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Use of the Porcine Intestinal Epithelium (PIE)-Assay to analyze early stages of colonization by the human fungal pathogen *Candida albicans*

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Virulence of *C. albicans* strains can be tested using a mouse model of haematogenously disseminated *Candida* cells. Initial steps of host-pathogen contact such as adhesion and colonization are not taken into account due to the injection of *Candida* cells into the blood stream. Here we describe an assay, based on the *ex vivo* usage of porcine intestinal epithelium (PIE), that is useful to monitor the early stages of a *C. albicans* infection. The ability of *C. albicans* to undergo morphogenetic switching between yeast and hyphal stages is thought to contribute to its virulence. We found that hyphal formation was required to allow cells to colonize the PIE. The non-filamentous mutant strains *efg1/cph1* which lacks two of the central transcription factors that are required to promote hyphal growth and *wal1* that carries a deletion of the *C. albicans* homolog of the human Wiskott-Aldrich Syndrome Protein and is deficient in endocytosis showed only weak adherence. Furthermore, the *wal1* mutant was found to be reduced in virulence using the mouse tail vein injection assay. We also analyzed the colonization properties of a variety of other mutant strains carrying deletions of either secreted aspartyl proteinase (SAP)-family genes or amino acid permease encoding genes (*GAP1, SSY1,* and *PUT4*). Interestingly, the *nag5* strain which lacks an N-acetylglucosamine kinase showed enhanced filamentation and invasive growth as well as increased resistance against farnesol.

The human fungal pathogen *Candida albicans*, which can cause life-threatening systemic infections in immuno-compromised individuals, is able to use several routes of entry into the host. This includes the oro-pharyngeal tract; the genito-urinary tract as well as the gastro-intestinal tract. In otherwise healthy individuals *C. albicans* may be found as a commensal on these mucosal surfaces as well as on the skin. Under certain circumstances, e.g. immuno-suppression or antibiotic treatment, *C. albicans* is able to invade the host and cause disease. Several virulence determinants that appear to aid in this process of colonization and infection have been described (YANG 2003). Among these is the ability of *C. albicans* cells to adhere to the host tissue and to plastic surfaces such as in catheters, the expression of secreted aspartyl proteinases (Saps) which are encoded by a large gene family, as well as the yeast-to-hyphal transition including the expression of hyphal specific genes (BERMAN and SUDBERY 2002, NAGLIK *et al.* 2003, SHEPPARD *et al.* 2004).

The ability of *C. albicans* to use amino sugars, e.g. N-acetylglucosamine (GlcNAc), as carbon sources was shown to depend on a catabolic pathway gene cluster which includes the GlcNAc-kinase Nag5 (KUMAR *et al.* 2000). Disruption of genes in this pathway has been shown to attenuate virulence and adhesion in *C. albicans* (SINGH *et al.* 2001). Specifically,

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deletion of *NAG5*, which encodes a GlcNAc-kinase, resulted in mutant strains that were avirulent in a mouse model of systemic infection (YAMADA-OKABE *et al.* 2001).

Similarly, defects in sensing amino acids or in the up-take of amino acids due to the deletions of the *C. albicans* homologs of *S. cerevisiae SSY1* or *SHR3* genes encoding an integral plasma membrane protein which is part of the amino acids sensor complex and a protein which is required for correct targeting of amino acid permeases, respectively, result in defects in amino acid uptake and in the inability to induce hyphal growth in response to amino acids (BREGA *et al.* 2004, MARTINEZ and LJUNGDAHL 2004).

Current models to analyze early stages of colonization and infection by *C. albicans* mutants use reconstituted human epithelia. The role of the transcription factors Efg1 and Cph1 during adhesion and invasion was examined in a model of human epidermis and in a reconstructed human intestinal model (DIETERICH *et al.* 2002). The involvement of Sap1 and Sap2 to promote tissue damage was demonstrated in a reconstituted human vaginal epithelium (SCHALLER *et al.* 2003).

