN-----GETDTRHMGKKGHNLMGRIGAAVYIETQLCEMDKFEASIPG-
Cas32 : AQYYDLPVINQRLPLVNHFAHFEQLVGPYFAQDWWG-----NPDTRHINSHGHRDLGMLVASFVKDVAEMISQPTFFVQS
Cas33 : ALYYDLPYLSMRKLMENHLYRPHSTAKTFEOPDLL-----HPNARGHRVLSDDLAYLDSLECMLSKYGLPPIAPP
Cas34 : LSFYDVPVSLSTRDVLVPLRIADPQQMRWFRTGDDVAMGDFKVKWEGGVVVMHISATGHSAAAGLVIRYLHEQIDRSTSYGLSALS
Cas35 : GQFYDVPHISTKGVLYEHLGTEDKARSAYYVDFN-----HPNFAGHDLADVLISYLSQICAGWSAINGHYD
Cap64 : AQFYDVPHISAKGVLYGDYLAEPAAARNTFTDPI-----LANPTGHGVADVLISYMQSQCAGWAATMGHAFD

          L in cas3-2
Cas3 : ADSMSIDQLYPPEP-----IPRMLNMKYDK-----
Cas31 : AKSMTIDQLYLPLEP-----IPRMLNMKYDK-----
Cas32 : PSFAEALSIVALAQPPTQEDKEDAKIEDELLAAEQKWWEQSRSWRKDPTEEKVPVGLMPGLWSTPIEYGLLPRLRVLEGWPHL-----
Cas33 : LQDTISTTHPTFDLIDVHVPLETLHLVDPAAAPPEG-----WEETFKTEPLEALSHEKRLFAVPLTPYSIPVGMFTPLSDVVPNTND
Cas34 : LTSTPKKPTVRIMD-----VPATTLTGLFNP-----
Cas35 : VPALGTEGDNVWAGTPELGGVG-----LRKMPGQDPGDGSSIGSSLSDRYQGLRVPQIRLADRPHD-----
Cap64 : VPYMG-----APALEDNLGAG-----IQS-----EEDLESEGGGLAAKLRAIKVPAQLMSDRPSD-----

Cas3 : -----DLVLPPTIKPQCFSANSEKHP-----LVFVENNGWRKWNWKE-----KHYLVADVPGSRVSFKLKTNMG
Cas31 : -----DLVLPPTIKPQCFLANGKKNP-----LVFVTNEGWRNWNWKE-----KHYLISDTPGSRITFKLITTVTG
Cas32 : -----DYSVPPFHEPCGLSTRAKEPHFN-----KPYLVARTPGARVTELETSVG
Cas33 : DPITGEHIAAALQPRLECADANDKKNP-----MKETQADGWEPFANNGE-----KHYVWSHKSRSRIVNIVKVSAG
Cas34 : -----DKADMRHAEVQRSONSGKTHGKVSLSLVDDFSEGYKGLTLAEGSHGSEWSWKE-----KHYLIAREPFSRAIFDFTISLP
Cas35 : -----VQAFRETEPFCVAASDLVNPLP-----PTLFFGNWATYHPPAKGNVVEDRHYWYADREPTARLRIFPKLGAG
Cap64 : -----ILKFREVAEFCVANSANDLVNPLP-----PSHFYGSQWHAHHP-SKGASDDEKHYWYSIDIGSKRVVPTLSAG

Cas3 : -----
Cas31 : -----
Cas32 : -----
Cas33 : -----
Cas34 : QEPLISDDPSAFLLDGADNITAEPTSQVHKTEGEDDMPVEQEFPFRGSLAHRSAGLRKWSSAISHQPRPTSPTLPGRYISQFNAKQNPQ
Cas35 : -----
Cap64 : -----

          Stop in cas3-5
          ↓
Cas3 : ---KELVQYLR-SYQYHQGSAKRWDDEEVEKAIKLDGYWKEP-YNIGRAVTIREGLEPGEHTLICELLKQTADPEG--GLEFRLISIMSI*
Cas31 : ---NELTHYLR-SYQYNIGSVKQWIDEDVDKAMRIDGYWKEP-YNIGRAATVGNLTPGEHKLICELLKGTADPSG--GKEFRLISVMSI*
Cas32 : ---IWMYALK-SKTEGLGTVEWADDEERRKAVTEGGYWDGNVNIYGRFAPIRDNLQEGKHTICELEETSDFGG--GREFRMISVMSV*
Cas33 : ---KWAVYFR-SQHYNDGAKQWDDNEKGAVTSSGYWTKQ-YNVAAAYIDEKVTPGDHYVVCVILHTSHPTNDPAHHRITAVMAT*
Cas34 : AGGSLDIGYQR-SAKLGLGSVWCWIDDDRDGGQRVDGWKLDKRNMGVKEIAVGLPEPKHTLICEILEDLDPMG--GTEFRLFALMHD*
Cas35 : ---DWGTYFLQSPDKPLDGVVVKWVDDNVNGAKEHGTAEVE-DVATLVMTDRGVERGSHFVCELCQGGPFGSS--PPFKILGTFMT*
Cap64 : ---DWALYYLH-PAENPLGRAAWDDNFASAVEISGGVSDVQ-EATPTLTLIDENVSTGSHYVCEVLLGKEGEKT--APFKMLGVFGT*

          ↑
          W in cas3-1

```

FIG. 2. Regions conserved between different members of the Cas3/Cap64 family. Sequences were aligned by using ClustalW 1.83 (47) and edited with Genedoc (38). The *TesA* conserved domain identified in the Cas33p sequence is underlined. The positions and natures of sequence alterations in the different *cas3* alleles are indicated by arrows.

