The role of Alg13 *N*-acetylglucosaminyl transferase in the expression of pathogenic features of *Candida albicans*.

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Abstract

Background: The pathogenic potential of *Candida albicans* depends on adhesion to the host cells mediated by highly glycosylated adhesins, hyphae formation and growth of biofilm. These factors require effective *N*-glycosylation of proteins.

Here, we present consequences of up- and down- regulation of the newly identified *ALG13* gene encoding *N*-acetylglucosaminyl transferase, a potential member of the Alg7p/Alg13p/Alg14p complex catalyzing the first two initial reactions in the *N*-glycosylation process.

Methods: We constructed *C. albicans* strain *alg13Δ::hisG/TRp-ALG13* with one allele of *ALG13* disrupted and the other under the control of a regulatable promoter, TRp. Gene expression and enzyme activity were measured using RT-qPCR and radioactive substrate. Cell wall composition was estimated by HPLC DIONEX. Protein glycosylation status was analyzed by electrophoresis of HexNAcase, a model *N*-glycosylated protein in *C. albicans*.

Results: Both decreased and elevated expression of *ALG13* changed expression of all members of the complex and resulted in a decreased activity of Alg7p and Alg13p and under-glycosylation of HexNAcase. The *alg13* strain was also defective in hyphae formation and growth of biofilm. These defects could result from altered expression of genes encoding adhesins and from changes in the carbohydrate content of the cell wall of the mutant.

General significance: This work confirms the important role of protein *N*-glycosylation in the pathogenic potential of *C. albicans*.

Keywords *Candida albicans*, *N*-glycosylation, Alg13 *N*-acetylglucosaminyl transferase, morphogenesis

1. Introduction

Candida albicans is a common component of human microflora and the most common cause of opportunistic fungal infections of immunocompromised patients, with a mortality rate around 30-50% [1, 2]. The pathogenic potential of *C. albicans* is attributed to several factors including expression of adhesins, the yeast-to-hyphae transition and biofilm formation. All these factors can be affected by changes in glycosylation of proteins which therefore plays an important role in *Candida* virulence [3, 4, 5, 6].

N-glycosylation is an essential protein modification highly conserved in evolution. In all eukaryotes, *N*-glycosylation is obligatory for viability since glycans have a common role in promoting protein folding, quality control, and certain sorting events and, finally, determination of protein activity [7, 8]. *N*-glycosylation can be divided into two phases: the first is the assembling of the core polysaccharide containing 14 monosaccharide residues which is then transferred *en bloc* to a growing polypeptide chain. The second step of *N*-glycosylation is further processing of the polycarbohydrate *N*-linked to the protein to the mature structures characteristic for the host [2, 9-13]. Glycosylation requires a phosphorylated isoprenoid lipid, dolichyl phosphate (Dol-P), as a carrier of the carbohydrates. During *N*-glycosylation the whole core polysaccharide is assembled on Dol-P [10]. Biosynthesis of the lipid-linked oligosaccharide (LLO) begins at the cytosolic side of the endoplasmic reticulum (ER) with a sequential addition of two *N*-acetylglucosamine (GlcNAc) residues

and five mannoses to Dol-P with nucleotide diphosphate sugars, UDP-GlcNAc

and GDP-mannose, as donors [14]. The resulting oligosaccharide,

Dol-PP-GlcNAc$_2$ Man$_5$, is then flipped to the lumen of the ER and four

mannosyl and three glucosyl residues from Dol-P-mannose and Dol-P-glucose

are added to form Dol-PP-GlcNAc$_2$ Man$_9$ Glc$_3$. The assembled core

oligosaccharide is transferred to the γ-amido group of asparagine residues

located in a highly conserved motif Asn-X-Ser/Thr (X can be any amino acid

except proline) of the modified protein.

The addition of the second GlcNAc residue to Dol-PP- GlcNAc is catalyzed by

a hetero-oligomeric GlcNAc transferase that in most eukaryotes comprises the

Alg13p and Alg14p subunits. Alg13p is the catalytic subunit recruited to the ER

by the membrane protein Alg14p [15-17]. Bickel et al. [15] have demonstrated

that yeast membranes depleted of Alg13p or Alg14p lack GlcNAc transferase

activity *in vitro* and accumulate Dol-PP-GlcNAc *in vivo*. This activity is also

present in bacteria, however, in *E.coli* the GlcNAc transferase (MurG)

catalyzing peptidoglycan biosynthesis is a single protein with Alg13/Alg14

homologous domains. A structural comparison of Alg13p, Alg14p and MurGp

based on the crystal structure of the latter has revealed that Alg13p corresponds

to the C-terminal part of MurG thought to bind the UDP-GlcNAc donor, and

Alg14p to the N-terminal part containing a glycine-rich motif postulated to be a

membrane association site involved in Dol-P recognition [18]. The interaction

between the two Alg13p/Alg14p subunits of UDP-GlcNAc transferase in yeast

is limited to a C-terminal α-helix (comprised of fifteen amino acids) of Alg13p and three amino acids of Alg14p [16, 17, 19]. Furthermore, the N-terminal region of Alg14p interacts directly with one more protein, Alg7p. This enzymatic protein catalyzes the formation of Dol-PP-GlcNAc, the acceptor of the second GlcNAc added by Alg13p [17, 20]. Thus in *S. cerevisiae* Alg7p is, in fact, the third member of the Alg7p/Alg13p/Alg14p (Alg7/13/14) complex indispensable for the initial reaction in LLO biosynthesis. The genes coding for the proteins forming this complex are essential.

Here, we cloned and analyzed a previously uncharacterized *orf19.6025* from *C. albicans*. This ORF was found by searching a *C. albicans* genomic data base with the Alg13p sequence from *S. cerevisiae* as probe.

To analyze the function of this hypothetical Alg13 protein we constructed a *C. albicans* strain *alg13Δ::hisG/TR*p-*ALG13* with one allele of *ALG13* disrupted and the other under the control of a regulatable promoter, TRp. TRp is a strong promoter which can be repressed by doxycycline. Thus, depending on the composition of medium the constructed strain expressed *ALG13* at a level exceeding that of the wild type (without doxycycline added) or markedly lower (with doxycycline).

The changes in expression of *ALG13* influenced the expression of *ALG7* and *ALG14* and altered Alg7p and Alg13p activities causing under-*N*-glycosylation of the model *N*-glycosylated protein HexNAcase.

The impaired glycosylation resulted in defects in hyphae formation and biofilm growth- the two invasive forms of *Candida*. The background of these changes included altered expression of *HWP1* (hyphae wall protein) and *ALS1* (agglutinin-like sequence protein) genes encoding factors contributing to biofilm formation. Changes in the cell wall composition and in the composition of the extracellular matrix of biofilm were also observed.

2. Materials and methods

2.1. Strains and growth conditions

C. albicans strain CAI4 (genotype: *ura3 Δ::imm434/ ura3 Δ::imm434*), an uridine auxotroph was used for deletion of *ALG13* gene (Table 1).

E. coli strain DH5α F' (genotype: *F' supE44 ΔlacU169 {φ80 lacZ ΔM15} hsdR17 recA1 endA1 ngyrA96 thi-1 relA1*) [21] was used for plasmid propagation.

C. albicans strains were routinely grown at 30° C in YPD medium (1% yeast extract, 1% Bacto-peptone, 2% glucose) or SD medium (0.67% yeast nitrogen base, 2% glucose). Uridine auxotrophic strains were grown on media supplemented with uridine (20 μg/ml). To repress the tetracycline promoter, doxycycline was added to the medium at concentrations from10 to100 μg/ml; 40 μg/ml was established as the optimal concentration and was then used throughout the study.

The ability to form hyphae was tested on Spider medium (1% nutrient broth, 1% mannitol, 0.2% K_2HPO_4 and 1.35% agar), YPSerum (1% yeast extract, 0.5% peptone, 10% horse serum and 2% agar) or YPD [22].