In this study we have used an *ex vivo* model of porcine intestinal epithelium (PIE) to assay adhesion and filamentation properties of selected *C. albicans* strains in a qualitative manner during the early steps of colonization. We found that the *nag5* mutant strain shows increased filamentation on the PIE and characterized this strain in more detail. With the PIE-assay one can compare localized assaults of *C. albicans* cells to haematogenously inflicted disseminated candidiasis in order to evaluate the virulence of *C. albicans* mutants under differing environmental conditions.

Materials and methods

Strains and media: Strains used in this study are listed in Table 1. Media used: YPD (1% yeast extract, 2% peptone, 2% glucose); CSM (complete supplement mixture; 6.7 g/l YNB with ammonium sulphate without amino acids, 0.69 g/l CSM base; 20 g/l glucose, 20 g/l agar, plus the required amino acids) or SD (6.7 g/l YNB with ammonium sulphate without amino acids, 20 g/l glucose, 20 g/l agar, plus the required amino acids). Growth conditions were used as described previously (WALTHER and WENDLAND 2004). Hyphal induction was carried out at 37 °C in minimal media containing either 10% serum, 0.5 g/l proline or 0.5 g/l N-Acetyl-glucosamine (GlcNAc), respectively.

Table 1
Strains used in this study

Strain[a]	Genotype	Reference
SC5314	*Candida albicans* wild type	GILLUM *et al.* (1984)
DYS459	*sap4::hisG/sap4::hisG, sap5::hisG/sap5:URA3, sap6::hisG/sap6::hisG*	SANGLARD *et al.* (1997)
HLC54	*cph1::hisG/cph1::hisG, efg1::hisG/efg1::URA3*	LO *et al.* (1997)
BWP17	*ura3::λimm34/ura3:λimm34, arg4::hisG/arg4::hisG, his1::hisG/his1::hisG*	WILSON *et al.* (1999)
M119	*sap1::hisG/sap1::hisG, sap2::hisG/sap2::hisG, sap3::hisG/sap3::URA3*	KRETSCHMAR *et al.* (2002)
Δsap9/Δsap10	*sap9::hisG/sap9::hisG, sap10::hisG/sap10::hisG,*	ALBRECHT *et al.* (2005)
CAT6	*wal1::HIS1/wal1::URA3, arg4*	WALTHER and WENDLAND (2004)
GC30	*nag5::HIS1/nag5::URA3, arg4*	This study
SIC1	*put4::HIS1/put4::URA3, arg4*	This study
SIC2	*gap1::HIS1/gap1::URA3, arg4*	This study
SIC3	*ssy1::HIS1/ssy1::URA3, arg4*	This study

[a] CAT6 and all strains produced in this study are derivates of BWP17 with the indicated genotypic alterations

Targeting of *C. albicans* genes: The *C. albicans GAP1, NAG5, PUT4*, and *SSY1* genes were identified in the genomic sequence (http://www-sequence.stanford.edu/group/candida). Deletions of the complete open reading frames of both alleles of each gene were performed by PCR-generated *URA3* and *HIS1* disruption cassettes containing 100bp of target homology region at both ends of the cassettes as described (GOLA *et al.* 2003, SCHAUB *et al.* 2006). Transformation was carried out using the lithium acetate protocol (WALTHER and WENDLAND 2003). For each gene at least two independent homozygous mutants were generated and analyzed. Disruptions were verified by PCR on whole yeast cells. Correct integration of the selectable marker genes into the target ORF was analyzed with the use of diagnostic primers located in the target locus and within the selectable markers used. Absence of the target ORF in homozygous mutants was also verified by PCR. Primers used for the construction of cassettes and the verification of deletions are available upon request.