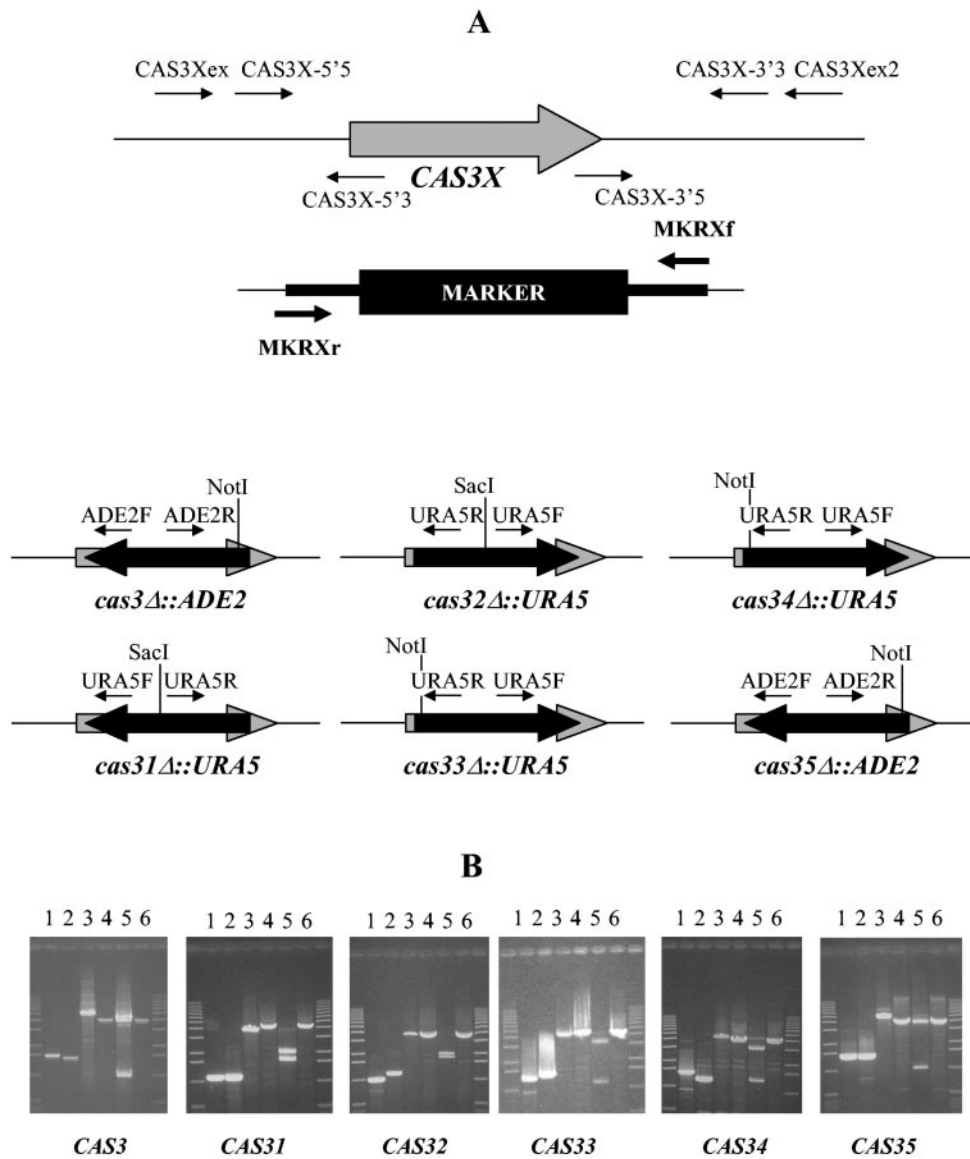


FIG. 3. Gene disruption. (A) Positions of the different primers used to construct the disruption cassettes and for screening the transformants (X indicates 1, 2, 3, 4, or 5). (B) PCR analysis of the gene disruptants. For *CAS3* and *CAS35*, the primers used were (lane 1) CAS3Xex-ADE2F, (lane 2) CAS3Xex2-ADE2R, and (lanes 3 to 6) CAS3Xex-CAS3Xex2. For *CAS31*, the primers used were (lane 1) CAS31ex-URA5F, (lane 2) CAS31ex2-URA5R, and (lanes 3 to 6) CAS31ex-CAS31ex2. For *CAS32* to *CAS34*, the primers used were (lane 1) CAS3Xex-URA5R, (lane 2) CAS3Xex2-URA5F, and (lane 3 to 6) CAS3Xex-CAS3Xex2. Genomic DNA of strain JEC21 (*MAT α*) (lanes 4 and 6) or of the corresponding *cas3X Δ* mutant (lanes 1, 2, 3, and 5) was used as the substrate. Lanes 5 and 6 the PCR amplification product were gel purified and digested with the adapted restriction enzyme (NotI or SacI).