For sensitivity assays solid YPD medium (1.5% agar) was supplemented with uridine (20 µg/ml), doxycycline (40 µg/ml), tunicamycin (1, 1.5, 2 µg/ml) or Congo Red (5, 10, 15 µg/ml) or Calcofluor White (5, 10, 15 µg/ml). To induce the excision of *URA3* gene, *C. albicans* transformants were grown on FOA plates (0.67% yeast nitrogen base, 2% glucose, 0.3% 5-fluoroorotic acid, 40 µg/ml uridine, 2% agar).

2.2. Construction of C. albicans *strain*

One copy of *ALG13* (*orf19.6025*) was deleted using the "URA-Blaster" method [23]. For the construction of the deletion cassette the following primers were used: ALG13-flank1-F/ALG13-flank1-R for amplification of the 5' region of homology (-494 to -46 upstream of the ORF) and ALG13-flank2-F/ALG13flank2-R for amplification of the 3' region of homology (+3 to +425 downstream of the AUG start codon) (Table 2). The obtained fragments were cloned to the p5921 plasmid in the SacI/BglII and BamHI/SalI sites, respectively. The SacI/SalI fragment was then excised and used for gene replacement in the CAI4 strain to obtain the *alg13Δ::hisG-URA3-hisG/ALG13* strain. The *URA3* selective marker was removed as above to obtain the *alg13Δ:hisG /ALG13* hemizygote [23].

To put the second copy of *ALG13* under the control of tetracycline promoter (TRp), primers ALG13-TRp-F and ALG13-TRp-R were used to amplify the cassette on the template of p2151c plasmid. The 4067-bp fragment contained: a 55-bp fragment homologous to region -162 to -107 bp upstream of the *ALG13* start codon, the *URA3* selection marker, the fusion transactivator – *tetR-ScHAP4AD*, the regulatable *tetO-ScHOP1* promoter and a 53-bp sequence homologous to region -4 to +49 bp of *ALG13*. The cassette was used for transformation of the *alg13Δ::hisG/ALG13* strain.

Proper construction of the strains was confirmed by Southern blot analysis (Fig.1S). DNA was isolated from the *alg13Δ::hisG/ALG13*, *alg13Δ::hisG/TR*p-*ALG13* and the CAI4 control strain, digested with BanI, BglII, or HindIII, electrophoresed, transferred to Hybond-N membrane and hybridized with a DIG-labelled 445-bp probe homologous to the coding region of *ALG13*. The probe was amplified by PCR with ALG13-flank2-F and ALG13-flank2-R primers on *C. albicans* genomic DNA and visualized with the NBT/BCIP system (Promega).

2.3. Molecular biology methods

Chromosomal DNA was isolated from *C. albicans* using the Promega Wizard Genomic DNA Purification kit. Total RNA was isolated using the single-step method described by Chomczynski and Sacchi [24]. Other molecular biology procedures were performed according to standard protocols [25].

2.4. Quantitative reverse transcription PCR (RT-qPCR)

Reverse transcription was performed using the Advantage RT-for-PCR Kit (Clontech Laboratories) with 1 µg of total RNA as a template. Gene-specific primers designed using Clone Manager 6 software, their calculated Tm and amplicon sizes are shown in Table 3. qPCR assays were performed in a Light Cycler 1.6 Instrument (Roche Life Science). For the amplification Light Cycler Fast Start DNA Master PLUS SYBR Green (Roche Life Science) mix was combined with 0.2 µM forward and reverse primers and cDNA diluted 1:5 with nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95° C for 10 min, followed by 40 cycles of denaturation at 95° C for 10 s, annealing at appropriate temperature (Table 3) for 10 s and elongation at 72° C for 1s per 25 bp. All primer pairs produced a single amplicon with a uniform melting curve as determined by the denaturation profile of the product. Two technical repeats were carried out for each data point. The expression ratios of the investigated transcripts were calculated against actin as a reference gene using Pair Wise Fixed Reallocation Randomization Test © and Relative Expression Software Tool REST ©. Each assay was repeated three times for independently isolated RNA.

2.5. Cell membrane preparation

C. albicans strains were cultured at 30°C in 1 l of YPD medium to OD$_{600}$=1-1.5; the cells were then harvested by centrifugation and resuspended in 2 volumes of 50 mM Tris/HCl buffer pH 7.4 containing 15 mM MgCl$_2$ and 9 mM 2-mercaptoethanol. The suspension was homogenized with 0.5-mm glass beads and the homogenate was centrifuged at 4 000 x g for 10 min to remove unbroken cells and cell debris. The supernatant was centrifuged for 1 h at 50 000 x g and the pelleted membrane fraction was used for enzymatic assay.

2.6. Determination of N-*acetylglucosaminyl transferase (Alg7p and Alg13p) activities*

Formation of Dol-PP-GlcNAc and Dol-PP-GlcNAc$_2$ was measured in the membrane fraction by incubation for 30 min at 30°C of 200 µg of membrane proteins in a total volume of 50 µl containing: 1 x 10^5 cpm UDP[^{14}C]*N*-acetylglucosamine (sp. act.: 300 mCi/mmol, American Radiolabeled Chemicals, Inc.) and 5 ng of Dol-P in 40 mM Tris/HCl buffer pH 7.4 with 10 mM MgCl$_2$ and 0.1% Nonidet P-40 [26, 27]. The reaction was stopped by addition of 4 ml of chloroform – methanol (3:2 v/v). After removing the denatured protein by centrifugation, the lipid phase (supernatant) was washed and the radioactive glycolipids were separated by TLC chromatography on silica gel 60 plates (Merck) in chloroform: methanol: water (65:25:4 v/v/v). Radioactive Dol-PP-GlcNAc and Dol-PP-GlcNAc$_2$ spots were located by autoradiography, scraped off the plates and their radioactivity was determined in a scintillation counter.

To analyze separately the activity of Alg7 or Alg13, the enzymes were solubilized as described [28,29]: membrane preparation (6 mg/ml proteins) was incubated in 1.25 M sodium chloride, 0.5 mM dithiothreitol, 20% glycerol and 6 mM diheptanoylphosphatidylcholine for 20 min on ice and centrifuged for 40 min at 160 000 x g.

To analyze the formation of Dol-PP-GlcNAc the reaction contained in a final volume of 60 µl: 28 mM Tris/HCl, pH 7.4, 20 mM $MgCl_2$, 0.7 mM DTT, 0.3% Nonidet P-40, 23% glycerol, 3.5 mM diheptanoylphosphatidylcholine, 0.7 M NaCl, 1 x 10^5 cpm UDP[^{14}C]N-acetylglucosamine, 2 µg Dol-P and solubilized enzyme (equivalent to 0.2 mg membrane protein). After incubation for 3 min at 30^oC the reaction was stopped by addition of 4 ml of chloroform – methanol (3:2 v/v) and processed as above.

To analyze the formation of Dol-PP-GlcNAc$_2$ the reaction contained in a final volume of 60 µl: 27 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.8 mM DTT, 0.45% Nonidet P-40, 27% glycerol, 0.2 mM UDP-GlcNAc, Dol-PP- [^{14}C] GlcNAc- (3000 cpm) and solubilized enzyme (equivalent to 0.1 mg membrane protein). After incubation for 6 min at 30^oC the reaction was stopped and processed as above.

2.7. Determination of cis-*prenyltransferase activity*

The enzyme activity was assayed in the membrane fraction by incubation for 90 min at 30° C of 100 µg of membrane proteins with 4 µg of farnesyl diphosphate (FPP), 50 mM sodium phosphate buffer pH 7.4, 0.5 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 10 mM KF and 3 x 10^5 cpm [^{14}C] isopentenyl diphosphate (IPP) (sp. act.: 55 mCi/mmol, American Radiolabeled Chemicals, Inc.) (final volume 50 µl). The reaction was terminated by addition of 4 ml of chloroform - methanol (3: 2 v/v). The protein pellet was removed by centrifugation and the supernatant was washed three times with 1/5 volume of 10 mM EDTA in 0.9 % NaCl. The organic phase was concentrated under a stream of nitrogen and subjected to thin-layer chromatography on HPTLC RP-18 plates developed in 50 mM H_3PO_4 in acetone. The zone containing the radiolabeled polyprenols was scraped off and the radioactivity was measured in a scintillation counter [30].