Porcine Intestinal Epithelium (PIE)-Assay: The PIE was obtained from freshly slaughtered pigs from the local slaughterhouse. Female donors were preferentially used based on availability and in order to avoid potential problems of variability based on gender. The animal organ parts were placed in Ringer's solution (9.0 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $CaCl_2$, 0.1 g/l $NaHCO_3$, 1 g/l glucose) and transported form the abattoir to the institute in ice-cooled glass beakers. Upon arrival the organ parts were cleared of food remains and rinsed with water to remove the resident microbiota from the mucosa. Pieces of the pig gut were cut to app. 20 cm^2 pieces and placed into petri dishes containing Ringer's solution without submerging the PIE. Ringer's solution in itself does not induce hyphal formation in *C. albicans*. *C. albicans* strains used for the PIE-assay were pre-grown overnight in full medium liquid cultures and applied to the PIE as yeast cells at cell densities of 1×10^6 to 1×10^7 cells/cm^2. The PIE-assay was then incubated at 37 °C without shaking for one to seven hours with or without application of 5% CO_2. Thus the total time that elapsed from slaughtering of the animal to the beginning of the incubation period was generally less than 2 h. After this incubation the PIE was washed rigorously three times either with buffer or water to remove all non-adherent cells from the epithelium. Surface cell layers containing epidermal and fungal cells were then scraped off and stained with Calcofluor to specifically stain the cell wall of *C. albicans* cells followed by fluorescence microscopy. Cells in the wash fraction were counted as cfu after plating on YPD and incubation for two days.

Microscopy and staining procedures: Images were acquired on a Zeiss AxioplanII-Imaging microscope (Zeiss, Jena and Göttingen) using the Metamorph4.6 software package (Universal Imaging Corp.) and a MicroMax1024 CCD-camera (Princeton Instruments). Chitin staining was done by directly adding 1 µl calcofluor (1 mg/ml) to a 100 µl cell suspension scraped off from the top epithelial layer of the PIE followed by an incubation of 15 min at room temperature and a subsequent washing step to optimize the signal-to-noise ratio.

Virulence assay: *C. albicans* strains were grown in complete medium at 30 °C, harvested, washed and resuspended in sterile water to the required cell density. Five mice (Swiss outbred strain, 6 week old females) per test and *C. albicans* strain were injected with up to 1×10^6 cells via the lateral tail vein. Survival of mice was checked three times daily for up to six weeks.

Hyphal induction in farnesol containing media: Cells were pre-grown in either YPD or minimal media and then inoculated at a cell density of $OD_{600} = 0.1$ in fresh media supplemented with GlcNAc and farnesol as indicated. (E,E)-Farnesol (trans-farnesol) was purchased from Sigma (St. Louis, MO.). Stock solutions of farnesol were prepared with ethanol and added at the given concentrations. Cell morphologies were counted as percent of yeast, pseudohyphal or hyphal cells (n = 150 to 300 cells for each condition).

Results

Setting up the PIE-Assay

Since the gastro-intestinal tract may serve as one of the main entry routes of *C. albicans* into the human host to elicit systemic infections we set out to establish a model system that very closely resembles this situation. Additionally, this *ex vivo* model should be readily acces-

sible, straightforward in all handling procedures and not being limited by difficulties in obtaining supplies. To this end we decided to analyze the early stages of colonization of *C. albicans* on porcine intestinal epithelia (PIE). Organ parts were made available by the local Jena slaughterhouse directly from freshly slaughtered pigs and for the course of these experiments epithelia derived from either colon or caecum were used (see Materials and methods for details).

Non-filamentous C. albicans strains show weak adherence to the PIE

To demonstrate the usefulness of this model we initially tested two strains on the PIE: the *C. albicans* wild type strain SC5314 and the non-filamenting *wal1* mutant, which lacks the *C. albicans* homolog of the human Wiskott-Aldrich Syndrome Protein. In a previous study we have already shown that *wal1* cells are unable to filament under all conditions tested *in vitro* (WALTHER and WENDLAND 2004). Yeast cells of both strains in exponential growth phase were applied to the PIE. At various time points epithelium was rinsed to remove non-adherent cells and then parts of the surface layer of the epithelium with the adherent cells were removed, stained with calcofluor to specifically mark fungal cells, and analyzed by fluorescence microscopy (Fig. 1).