Similar procedures were used for *CAS31*, *CAS32*, *CAS33*, *CAS34*, and *CAS35* to isolate different sets of strains. Finally, a large set of double mutants were selected by using the different auxotrophic markers. For example, strain NE53 (*MAT α cas3 Δ ::ADE2 ura5*) was crossed with strains NE195 (*MAT α cas31 Δ ::URA5 ade2*), NE197 (*MAT α cas32 Δ ::URA5 ade2*), NE152 (*MAT α cas33 Δ ::URA5 ade2*), and NE156 (*MAT α cas34 Δ ::URA5 ade2*). Prototrophic progeny strains were then selected on SD medium, and their genotypes were confirmed by using PCR. Strains of each possible genotype (*MAT α cas3 Δ ::ADE2 cas3X Δ ::URA5* or *MAT α cas3 Δ ::ADE2 cas3X Δ ::URA5*) were stored in duplicate for further studies (Table 1).

Phenotypic analysis. (i) Growth rate, colony morphology and sensitivity to drugs. The growth rates of all of the strains inoculated on yeast-peptone-dextrose and incubated at both 30 and 37°C were compared, and no significant differences were found (data not shown). In addition, the colony morphologies of mutants were indistinguishable from that of the wild type, except that the *cas35 Δ* and *cap64 Δ* strains produced drier colonies (data not shown). We tested the mutant and wild-type strains for their sensitivities to caspofungin and amphotericin B and found no significant differences between the mutants and the parental strains (data not shown).

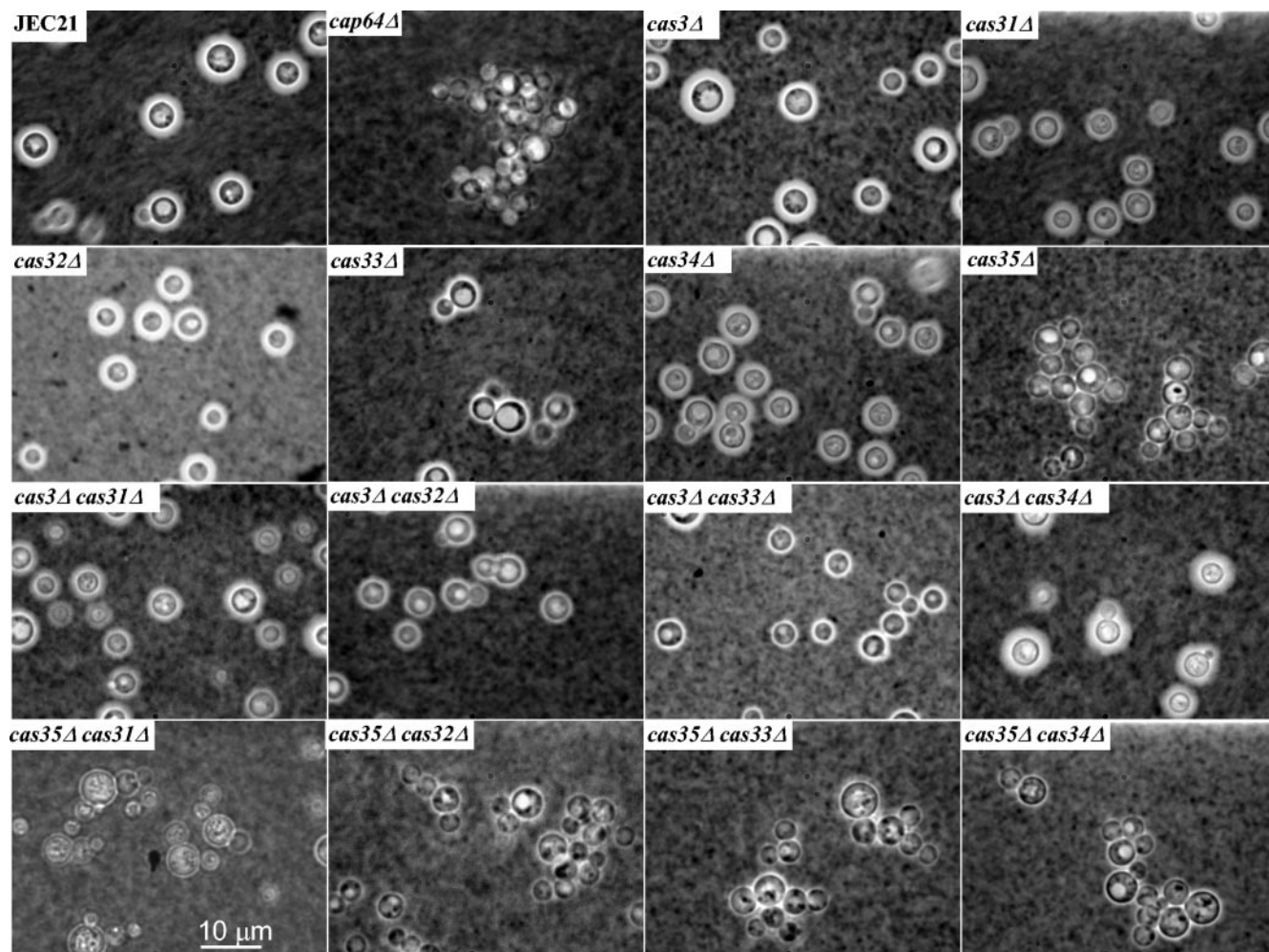


FIG. 4. Analysis of capsule size. Cells were grown to stationary phase in capsule-inducing medium (see Materials and Methods), washed with sterile water, and stained with India ink.