2.8. Immunodetection of Alg7 protein

Total membrane fraction use for activity assay was also examined for the amount of the Alg7 protein by immunostaining with monoclonal antibody against human Alg7p (OriGene, Herford, Germany). Membrane proteins were solubilized in 0.1% Nonidet P40, subjected to the Immobilon P membrane (Milipore) and *C.albicans* Alg7 protein was detected by immunological reaction with human antibody. Immunoreactive material was detected using an anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich). Bovine serum albumin was used as a negative control.

2.9. Determination of N-*acetylglucosaminidase (HexNAcase) activity* in situ

The HexNAcase *in situ* activity staining was performed as described [3]. To induce HexNAcase production strains were grown for 16 h in SC medium supplemented with 25 mM *N*-acetylglucosamine (GlcNAc) in the presence or absence of doxycycline. Cells were disrupted by vortexing with glass beads in 10 mM Tris/HCl, pH 8, containing protease inhibitor cocktail (Sigma-Aldrich). After lysate clarification by centrifugation, samples were mixed with native loading dye and run on a Tris/acetate 3–8% gradient polyacrylamide gel (Invitrogen) under non-denaturing conditions. The gel was washed in 0.1 M citrate/KOH buffer, pH 4, for 10 min at room temperature and then incubated in substrate solution (0.18 mM naphthyl-GlcNAc (Glyco-synth Ltd., Warrington, UK) in 0.1 M citrate/KOH buffer, pH 4) for 30 min at 37°C. The reaction was visualized by incubation in the substrate solution plus 0.7 mM Fast Blue at 60°C until the color developed.

2.10. Cell wall preparation

C. albicans was cultivated in YPD medium, washed with 10 mM Tris/HCl, pH 7.5, suspended in the same buffer, disintegrated with 0.5 mm glass beads in the presence of a protease inhibitor coctail (Sigma-Aldrich) and centrifuged at 1500 x g for 10 min. The resulting pellet containing cell walls was washed with ice-cold 1 M NaCl until disappearance of absorbance at 260-280 nm [31].

For the cell wall analysis during biofilm growth, biofilm was cultivated for 12 h, cells were scraped from the surface and used for cell wall preparation.

2.11. Determination of cell wall carbohydrates

Lyophilized cell wall was hydrolyzed o/n in 4 M trifluoroacetic acid (TFA) at 100° C. After cooling on ice, samples were centrifuged at 17 000 x g for 5 min at 4° C. The supernatant was dried under N_2 and washed twice with pure methanol. After removing methanol with N_2, the pellet was resuspended in miliQ water and purified on a Millipore Filter Device (0.45 µm pores) by centrifugation at 16 000 x g for 4 min. Samples were stored at -20° C. Monosaccharides were determined by high performance anion-exchange chromatography using a Dionex ICS-3000 Ion Chromatography System with a Carbo Pac PA10 analytical column. Neutral sugars were eluted with 18 mM NaOH at 0.25 ml/min [32].

2.12. Biofilm formation

C. albicans biofilm development was documented under a light microscope [33-35].

Strains were grown for 16 h at 30^oC in liquid YPD medium supplemented with 20 µg/ml of uridine (if necessary), centrifuged and the cell pellet was resuspended in YPD with 20 µg/ml of uridine and 40 µg/ml of doxycycline (if necessary) and adjusted by measuring optical density at 600 nm to a final

concentration of 1×10^6 cells per ml. Three-milliliter portions of the standardized cell suspension were transferred into sterile 55-mm Petri dishes and incubated at 37°C for 1.5, 6, 12, and 48 h. Subsequently, non-adhered cells were washed away from the biofilm with two 2-ml portions of phosphate buffered saline (PBS) and the biofilm was allowed to dry and examined under a light microscope (Delta Optical). The assay was carried out in triplicate for each strain.

2.13. Isolation of extracellular DNA (eDNA) from biofilm cultures

Strains were cultivated for biofilm formation for 12 h. Culture medium was decanted and the biofilm was washed twice with 5 ml of water. The liquids were pooled, centrifuged to remove cell debris and dialyzed o/n at 4°C against 2 l of water and lyophilized. Ten milligrams of the resultant powder was dissolved in 0.5 ml of water, extracted with phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) and eDNA was then precipitated with isopropanol. The eDNA was dissolved in 20 µl of water and analyzed using a Colibri Microvolume Spectrophotometer (Bertold Titertek).

2.14. Isolation of extracellular glucan from biofilm cultures

The same powder which was used for eDNA preparation was also analyzed for extracellular glucan. Ten milligrams of the powder was hydrolyzed o/n in 4 M TFA at 100° C and analyzed as described for the cell wall carbohydrate analysis.

3. Results

3.1. Disruption of putative ALG13 *gene in* C. albicans

The aim of this study was to find a homologous gene in *C. albicans* and to characterize its function. A putative *ALG13* gene (*orf19.6025*) was found in the genomic data base of *C. albicans* (http://www.candidagenome.org/) using the amino acid sequence of Alg13 protein from *S. cerevisiae* as probe. The 879-bp *orf19.6025* potentially encoded a protein of 293 amino acids, a predicted catalytic subunit of *N*-acetylglucosaminyldiphosphodolichol: *N*-acetylglucosaminyltransferase Alg13p (GlcNAc-T) [EC 2.4.1.141] from *C. albicans*. This hypothetical protein showed 37% identity with the 202-amino acid Alg13p from *S. cerevisiae* (E value 8e-32) (Fig.1). We also found a sequence potentially encoding a homologue of the *S. cerevisiae* Alg14p anchor protein. The identity between these proteins from *S. cerevisiae* and *C. albicans* was 32% (E value 7e-30).

To confirm the function of the predicted Alg13p in *C. albicans* one copy of *orf19.6025* was disrupted and the second one was put under the control of the regulatory tetracycline promoter TRp (see Materials and methods). These changes made in the CAI4 genome were confirmed by Southern blot analysis (Fig.1S). DNA from strains *alg13Δ::hisG/ALG13*, *alg13Δ::hisG/TRp-ALG13* and the control strain CAI4 was cut with BanI, BglII or HindIII restriction enzymes (not cutting inside the *ALG13* gene) and hybridized with a 445-bp

DNA probe homologous to the *orf19.6025* coding region. The analysis using BanI revealed two hybridizing bands in the wild type strain CAI4, suggesting that the two *orf19.6025* copies are localized differently in the homologous pair of chromosomes or that a BanI restriction site is mutated in one of the chromosomes. To elucidate this finding we cut the genomic DNA with BglII or HindIII. Both enzymes gave a single hybridizing band which proved that the localization of the *ALG13* gene was the same in both chromosomes. Thus a BanI restriction site close to *orf19.6025* had to be absent in one copy of the genome. This finding was not surprising since a particularly high variability of the upstream regions of genes has been reported for *C. albicans* [36].

3.2. Expression of orf19.6025 *and its effect on UDP*-N-*acetylglucosaminyl:DolPP*-N-*acetylglucosaminyl transferase [EC 2.4.1.141] activity*

To quantify *orf19.6025* expression in the wild type and the mutant strain total RNA was isolated and reverse transcription qPCR analysis was performed. A significant reduction of *orf19.6025* expression was observed in the *alg13Δ::hisG/TR*p-*ALG13* strain cultivated with doxycycline. Consequently, cultivation without doxycycline, when the TRp promoter was fully active, resulted in an uncontrolled expression exceeding over eight-fold the expression in the CAI4 wild type control strain (Fig.2).

Since the activity of UDP-*N*-acetylglucosaminyl:DolPP-*N*-acetylglucosaminyl transferase depends on the proper localization of the Alg13p catalytic subunit of the enzyme in the ER we examined expression of *orf19.5363*, a putative *ALG14* gene coding for the protein anchoring Alg13p in the ER.