In the PIE-assay the wild type demonstrated strong adherence to the epithelium and vigorously initiated filamentous growths. After 4.5 h hyphae of the wild type were elongated and septate. Wild type cells also were found to clump together quite tightly. This indicated a strong response of wild type cells to this epithelial environment allowing fast colonization and spreading via the initiation of mycelium formation. In contrast, *wal1* cells produced a much weaker response on the epithelium. Hyphal development was impaired under these conditions corroborating our previous *in vitro* results using liquid and solid media. Cell clumping was not observed in contrast to the wild type. Adhesion was also much weaker with *wal1* cells since after six hours a large fraction of cells could be found in the wash frac-

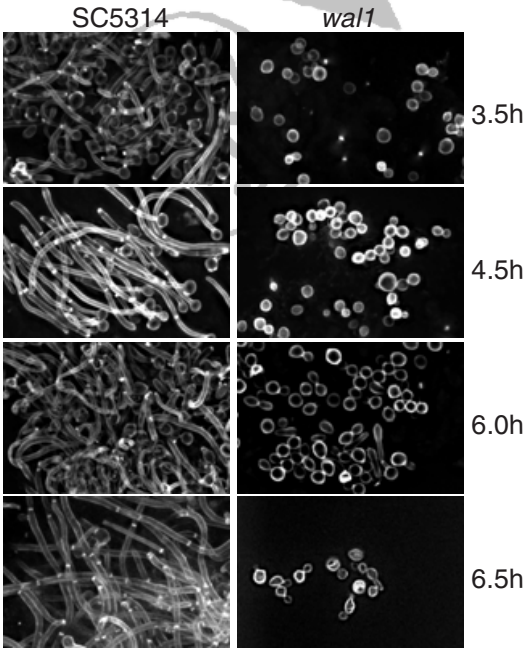

Fig. 1
Filament formation results in strong adherence to the PIE
The PIE-assay was performed using the *C. albicans* wild type strain SC5314 and the *C. albicans wal1* mutant. Incubation of the PIE with the indicated strains was done for several hours. Subsequent preparations and staining were done as described (see Materials and methods)

tion. Since during this time fungal cells also proliferated, quantification of the percentage of cells that did not adhere to the epithelium was compared to the wild type based on colony forming units present in the wash fraction. This indicated that less than 20% of *wal1* cells were able to adhere to the epithelium. Therefore, the ability to adhere to the porcine epithelium is drastically increased during the hyphal growth phase.

These results demonstrated the usefulness of this assay to evaluate general adhesion, filamentation and agglutination properties of *C. albicans* strains. The *wal1* mutant was afilamentous and showed low adhesion abilities on the PIE. Thus penetration of *wal1* cells into the tissue and dissemination in the bloodstream is unlikely to occur. To analyze the virulence potential of *wal1* cells in a standard assay we used this strain in a systemic murine tail-vein infection assay (Fig. 2). Injecting *C. albicans* cell directly into the bloodstream eliminates the need to cross epithelial barriers that normally could withstand such an infec-