(ii) **Capsule sizes.** The capsule size of each strain was analyzed by using India ink negative staining and microscopic examination. As shown in Fig. 4, only mutations in *CAP64* and *CAS35* seemed to affect the size of capsule. Rigorous measurements of capsule sizes confirmed these results ($1.5 \pm 0.48 \mu\text{m}$ for JEC21, $1.7 \pm 0.49 \mu\text{m}$ for the *cas3Δ* strains, $1.80 \pm 0.43 \mu\text{m}$ for the *cas31Δ* strains, $1.25 \pm 0.50 \mu\text{m}$ for the *cas32Δ* strains, $1.8 \pm 0.4 \mu\text{m}$ for the *cas33Δ* strains, $2.0 \pm 0.47 \mu\text{m}$ for the *cas34Δ* strains, and $0.71 \pm 0.40 \mu\text{m}$ for the *cas35Δ* strains). Strains with the *CAS35* gene disrupted were thus hypocapsular, and an analysis of 32 progeny strains obtained from a cross between NE234 (*MAT α cas35Δ::ADE2 ura5*) and JEC32 (*MAT α lys2*) showed linkage between the hypocapsular phenotype and the *cas35* deletion. Thus, all of the progeny strains containing the disruption cassette were hypocapsular, while all of the encapsulated ones contained the wild-type allele. The hypocapsular phenotype was, as expected, associated with a strong virulence defect of the *cas35Δ* strains in a mouse model (Y. C. Chan and K. J. Kwon-Chung, unpublished data). Double mutants deficient in *CAS35* and any other genes displayed no additive defects in capsule production.

(iii) **Antibody analysis of capsule structure.** We previously performed a dot blot assay with different GXM-specific monoclonal antibodies to discriminate between modifications in capsule structure (27, 37). We used the same assay to analyze the different mutant strains obtained in this study, and the results are presented in Fig. 5. Mating type does not appear to influence capsule structure, as *MAT α* strains were identical to *MAT α* strains in the dot blot assay (data not shown). The phenotypes of the *cas3Δ* strains constructed for this report were the same as those of *Cas3* mutant strains (37). Moreover, backcrosses with the *cas3Δ* mutants and the original strain demonstrated that this