Expression of *ALG14* was decreased by the uncontrolled high expression of *ALG13* gene in the *alg13Δ::hisG/TR*p-*ALG13* strain cultivated without doxycycline. In the *alg13Δ::hisG/TR*p-*ALG13* strain cultivated with doxycycline expression of *ALG14* was the same as in the control strain.

To gain a complete picture of the expression of all components of the *N*-acetylglucosaminyl transferase complex we also examined expression of *orf19.2187* encoding Alg7p [EC.2.7.8.15] [37]. Expression of *ALG7* was decreased in the *alg13Δ::hisG/TR*p*ALG13* strain irrespective of the cultivation conditions (Fig.2). Lower expression of *ALG7* resulted in lower amount of Alg7 protein in the membrane fraction of the *alg13Δ::hisG/TR*p*ALG13* mutant compared to the control strain (Fig.3).

Next, we examined how the changes in the expression of *ALG7*, *ALG13* and *ALG14* influenced the rates of formation of Dol-PP-GlcNAc and Dol-PP-GlcNAc$_2$, products of the activity of Alg7p and Alg13p *N*-acetylglucosaminyl transferases, respectively. The decreased expression of *ALG7* combined with the increased expression of *ALG13* in the *alg13Δ::hisG/TR*p*ALG13* strain cultivated without doxycycline resulted in a production of Dol-PP-GlcNAc 36% lower than in the control (Fig.4A). A further decrease of Dol-PP-GlcNAc formation by

65% was observed when the *alg13Δ::hisG/TR*p*ALG13* strain was cultivated with doxycycline.

The presence of doxycycline in the cultivation medium of the *alg13Δ::hisG/TR*p*ALG13* strain resulted in a significant decrease of combined Alg7p and Alg13p activity (as measured by the formation of Dol-PP-GlcNAc$_2$) by 84% compared to the control, while without doxycycline this activity was lower by 55% than in the control strain (Fig.2, 4A).

Those activity assays performed in the membrane fraction showed how these enzymes worked in the enzymatic complex when the amount of substrate for Alg13p was limited by Alg7p activity and the Alg7p product was consumed by the action of Alg13p. To avoid this limitation we liberated both enzymes from the membrane fraction and analyzed their activity separately. The decreased expression of *ALG7* and decreased amount of Alg7 protein in the mutant strain cultivated without doxycycline resulted in a 74% lower activity of Alg7p compared to the control strain and 80% lower when doxycycline was added (Fig.2, 3, 4B).

The activity of Alg13p was nearly the same in the mutant strain in both cultivation conditions as in the control.

3.3. Cis-*prenyltransferase (*cis-*PT) activity*

The Alg7p *N*-acetylglucosaminyl transferase requires dolichyl phosphate (Dol-P) as the acceptor of the carbohydrate residue, therefore the amount of Dol-P

available affects the velocity of the Alg7p-catalyzed reaction, and, consequently, also that catalyzed by Alg13p. Dolichol is synthesized by *cis*-prenyltransferase (*cis*-PT) which uses monomers synthesized via the mevalonate pathway. To establish the possible regulatory role of the availability of the Dol-P acceptor for the reaction catalyzed by Alg7p we measured the activity of *cis*-PT in the studied strains and found it was decreased in the mutant. The lowest activity was observed for the *alg13Δ::hisG/TR*p*ALG13* strain cultivated without doxycycline (Fig.5).

*3.4. Defective Alg13p activity results in under-*N-*glycosylation of proteins*

A lower activity of Alg13p should result in defects in protein glycosylation. To verify this assumption we analyzed in the *alg13* strain the *in situ* activity of HexNAcase, which had previously been used to gauge protein glycosylation level in *C. albicans* [3, 38]. *C. albicans* was cultivated in a medium containing GlcNAc as the sole carbon source to induce HexNAcase production. Cell extracts were then run in a native gel, incubated with an artificial substrate (naphthyl-GlcNAc) and with tetrazolium salt for visualization. The mobility of HexNAcase on the gel should be increased by defective glycosylation. Indeed, the enzyme from the mutated strain displayed an increased electrophoretic mobility compared to that from the control strain CAI4, the most pronounced glycosylation defect being revealed for HexNAcase from the *alg13Δ::hisG/TR*p*ALG13* strain cultivated with doxycycline (Fig.6). Thus, the

lower activity of Alg13p resulted in under-glycosylation of proteins, as expected.

3.5. Sensitivity to tunicamycin

Tunicamycin specifically inhibits the activity of Alg7p [39]. Since the activity of Alg7p was inhibited to various extents in the *alg13Δ::hisG/TRpALG13* strain cultivated in the presence or without doxycycline, different sensitivity of this strain to tunicamycin could be expected. In the presence of tunicamycin growth of the control strain was partially inhibited while the *alg13Δ::hisG/TRpALG13* strain could not grow regardless of the doxycycline presence in the medium (Fig.7). Notably, addition of doxycycline to the medium partially inhibited growth of the *alg13Δ::hisG/TRpALG13* strain even in the absence of tunicamycin.

3.6. Defective activity of Alg13 alters morphology of C. albicans

C. albicans can grow in the unicellular form but also as pseudohyphae and true hyphae. It has previously been shown that *C. albicans* mutated in DPM synthase subunits cannot grow in the filamentous (hyphal) form [40]. Since Alg13p, similarly as DPM synthase [10, 41], is required for *N*-glycosylation we checked the influence of Alg13p depletion for hyphae formation. The mutant and the control strain were cultivated on horse serum medium (YPSerum), Spider medium and on YPD medium. As presented in Fig. 7 the CAI4 wild type strain

formed filaments on Spider medium and YPSerum while the

*alg13Δ::hisG/TR*p*ALG13*strain was unable to assume filamentous form with or

without doxycycline in the medium. In contrast, all the strains formed filaments

on YPD medium, although they were less frequent for

*alg13Δ::hisG/TR*p*ALG13*, especially when it was cultivated with doxycycline

(Fig.8).

3.7. Defective activity of Alg13p alters biofilm formation

Studies of *Candida* morphology indicate that filamentous growth is required for

stable biofilm formation [42]. Since the *alg13* strain revealed defects in

filamentation (Fig.9), we studied its ability to form biofilm.

Following cultivation on Petri dishes as described in Methods, non-adherent

cells were washed away and the biofilm left was examined under a light

microscope. Our analysis revealed that CAI4 needed 6 h to start forming the

biofilm while the *alg13Δ::hisG/TR*p*ALG13* strain cultivated without

doxycycline required more than 12 h (Fig.9). With doxycycline biofilm

formation took at least 48 h and still the biofilm did not overgrow the whole

surface of the plate.

The adhesion of *Candida* cells to synthetic materials has been shown to be

preceded by expression of adherence molecules, such as agglutinin-like proteins

(Als), hyphal wall proteins (Hwp) and cell wall glycoproteins [43-46].

Since we observed delayed biofilm formation, expression of *ALS1* and *HWP1* genes was analyzed in our strains after 6, 12 and 48 h of cultivation. Expression of *ALS1* was significantly lower in the *alg13Δ::hisG/TR*p-*ALG13*strain after 6 h of biofilm formation with or without doxycycline compared to the control strain (Fig.10). After 12 h the expression in the *alg13Δ::hisG/TR*p-*ALG13*strain cultivated without doxycycline was similar to the control while during cultivation with doxycycline it remained slightly lower. After 48 h of biofilm cultivation *ALS1* expression was undetectable in all the strains.

Expression of *HWP1* showed a strikingly different pattern. In in *alg13Δ::hisG/TR*p-*ALG13*strain cultivated without doxycycline it was ca. 1.5-fold higher than in the control during the first 12 hours and increased even further after 48 h of biofilm cultivation. Repression of *ALG13* gene by doxycycline reduced the expression of *HWP1* to ca. half of the control value throughout biofilm cultivation.