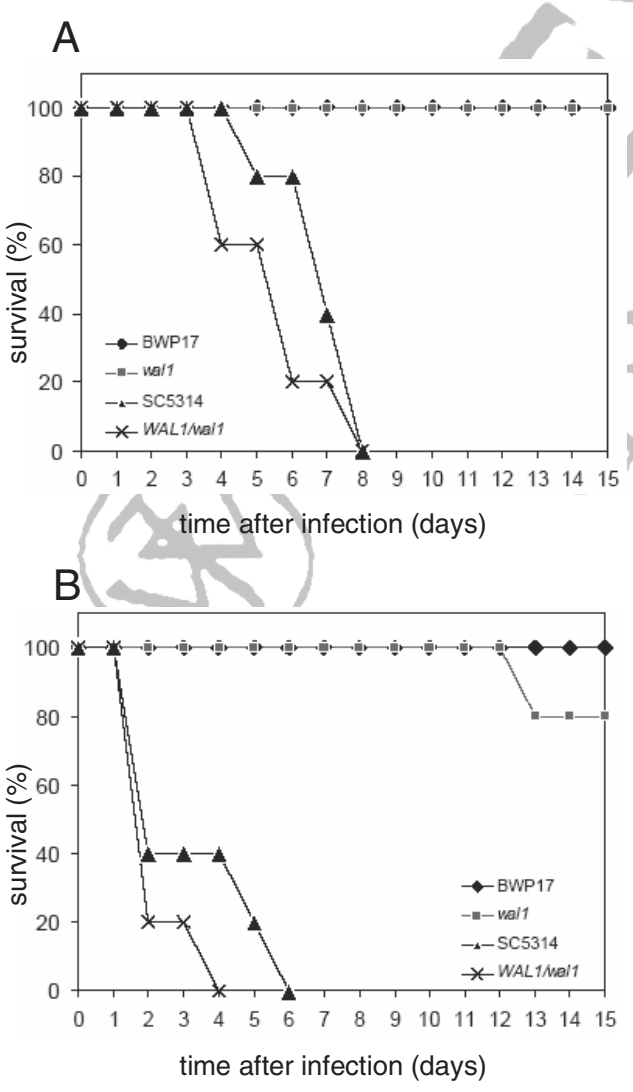

Fig. 2
In vivo mouse model of systemic infection to monitor the virulence of *wal1* cells
Survival curves of mice (5 in each group) that were intravenously infected with either 5×10^5 (A) or 1×10^6 (B) cells of the indicated strains

tion. Therefore, we assumed that the *wal1* mutant with its filamentation defects might also be attenuated in its virulence. Using low doses of *C. albicans* showed that the *wal1* strain was in fact severely reduced in its virulence. Higher doses (1×10^6 cells) still showed highly attenuated virulence but revealed some lethality in the mouse assay (Fig. 2).

Analysis of morphological parameters of C. albicans mutant strains in the PIE-assay

Next we went on to generate several mutant strains with the aim to analyze their colonization behaviour in the PIE-assay. To this end we deleted the *C. albicans PUT4, GAP1,* and *SSY1* genes encoding a proline transporter, a general amino acids permease, and a component of the amino acid sensor system consisting of Ssy1, Ptr3, and Ssy5, which senses external amino acid concentration, respectively. These strains were used to monitor the impact of interfering with amino acid sensing or uptake on the colonization of the PIE.

N-acetylglucosamine (GlcNAc) is one of the major compounds of the mucus gel layer that covers intestinal epithelia (DEPLANCKE and GASKINS 2001). *C. albicans* encodes several genes that allow the utilization of GlcNAc as sole carbon source. Deletion of *NAG5* was shown to markedly reduce the virulence of *C. albicans* in the mouse systemic infection model (YAMADA-OKABE *et al.* 2001). To test whether deletion of *NAG5* also plays a morphogenetic role during growth on the PIE we generated a *nag5* deletion strain in the BWP17 strain background. All deletions were done by PCR-based gene targeting approaches and the mutant strains were verified by diagnostic PCR (see SCHAUB *et al.* 2006). The *efg1/cph1* mutant was used as an afilamentous control strain. Additionally, we included various *sap* mutant strains (provided by B. HUBE, Berlin) carrying deletions in genes encoding secreted aspartyl proteases in this assay since the mucus also consists of glycoproteins secreted by goblet cells (DEPLANCKE and GASKINS 2001). All of the mutant strains that were capable of filament formation also adhered to the epithelium (Fig. 3). This suggests that the mutants tested were neither experiencing any nutritional shortages nor were they affected in their morphogenetic responses on the epithelium. Rather we found that the *nag5*