phenotype was genetically linked with the *CAS3* disruption. We could not detect any difference in capsule structure in the other single mutant capsular strains. Although lighter signals were obtained with all of the MAbs and the *cas35Δ* strains, the decreased binding could have been due to the smaller size of the capsule in these mutants. As expected, the acapsular *cap64Δ* strain did not react with any of the antibodies tested. With the exception of the *cas3Δ cas31Δ* double mutant strains, a synthetic phenotype associated with a combination of gene deletions was not observed. Strains lacking *CAS3* and *CAS31* were not recognized by any of the anti-

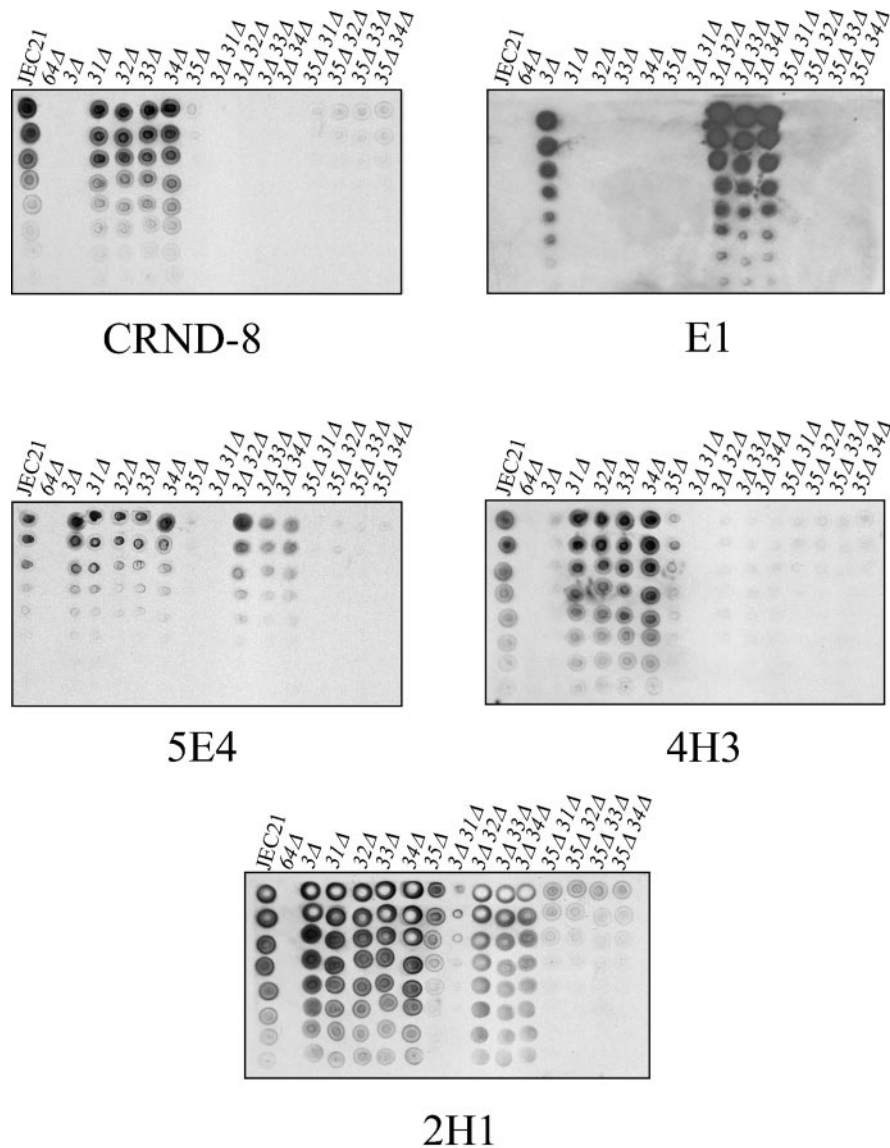


FIG. 5. Antibody reactivities of different GXM-specific MAbs with the mutant strains. Serial twofold dilutions of cell suspensions were spotted (starting with 3×10^4 cells on the first spot at the top of the lane) on a nitrocellulose membrane and probed with a given concentration of each MAb (37).

bodies used in this study which correspond to a phenotype previously observed with the *cas1Δ* strains (27).

(iv) **Analysis of polysaccharide structure.** We purified GXM from all of the single capsular mutant strains, as well as the double mutant deficient in *CAS31* and *CAS3*. The chemical structures of their polysaccharide were analyzed by NMR. Due to strong resonance overlap in the two-dimensional correlation spectra for minor residues in particular, not all chemical shifts could be determined unequivocally. Connectivity was determined for the smallest detectable repeating moiety. The smallest repeating moieties (Fig. 6) were determined by the assessment of connectivity between carbohydrate residues, using (^1H , ^{13}C) HMBC spectra and the chemical shifts in mutant strains compared to that of the parental strain as well as to those of previous work (1, 27, 37, 41). The structure of the GXM isolated from the parental strain was identical to that of

the chemotype Chem1, which is dominant in serotype D isolates (12, 43) (moiety 1 in Fig. 6).