The *C. albicans* biofilm has a highly heterogeneous structure in which adherent cells are covered by extracellular matrix [42] containing extracellular DNA (eDNA), proteins and polysaccharides [47-49].

We studied the content of those components in the biofilm matrix of the *C. albicans* strains. After 12 h of biofilm formation its surface was rinsed to remove non-adherent cells and extracellular material, the suspension was centrifuged and supernatant was used for eDNA and glucan quantification. The

eDNA content in the extracellular material of the *alg13Δ::hisG/TR*p-*ALG13*
strain cultivated with or without doxycycline was, respectively, twice and 1.7
times that of the wild-type strain (Fig.11A).

The extracellular glucan content, determined as glucose following hydrolysis,
was also elevated in the *alg13Δ::hisG/TR*p-*ALG13*strain – by 45% and 22%,
respectively, with and without doxycycline compared to the control strain
(Fig.11B).

3.8. Defective activity of Alg13p influences cell wall structure

The *C. albicans* cell wall composition is similar to that of *S. cerevisiae* and
contains four classes of interacting components: chitin, β1,3 and β1,6 glucan,
and highly glycosylated mannoproteins [50,51].

To establish the influence of a decreased activity of Alg13p on the cell wall
composition it was isolated from the *C. albicans* strains and subjected to acidic
hydrolysis and the monocarbohydrate content was analyzed. The
*alg13Δ::hisG/TR*p-*ALG13* strain cultivated with or without doxycycline had a
lower concentration of mannose in the cell wall compared to the control strain
(Fig.12). The lowest mannose content, 49% of that in the control, was found in
the cell wall of *alg13Δ::hisG/TR*p-*ALG13* strain cultivated with doxycycline.
The amount of cell wall glucose was similar to that in the control strain. The
small differences observed for the *alg13Δ::hisG/TR*p-*ALG13*strain cultivated
with or without doxycycline were not statistically significant (Fig.12).

The content of the cell wall *N*-acetylglucosamine was decreased in the mutant, however, only the 50% decrease found for the *alg13Δ::hisG/TR*p-*ALG13*strain cultivated with doxycycline was statistically significant (Fig.12). Under these conditions this strain was also hypersensitive to Calcofluor White and Congo Red, agents affecting the cell wall integrity (Fig.7).

The cell wall composition was also analyzed after 12 h of biofilm formation. No differences in the glucose or mannose content were found between the control and the mutant (supplementary Fig.2S), and the slight decrease of the *N*-acetylglucosamine content in the cell wall of the mutant compared to the control strain was not statistically significant (supplementary Fig.2S).

4. Discussion

N-and *O*-glycosylation of proteins has been demonstrated to be important in the host-fungus interaction and for the expression of virulence of *Candida* [2, 3, 6, 52, 53].

In this work we have identified and characterized an essential *ALG13* gene from *C. albicans*, encoding Alg13p catalyzing the first step of LLO formation during *N*-glycosylation. We presented consequences of decreased expression and overexpression of *ALG13* in the heterozygous *alg13* strain cultivated, respectively, in repressing and derepressing conditions.

It is known that Alg13p forms an active complex with Alg7p and Alg14p with the 1:1:1 stoichiometry [16]. Hetero-oligomerization of the ER

glycosyltransferases contributes to the efficiency of LLO synthesis. LLO intermediates are channeled from one enzyme to the other without diffusion [16]. Such an organization results in feedback relationship between the products of the reactions catalyzed by Alg7p and Alg13p, where the second intermediate, Dol-PP-GlcNAc$_2$, inhibits the formation of the first, Dol-PP-GlcNAc [55]. In our study, cross-regulation of Alg7p, Alg13p and Alg14p expression and activity was also found.

As expected, an altered expression of *ALG13* affected the expression of the two other members of the predicted Alg7/13/14 complex. Overexpression of *ALG13*failed to increase activity of Alg13p most likely due to the compensatory lower expression and activity of Alg7p and lower expression of *ALG14*. It was shown that overexpression of Alg13p in *S. cerevisiae* led to its cytosolic partitioning, as did reduction of Alg14p level [55]. Potential shortage of Alg14p anchor protein during overexpression of *ALG13*could lead to excess of cytosolic Alg13p, whereas only membrane-associated Alg13p was reported to be stable [56]. Proteasomal degradation of excess unassembled Alg13p was found as an important quality control mechanism that ensured proper Alg7/13/14 complex assembly [56].

A low expression of *ALG13* in the regulatory strain cultivated with doxycycline where simultaneously expression of both *ALG7* and *ALG13* genes was decreased resulted in a further decrease of enzymatic activity.

The combined lower activity of the first two enzymes creating LLO limited protein *N*-glycosylation, as was shown for the model enzyme HexNAcase used earlier to monitor the *N*-glycosylation level in *Candida* [3, 38]. Glycosylation of this enzyme decreased with the decreasing activity of Alg13p. This effect was enhanced by the lower activity of *cis*-prenyltransferase in *alg13Δ::hisG/TR*p-*ALG13* strain and the consequently diminished dolichol level additionally decreasing the glycosylation [38].

The compromised protein *N*-glycosylation affected the pathogenicity attributes of *C. albicans*. It has previously been observed that the cellular processes and structures involved in *C. albicans* pathogenicity are connected with glycosylation (reviewed in [1]). They include molecules mediating adhesion to the host cell, secretion of hydrolases, and hyphae and biofilm formation [33, 42, 46, 57-59]. The adhesion is mediated by adhesins, glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins that are heavily *N-* and *O*-glycosylated [60]. *C. albicans* has a specialized set of adhesins (Als1-7 and Als9) which mediate adherence to other cells and to abiotic surface [1, 61]. An increased expression of adherence genes is a property of biofilm-forming cells. Various adhesin genes were differentially expressed in *C. albicans* under different host and model conditions [61], probably reflecting the preferential expression of only the appropriate, most effective adhesins in any given situation. *ALS1* was the most upregulated adherence gene of *C. albicans* under biofilm-inducing

conditions [62]. In the strains studied here with impaired glycosylation, processing of these glycoproteins could be altered.

Notably, the expression of *ALS1* gene was lower in the Alg13-deficient cells compared to the control strain. Since biofilm formation is connected with hyphal growth we also investigated the expression of *HWP1* gene encoding a hyphae-associated GPI-linked protein, known for its role in host cell attachment and biofilm formation [1, 45, 63, 64]. The *alg13Δ::hisG/TR*p-*ALG13* strain cultivated without doxycycline revealed a gradual increase of *HWP1*expression during 48 h of cultivation. Since a delayed biofilm formation was observed it seems likely that this was a consequence of the delayed expression of hyphal proteins and thus the formation of hyphae. Cultivation of the *alg13Δ::hisG/TR*p-*ALG13* strain with doxycycline resulted in very low expression of *HWP1*gene coincident with only sporadic production of hyphae. This in turn prevented or greatly delayed biofilm formation.

In this study we demonstrated that both increased and decreased expression of *ALG13* resulted in filamentation defects. It has been shown that inhibition of the first reaction in LLO assembly by tunicamycin alters filamentation and yeast bud formation in *Candida* [65, 66]. Richard et al. [34] have shown that defects in hyphae formation hamper retention of cells in the biofilm since hyphae provide an adherent scaffold stabilizing the biofilm structure. In our study the control strain CAI4 formed filaments independently of the medium (YPSerum, Spider medium and YPD) used. In contrast, the residual capacity of the

*alg13Δ::hisG/TR*p-*ALG13* strain to produce filaments could only be revealed on the glucose-containing medium YPD. It has been reported [33] that a lack of glucose in the cultivation medium limits cells adhesion and biofilm formation, both significantly inhibited by the impaired *N*-glycosylation in the *alg13Δ::hisG/TR*p-*ALG13* strain.