The GXM isolated from *cas3Δ* strains contained partly 6-O-acetylated mannosyl residues. The acetylation ratio relative to the amount of mannosyl residues was only half of that in the parental strain (Table 3). The ^1H , ^{13}C HSQC and HMBC spectra showed that the chemical shifts of H-5/C-5 and H-6/C-6 of the mannosyl residue in moiety 1 have shifted from 4.08/73.6 ppm and 4.30 to 4.35/66.5 ppm in the wild type to 3.91/75.2 ppm and 3.78 to 3.84/62.8 ppm in the *cas3Δ* strain, indicating that this residue is not 6-O acetylated in the mutated strain (27). In contrast to the previously reported 6-O-deacetylated GXM from the *cas1Δ* mutant (27), a second minor carbohydrate moiety was found in *cas3Δ* strain GXM. The most obvious difference in this minor repeating carbohydrate moiety was the presence of an additional xylosyl residue. The HMBC

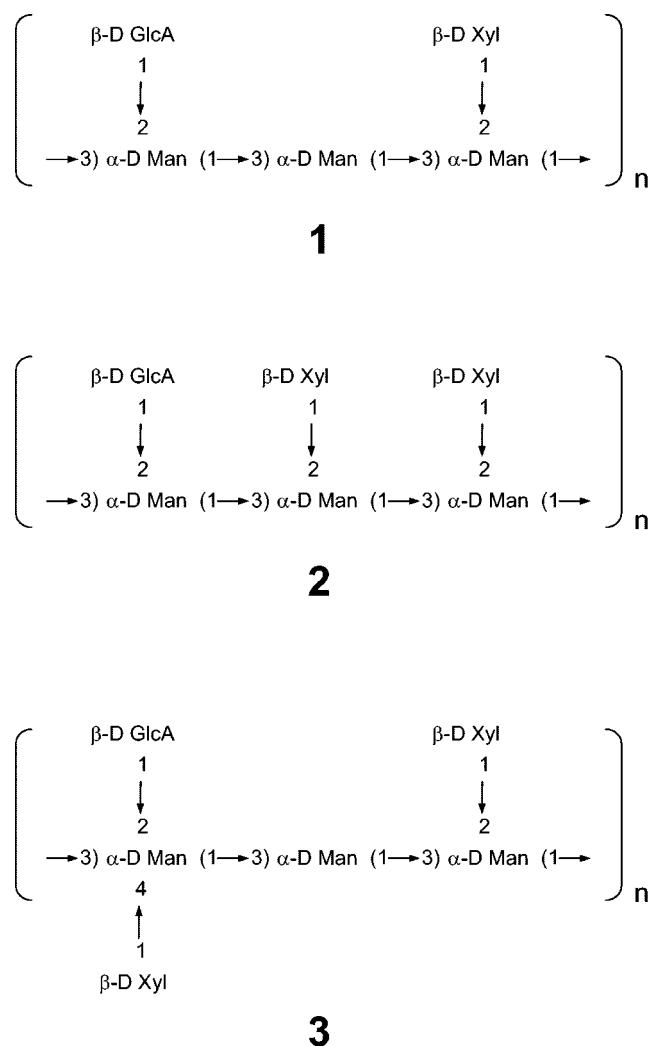


FIG. 6. Smallest repeating carbohydrate moieties identified in GXMs isolated from the mutated strains. Additional variability was due to different 6-O acetylations of the mannosyl residues.

spectra indicated that the xylosyl residue was 1→2 connected to a mannosyl residue, which was 1→3 connected to another similar mannosyl residue (1→2 xylose substituted) and 3→1 connected to a mannosyl residue with a 1→2 glycuronic acid substitution (moiety 2 in Fig. 6). This structure has been previously defined as the SRG M2 (12). Based on the (¹H, ¹³C) HSQC spectrum and integration of the H-1/C-1 cross-peaks of the xylosyl residues in the two moieties, the amount of the second moiety 2 relative to the major moiety 1 (also present in the wild type) was estimated to be 10 to 15% (Table 3). The structure and chemical shifts of the additional repeating carbohydrate moiety 2 were similar to those reported for 6-O-deacetylated GXM of serotype A (41). However, due to extensive resonance overlap between the two repeating sequences, it was not possible to determine whether the two sequences were present in parallel or whether the actual smallest repeating sequence contained more than three mannosyl residues, incorporating both repeating sequences. When *CAS3* was reintroduced into the *cas3Δ* strain, the O-acetylation level was fully restored and the additional minor repeating

carbohydrate disappeared in the GXM structure (data not shown).

The GXMs of all other *cas* mutants were 6-O acetylated, similar to what has been reported for the GXMs from wild-type strains (27). The acetyl/mannosyl ratios varied between 1.8 and 2.3 (Table 3). GXM 1, which is the dominant GXM in serotype D isolates, was also dominant in all other mutant strains. As in the *cas3Δ* mutant, the repeating sequence 2 was identified in all other mutant strains. Table 3 shows the ratio of moiety to moiety 2. Structure 2 was confirmed by the (¹H, ¹³C) HMBC spectra. In addition, the mannosyl residue, to which the additional xylosyl residue is 1→2 linked, showed changes in the H-1/C-1 and H-2/C-2 chemical shifts, similar to those for *cas3Δ*. Interestingly, the mannosyl residue, which is not xylosylated in moiety 1 but is xylosylated in the minor moiety 2, was 6-O acetylated in moiety 1 but not in moiety 2, confirming previous findings that mannosyl residues that are 2-xylosylated are not 6-O acetylated (27). Most likely, this has contributed to the minor changes in the acetylation ratio that correlated with the amount of the repeating moiety 2 in the respective GXM. Only minor differences in the GXM composition were found between the *cas31Δ*, *cas32Δ*, *cas33Δ*, and *cas34Δ* mutants, which concern the ratio of the repeating moieties 1 and 2 (Table 3).