Another factor among biofilm stabilizers is the extracellular matrix containing, i.a., eDNA and glucan [48, 49]. Our study showed that the *alg13Δ::hisG/TR*p-*ALG13* strain poorly forming biofilm actually released higher amounts of the components of the extracellular matrix. eDNA is required for the initial attachment as well as for subsequent early phases of biofilm formation in several bacterial species, but only for few species the DNA is an important structural component of mature biofilms [67]. Since the *alg13Δ::hisG/TR*p-*ALG13* strain showed a significant delay in biofilm formation, the release of large amounts of eDNA and glucan could represent a compensatory reaction necessary for the initial establishment of the biofilm.

The defective *N*-glycosylation also altered the cell wall composition. In general, the Alg13p-deficient strain cultivated in liquid medium contained less mannose and *N*-acetylglucosamine in its cell wall compared to the control strain. *O*- and *N*-linked mannans, along with β-glucans, represent the main *C. albicans* pathogen-associated molecular patterns recognized by the innate immune system. Mannans play important roles in cell wall integrity, adhesion to

host cells and tissues, virulence, and the establishment of a response by immune cells [5, 68].

On the other hand, during biofilm growth the carbohydrate content was nearly the same in the cell wall of the mutant and the control strain. This reflected differences in the cell wall composition of the strains growing in the biofilm form and in the liquid medium. We observed lower amounts of *N*-acetylglucosamine in the strains growing in biofilm conditions compared to the liquid cultures and some differences in mannose content. It seems interesting that under biofilm inducing conditions the cell wall composition of the surface associated cells of all the strains became similar to each other.

In summary, we showed that *C. albicans orf19.6025* identified as homologous to *S. cerevisiae ALG13* gene encodes an Alg13p *N*-acetylglucosaminyldiphosphodolichol: *N*-acetylglucosaminyltransferase, a member of potential Alg7/13/14 complex. Altered expression of this gene influenced the expression of the other members of the complex. A decreased activity of Alg7p and Alg13p caused under-glycosylation of proteins, hypersensitivity to Calcofluor White and Congo Red, changes in cell wall composition, hyphae formation and biofilm growth.

These results confirm the important role of protein *N*-glycosylation in the pathogenic potential of *C. albicans*.

Acknowledgements

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Figures Legend

Figure 1

Deduced amino acid sequences of *C.albicans* Alg13 and Alg14 proteins and their alignment with sequences of homologous proteins of *S. cerevisiae*. Potential membrane spanning regions of *Candida* proteins are indicated by black line above the sequence.

Figure 2

Transcript levels of *ALG7*, *ALG13* and *ALG14* genes determined by RT-qPCR. *C. albicans* strains were grown for 16 h in YPD medium, RNA was extracted and cDNA synthesized. *alg13Δ::hisG/TRpALG13* strain was cultivated in repressive (plus doxycycline) or derepressive (without doxycycline) conditions. qPCR reactions were performed using a LightCycler 1.6 instrument. The crossing point (Cp) value (cycle number in the log-linear region) was calculated using the LightCycler quantification software. Data obtained from three independent experiments each determined in triplicate.

Figure 3

Immunodetection of *C. albicans* Alg7 protein in the membrane fraction of *alg13Δ::hisG/TRpALG13* strain and the control with human anti-Alg7 protein antibody.

Serial 1:10 dilutions of membrane proteins were applied onto Immobilon P membrane.

CAI4 - control strain,

alg13Δ::hisG/TRpALG13 strain cultivated without (-Dx) or with (+Dx) doxycycline

Figure 4

Accumulation of products of *N*-acetylglucosaminyl transferases Alg7 and Alg13.

A - combined enzymatic activity in membrane fraction

B - individual enzymatic activity after solubilization

CAI4 - control strain,

alg13Δ::hisG/TRpALG13 strain cultivated without (-Dx) or with (+Dx) doxycycline

Data are mean ± standard deviation from three independent experiments each determined in triplicate.

Stars indicate statistically significant differences (P<0.05; *t* test)

Figure 5

Activity of *cis*-prenyltransferase in membrane fraction.

CAI4 - control strain,

*alg13Δ::hisG/TR*p*ALG13* strain cultivated without (-Dx) or with (+Dx)

doxycycline

Data are mean ± standard deviation from three independent experiments each

determined in triplicate.

Stars indicate statistically significant differences (P<0.05; *t* test)

Figure 6

Activity of HexNAcase in native gel.

Differences in mobility result from different extents of glycosylation of the

enzyme. Strains were cultivated in the presence of *N*-acetylglucosamine to

induce production of HexNAcase. Fifty micrograms of cell free extract proteins

was run on native gel. The activity was developed as described in the Methods.

1- CAI4; 2- *alg13Δ::hisG/TR*p*ALG13, -*Dx; 3 - *alg13Δ::hisG/TR*p*ALG13, +*Dx

Figure 7

Effect of *ALG13* up- and down-regulation on *C. albicans* sensitivity to selected

agents.

Control strain CAI4 and the *alg13Δ::hisG/TR*p*ALG13* strain were cultivated

with or without doxycycline. Serial 1:10 dilutions were plated on YPD plates

supplemented with tunicamycin, Congo Red or Calcofluor White and cultivated

at 30°C for 72 h.

Figure 8

Hyphal growth of *C. albicans* wild-type strain CAI4 and *alg13* strain.

Hyphae formation was induced for 7 days at 30°C on YPSerum, Spider medium and YPD medium plates supplemented or not with doxycycline as indicated and colonies were photographed under a light microscope.

Magnification used is indicated on the right.

Figure 9

Biofilm formation by *C. albicans* wild-type strain CAI4 and *alg13* strain on YPD medium. Three milliliters of cell suspensions (1 x 10^6 cells per ml) were transferred into sterile 5.5-mm Petri dishes and incubated at 37°C. Non-adherent cells were washed away, biofilm was dried and photographed under a light microscope.

Magnification, x10.

Figure 10

Transcript levels of *ALS1* and *HWP1* genes determined by RT-qPCR.

Biofilm of the control strain CAI4 and *alg13* mutant was grown for 6, 12 and 48 h on YPD medium, RNA was extracted and cDNA synthesized.

alg13Δ::hisG/TRpALG13 strain was cultivated in repressive (plus doxycycline) or derepressive (without doxycycline) conditions. qPCR reactions were performed using a LightCycler 1.6 Instrument. The crossing point (Cp) value (cycle number in the log-linear region) was calculated using the LightCycler quantification software. Data obtained from three independent experiments each determined in triplicate.

Figure 11

eDNA and glucan content in extracellular matrix of biofilm.

Culture medium was decanted and the biofilm was washed twice with 5 ml of water. The liquids were pooled, centrifuged to remove cell debris, dialyzed against water and lyophilized. eDNA was extracted and its content was measured spectrophotometrically.

Extracellular glucan was hydrolyzed and glucose content was determined by High Performance Anion-Exchange Chromatography.

A- eDNA content in matrix

B- amount of glucose obtained by acidic hydrolysis of extracellular glucan

Data are mean ± standard deviation from three independent experiments each determined in triplicate.

Black star indicates statistically significant differences (P<0.05; *t* test)

Figure 12

Cell wall composition of wild type CAI4 strain and the *alg13* strain.

C. albicans strains were grown in liquid YPD medium supplemented (+Dx) or not (-Dx) with doxycycline. Major carbohydrates were determined using High Performance Anion-Exchange Chromatography in cell wall preparation hydrolyzed with 2 M TFA.

Glucose (Glc), mannose (Man) and glucosamine (GlcN) content in the cell wall

Data are mean ± standard deviation from three independent experiments each determined in triplicate.

Stars indicate statistically significant differences (P<0.05; *t* test)

Figure 1S

ALG13 organization in *C.albicans* genomic DNA.

A-southern blot of *ALG13*

BanI, BglII and HindIII digested DNA of the control strain CAI4 (lanes 1,2,3),

hemizygote *alg13Δ::hisG/ALG13* (lanes 4,5,6) and the

*alg13Δ::hisG/TR*p*ALG13* strain (lanes 7,8,9)

The length indicated under each band represents a predicted DNA size.