GXMs isolated from the *cas35Δ* strain showed the most deviated form of polysaccharides. The *cas35Δ* mutant contains a third repeating moiety, shown in Fig. 6 (moiety 3), in addition to moieties 1 and 2. As in the other strains, it was not possible to determine whether the smallest repeating carbohydrate sequences were part of a larger repeating sequence or if they were separate entities present in parallel. This additional moiety varied from the GXM of the wild type by an additional 1→4 substitution of a xylose residue to the 1→2 GlcA substituted mannosyl residue. Although resonance overlapping made assignment difficult, the most likely explanation is a repeating sequence 3, which is identical to one that was found in a Cap70 mutant (1). However, it cannot be fully excluded that the

TABLE 3. Concentration ratios of carbohydrate residues as determined from the NMR spectra

Strain	Concn ratio (mean ± SD)		
	O-acetyl/Man ^a	(Xyl + GlcA)/Man ^b	Xyl 1/2/3 ^c
JEC21	2.31 ± 0.2	1.85 ± 0.1	10:0:0 ^d
<i>cas3Δ</i>	1.23 ± 0.1	1.83 ± 0.1	10:1:0
<i>cas31Δ</i>	2.12 ± 0.1	1.97 ± 0.1	10:1.5:0
<i>cas32Δ</i>	2.07 ± 0.1	2.06 ± 0.1	10:2:0
<i>cas33Δ</i>	2.33 ± 0.2	1.87 ± 0.1	10:1:0
<i>cas34Δ</i>	2.01 ± 0.2	2.26 ± 0.1	10:3:0
<i>cas35Δ</i>	1.86 ± 0.1	2.33 ± 0.2	10:3:1
<i>cas3Δcas31Δ</i>	0.00	2.44 ± 0.1	10:4:0

^a The acetyl/mannosyl ratio was determined from the ID ¹H NMR spectra by integration of the acetyl resonances (2.1 to 2.3 ppm) and the H-1 mannosyl resonances (5.1 to 5.4 ppm).

^b The (xylosyl + GlcA)/mannosyl ratio was determined from the 1D H NMR spectra by integration of the Xyl H-2, H-3, and H-5a resonances and the GlcA, H-2 and H-3 resonances (3.1 to 3.55 ppm) relative to the H-1 mannosyl resonances (5.1 to 5.4 ppm).

^c The ratio of the xylosyl residues from structures 1, 2, and 3 was determined by integration of the (¹H, ¹³C) HSQC cross peaks for H-1/C-1 of the respective xylosyl residues.

^d Resonances from minor unassigned residues were present (<5%).

Man[1→2GlcA, 1→4Xyl] residue is 1→3 linked to another 1→2 substituted mannosyl residue (found in serotype B and C isolates [1, 44]), in contrast to the unsubstituted mannosyl residue in structure 3. The chemical shifts of H-5/C-5 and H-6/C-6 indicate that Man^{GlcA,Xyl} is 6-O deacetylated in moiety 3.

The *cas3Δ cas31Δ* double mutant was the only one that exhibited a synthetic phenotype in the dot blot assay. Chemical analysis of the purified GXM from this mutant strain confirmed these results: the *cas3Δ cas31Δ* GXM was completely de-O acetylated, and a cumulative effect on the ratio of xylosyl residues associated with structure 2 was observed (Table 3).

DISCUSSION

In *C. neoformans*, four *CAP* genes have been demonstrated to be necessary for capsule production (8–11). The *CAP59* and *CAP60* genes, which have been identified by functional complementation of acapsular mutants, encode homologous proteins and share a significant degree of homology with the recently identified α -1,3-mannosyltransferase Cmt1p (8, 9, 44). Moreover, the recently released complete genome sequences of a serotype D and a serotype A strains of *C. neoformans* have allowed us to identify the fourth member of this protein family (26). Likewise, four proteins homologous to Cap10p (among them, Cap1p, encoded by a gene located in the mating type locus [34]) could also be identified in these genomes (26). In the present study, we demonstrated the existence of six proteins that are homologous to Cap64p. The existence of other Cap protein families orthologous to these proteins could be found in the proteomes of noncapsulated organisms, suggesting that the activities of the *CAP* gene products are not likely to be restricted to capsule biosynthesis (4). The Cas3p/Cap64p family, however, appears to be restricted to Homobasidiomycetous fungi. Among all the available genome data, *Pleurotus ostreatus* and *Phanerochaete chrysosporium* were the only organisms from which we were able to identify the orthologous genes. *U. maydis*, a Heterobasidiomycete, did not contain any ortholog of this family in its genome.

As for the other Cap proteins, the role of Cap64p in the capsule biosynthesis remains unclear, although several nonexclusive hypotheses have been made as to their function (capsule secretion, capsule biosynthesis regulation, or direct involvement in capsule biosynthesis) (4, 36). All of the mutant strains presented here except the *cap64* mutant produce capsule, and we demonstrated that Cas3p, Cas31p, Cas32p, Cas33p, Cas34p, and Cas35p are involved in xylose branching and/or O acetylation of the capsule. These results rule out the possibility of secretory function and seem to support the notion that this Cap protein family is directly involved in capsule synthesis. The presence of an esterase motif in the conserved domain of the Cas33p sequence also supports this hypothesis. We cannot, however, eliminate the possibility that these genes regulate the transcription or the activities of some genes directly involved in capsule biosynthesis.

Families of genes encoding partially or completely redundant functions are common in polysaccharide synthesis pathways. Thus, the mannosyltransferase activities are encoded by different families of genes in *Saccharomyces cerevisiae* (35, 45). Consequently, single gene mutations are often either silent or barely express a phenotype, as proteins encoded by other

paralogous genes tend to compensate for the lack of the activity of the mutated gene. Multiple mutations in different members of the gene family are therefore necessary to produce a phenotype. In this study, we constructed a set of single and double mutant strains to identify the ones which might be involved in similar functions. We hypothesized that a double mutation in genes encoding similar functions would have a synthetic phenotype, whereas a double mutation in genes acting at different steps of the biosynthesis would have a simple additive effect. Even though we observed a synthetic phenotype for only one double mutant strain (the *cas3Δ cas31Δ*), the phenotypes of the single mutants and the sequence similarities of the different proteins helped us to draw several hypotheses on their functions.

The GXM from the *cas31Δ* strain has the same O acetylation as that of the parental strain, the GXM from the *cas3Δ* strain has 30% of the O-acetyl residues, and the *cas3Δ cas31Δ* double mutant is completely de-O acetylated. These genes also appear to be the only ones of this family that are involved in O acetylation of the capsule. However, we cannot rule out that other combinations of double or triple mutations could reveal importance of the other genes in O acetylation. The Cas31p sequence is also most similar to that of Cas3p, suggesting that these two proteins have the same function but are differentially regulated. Indeed, Northern blot analysis demonstrated significantly weaker expression of *CAS31* compared to *CAS3* in rich medium (A. Alspaugh, personal communication).

Cas35p is most similar to Cap64p in its amino acid sequence, and while Cap64p is essential for capsule biosynthesis, *cas35Δ* mutant strains are hypocapsular. Moreover, the GXM of *cas35Δ* strains contained a unique repeating carbohydrate moiety (structure 3). Two hypotheses can be drawn to explain these results. First, Cas35p influences the substitution of the mannosyl backbone by directing an additional xylose residue to the Man^{GlcA} residue. Second, this triad exists in the parental strain but at a very low concentration. It is detectable only due to a down-regulation of the M1 synthesis. Cas35p would be then necessary for the full expression of the major triad (M1) in serotype D. In the mutant strain, the synthesis of M1 would be strongly repressed, and the relative concentration of the *cas35Δ*-specific triad would be artificially increased. The identification of the same triad in another hypocapsulated mutant, Cap70 (1), supports the latter hypothesis, even though we cannot rule out that this strain has a mutation within *CAS35*.

The three remaining single mutant strains have very similar capsular phenotypes. A mutation in each gene does not modify the size or the O acetylation of the capsule directly. They do not have any synthetic phenotype associated with a mutation in *CAS3* or *CAS35*. Finally, all three mutations increased the proportion of the minor SRG (M2) marginally without increasing that of the *cas35Δ*-specific triad. The only difference between M1 and M2 is the presence a β -1,2-xylose residue on the second mannose residue of the triad (Fig. 6). It remains to be demonstrated whether these proteins promote the addition of a β -1,2-xylose in position 3 of the triad or inhibit the β -1,2-xylosylation of the second mannose.

Sommer et al. have demonstrated recently that the protein Cmt1p, which has an α -1,3-mannosyltransferase activity, copurified with a Cap64p-paralogous protein (44). Here, we identified this protein as being Cas31p. This result strongly suggests

the existence of protein complex involved in capsule biosynthesis. Such protein complexes have been observed in *S. cerevisiae* for the synthesis of the mannan moiety of the glycoproteins (28, 30). Thus, two multiprotein complexes (M-Pol I and M-Pol II) are necessary for the synthesis of the mannan backbone of the N-linked glycan (29), whereas Pmt proteins initiate the assembly of *O*-mannosyl glycans from heteromeric and homomeric complexes (23). In this study, we demonstrated that Cas31p is involved in capsule O acetylation and xylosylation, suggesting that this potential protein complex is multifunctional.

As the proteins from this family, except for Cap64p, are involved in the position and linkage of xylose and *O*-acetyl residues on the mannan backbone, they represent good candidates for the source of *C. neoformans* serospecificity. We indeed identified the seven members of this protein family in the serotype A and serotype B genomes (http://www.bcgs.cbc.ca/cgi-bin/crypto_data/blast_wm276.pl). Whether these homologous genes have analogous functions in different serotypes or are regulated differently remains to be determined. The molecular and biochemical research on capsule formation thus far reported is beginning to uncover the complexity of the GXM biosynthetic pathways. Comprehensive studies on genetics, cell biology, enzymology, and glycochemistry are required for an understanding of the prospective function of each component.

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