B-schematic representation of restriction analysis of *ALG13*

C-schematic representation of *ALG13* under the regulatable promoter

Figure 2S

Cell wall composition of biofilm of wild type CAI4 strain and the *ALG13*

defective strain.

C. albicans strains were grown in liquid YPD medium supplemented (+Dx) or

not (-Dx) with doxycycline. Major carbohydrates were determined using High

Performance Anion-Exchange Chromatography in cell wall preparation

hydrolyzed with 2 M TFA.

glucose (Glc), mannose (Man) and glucosamine (GlcN) content in the cell wall

Data are mean ± standard deviation from three independent experiments each

determined in triplicate.

Alg13p

```
                        *        20        *        40        *        60        *
C.albicans   : MFDWRRLKPLRPHQIFFFYTTIQNYSDNGYQYSYKSNQIKSNQIKLNQQQQTSRQLIRINNQLPPSVVLH :  70
S.cerevisiae : ------------------------------------------------------------------MG :   2

                        *        80        *       100        *       120        *       140
C.albicans   : NTIMKSILITTGATITESLIQIIVSPQBLNNLIRLKINFLIIQYGHEIKNSINLSESFFNFTINKYDLI : 140
S.cerevisiae : ILEEKALFVTCGATVPFPKLVSCVLSDEPCQPLIQYGFVRLIIQFGRNYSSEFEHLV---QFRGG---QR :  66

                        *       160        *       180        *       200        *
C.albicans   : NLFNLELEPTPIGDDDDDEGIRLFKNSDIEILAFSYSSNINKYIE-NVDLIISHAGTGSIIDQLHLNKPI : 209
S.cerevisiae : ESQKIPIDQFGCGI---TARQYVLMNGKLKVIGRDFSTFMQSIIRDYSDLVISHAGTGSILDSLRLNKPI : 133

                        *       220        *       240        *       260        *       280
C.albicans   : IVIVNDELMDNHQLEIAQQFTRLNYCIYYSIPELEQYVNNNDNNKDSRFWNQLNQLINGELQINKLFQTD : 279
S.cerevisiae : IVCVNDSLMDNHQQQIADRFVELGYVWSCAPTETGLIAGLRASQ---------------TEKLKPFPVSH : 188

                        *
C.albicans   : GSIIPTIICPELEK : 293
S.cerevisiae : NPSFPRLLVETIYS : 202
```

Alg14p

```
                        *        20        *        40        *        60        *
C.albicans   : MDIETAACFSIAFIATBILIV----LVRLLFILESLRLPTSVK-----------KKFKLIQECQLSILLG :  55
S.cerevisiae : --------MKTAYLASLVLIVSTAYVIRLIAILEFFHTQAGTEKDTKDGVNLLKIRKSSKKPLKIFVFLG :  62

                        *        80        *       100        *       120        *       140
C.albicans   : SGGHTGEMMRIISKLDMGKVSRTAIYTSG-DNASLARAQDYFEKSE-TSSQKIPIPRARIVGQSYTSSIP : 123
S.cerevisiae : SGGHTGEMIRLLENYQDLLLGKSIVYLGYSDPASRQFAHRIKKFGHCKVFYYEFMKARFVKATLLQSVK : 132

                        *       160        *       180        *       200        *
C.albicans   : TTLYSPFFS------AIAMLKHRFAVILLNGPGTCVFVAYILFLYKLL--GLCNTFIIYIESLARVNKLS : 185
S.cerevisiae : TIIGLLVQSFVHVVRIRFAVCGSRHLFLLNGPGTCCIISFALFLMFLLFPLLGSSHIVYVESLARINTFS : 202

                        *       220        *       240        *
C.albicans   : LSGLLLLPTSDRFIVQWFSLYQQNS-RVFVYGILI : 219
S.cerevisiae : LTGKILYWVVDPFIVQWQFLRDNYLFRSWNFGILV : 237
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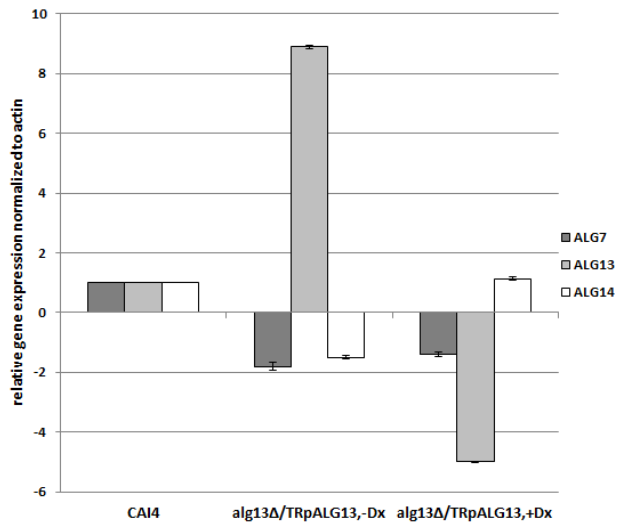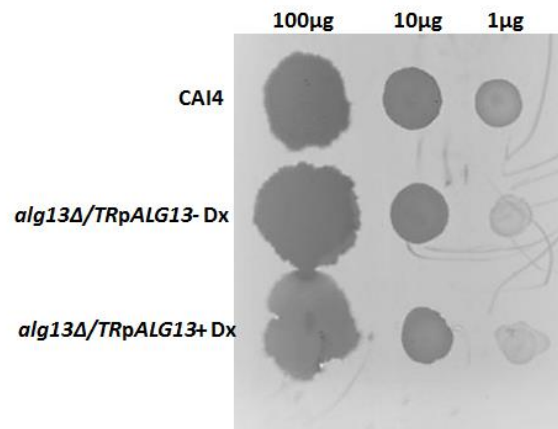
Fig1

Fig2

	100μg	10μg	1μg
CAI4			
alg13Δ/TRpALG13- Dx			
alg13Δ/TRpALG13+ Dx			

Fig3
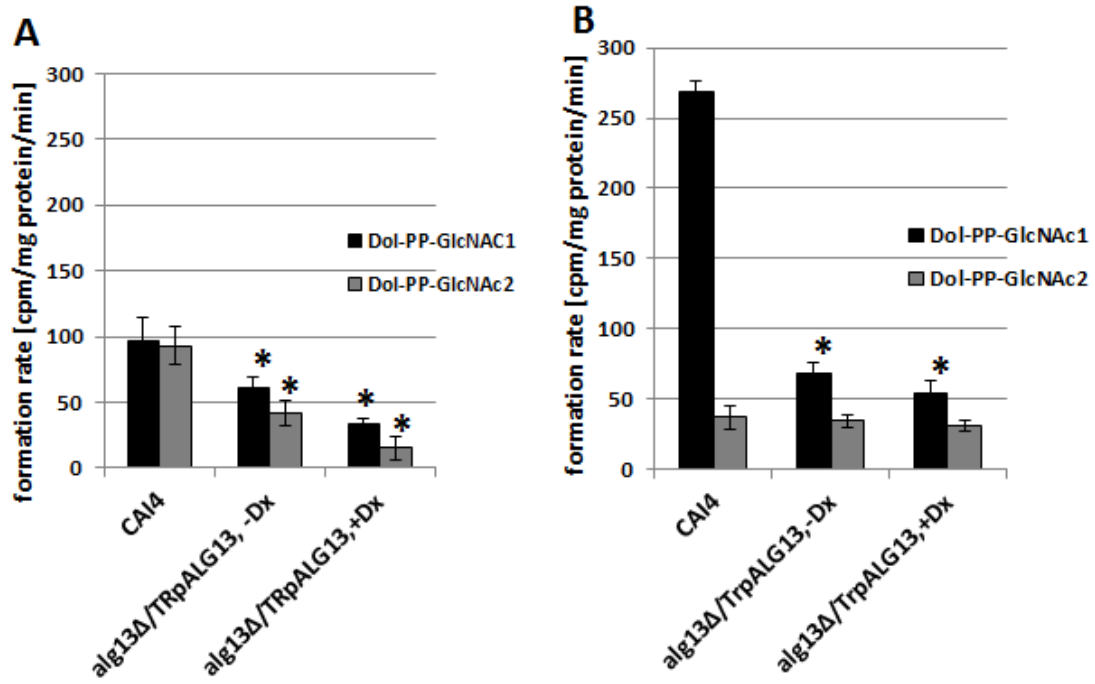
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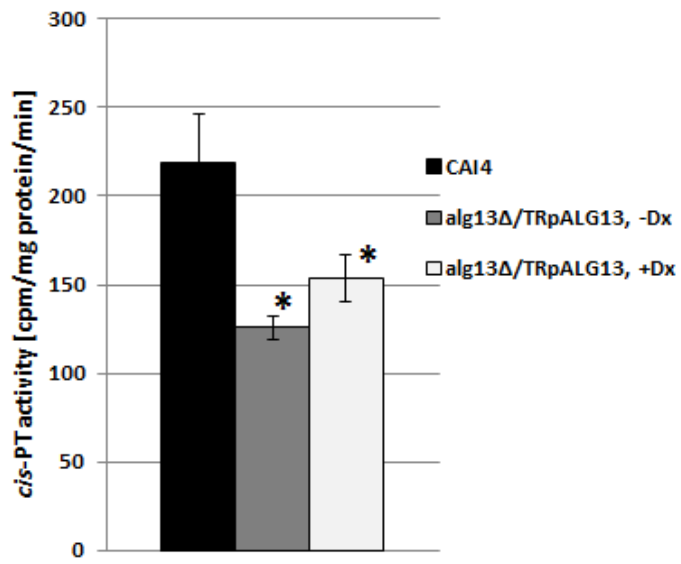
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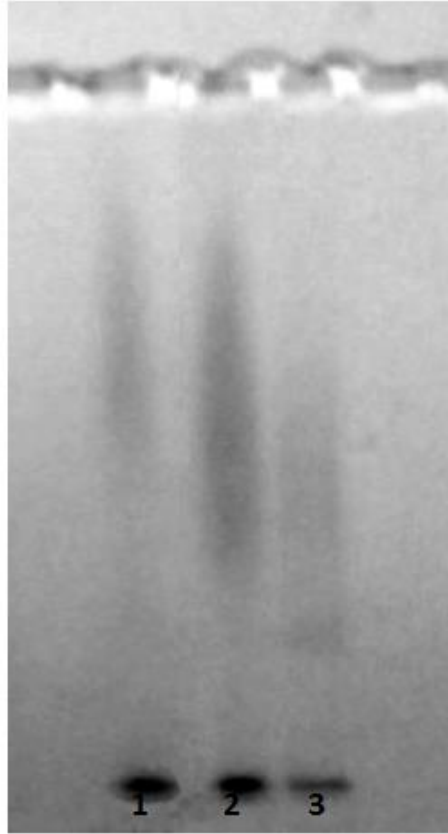
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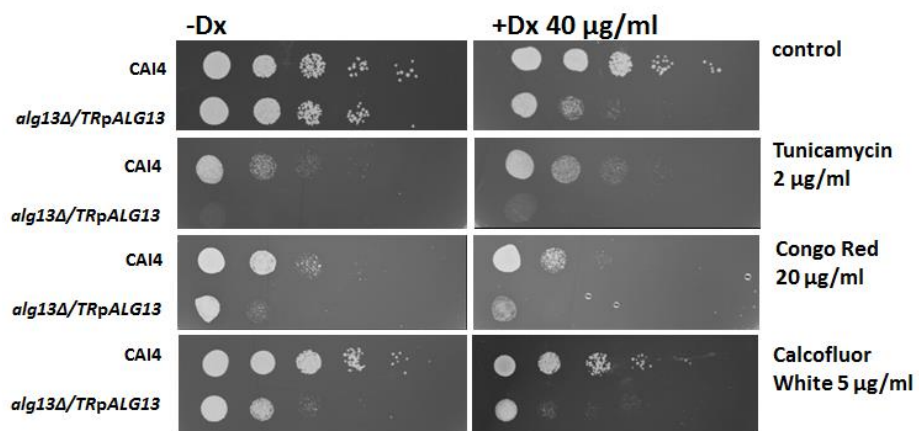
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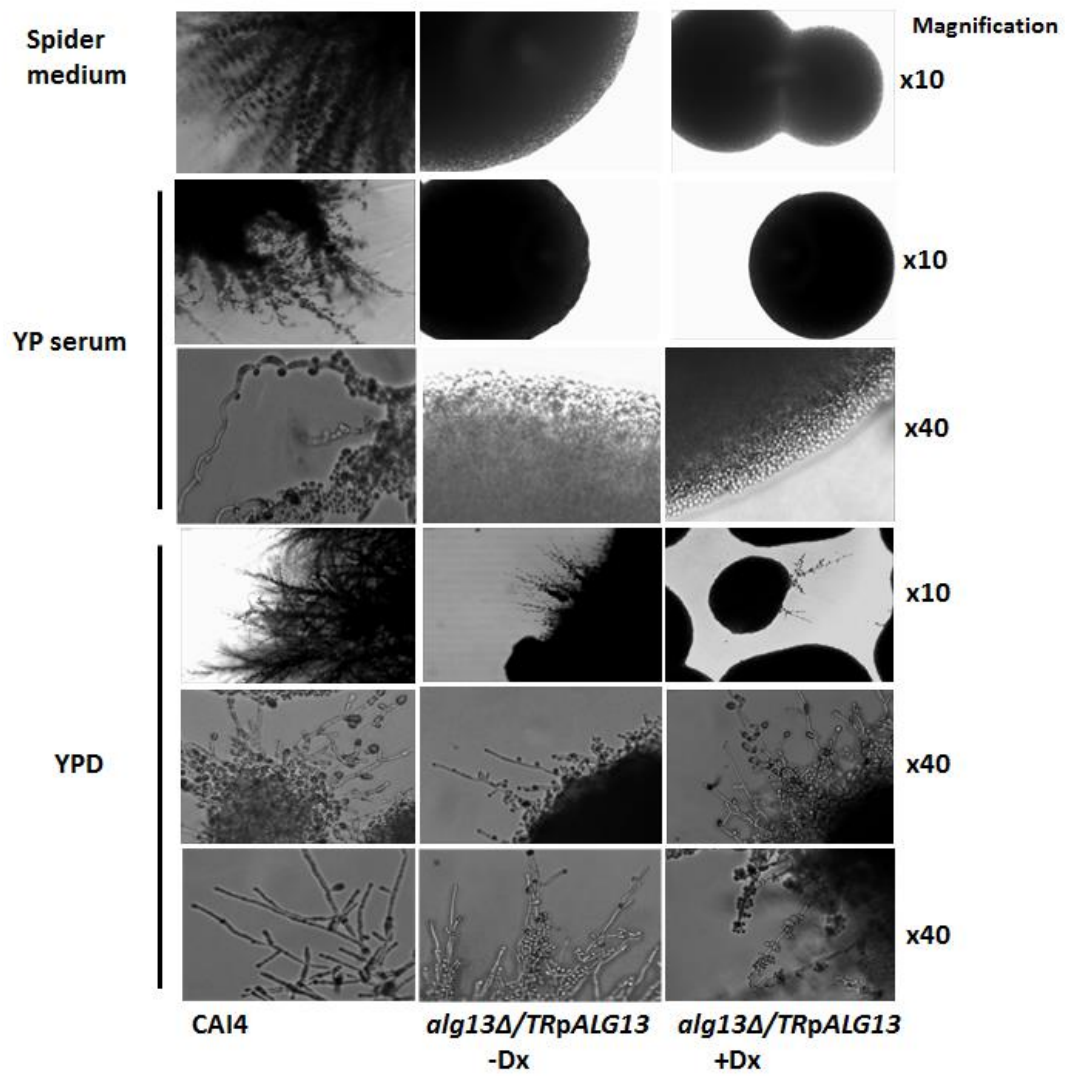
Fig8

Fig9

Fig10

Fig11

Fig12