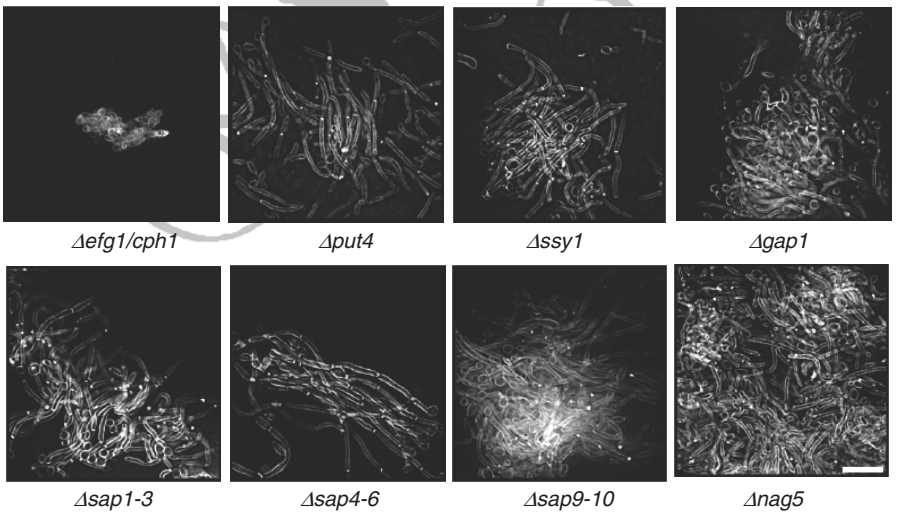

Fig. 3
Analysis of several mutant strains in the PIE-assay
The PIE assay was performed with a collection of mutant strains that were defective in either filamentation, Sap-production, amino acids uptake, or in the GlcNAc-metabolism. *C. albicans* cells were incubated on the PIE for six hours. Scale bar is 25 μm

mutant strain was filamenting apparently more vigorously than the wild type. Only the non-filamentous *efg1/cph1* mutant showed weak adherence similarly as was observed in the *wal1* strain.

Analysis of the filamentation properties of the nag5 strain

In order to test the colony growth phenotype of the *nag5* strain cells were plated on YPD and on minimal media, respectively, and incubated for several days at 30 °C. The wild type showed smooth colony surfaces on YPD and SD media while after prolonged incubation (eight days) wrinkled colonies appeared on CSM medium (Fig. 4; top row). In contrast the *nag5* strain showed a strong increase of filament formation at the colony edges on all media (Fig. 4, bottom row). This filamentation was accompanied by invasion of hyphae into the agar as was observed after washing the none-invasive cells off the colonies (not shown). This suggested that the *nag5* strain shows an intrinsic property to filament on solid media that is not specific to the PIE. In liquid SD-medium *nag5* was found not to filament.

Farnesol is a known quorum sensing compound that inhibits hyphal development in *C. albicans* (HORNBY *et al.* 2001, MOSEL *et al.* 2005). To test whether farnesol is able to shut off *nag5*-filamentation we compared the responses of the wild type and the *nag5* strain on YPD and minimal medium containing GlcNAc (Fig. 5). The wild type shows yeast-like growth on both media at 30 °C and also on YPD at 37 °C, but is induced to form hyphae at 37 °C in the presence of GlcNAc. This filamentation can be eliminated using 100 μM farnesol. Filamentation of the *nag5* strain at 30 °C was readily abolished using 100 μM of farnesol. However, at 37 °C in the presence of GlcNAc a basal level of filamentation was present even at highest concentration of farnesol that can be applied to the cells.

Discussion

The virulence of *C. albicans* is based on several factors, which include adhesion to host epithelia and filamentation to colonize surface niches and to subsequently invade these epithelia. Several genes have been characterized that influence these characteristics. For ex-

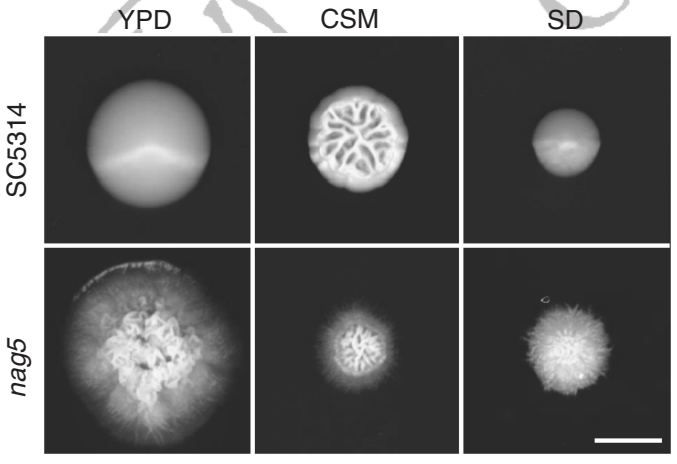

Fig. 4
Colony morphology of the *nag5* strain
The SC5314 wild type and the *nag5* mutant strain were incubated on different solid media for 8d at 30 °C. While the wild type does not filament on YPD-plates at 30 °C the *nag5* strain is strongly filamenting and also invades into the agar. On complete minimal media growth and the filamenting properties of *nag5* cells are reduced but still more pronounced than in the wild type. Scale bar is 5 mm

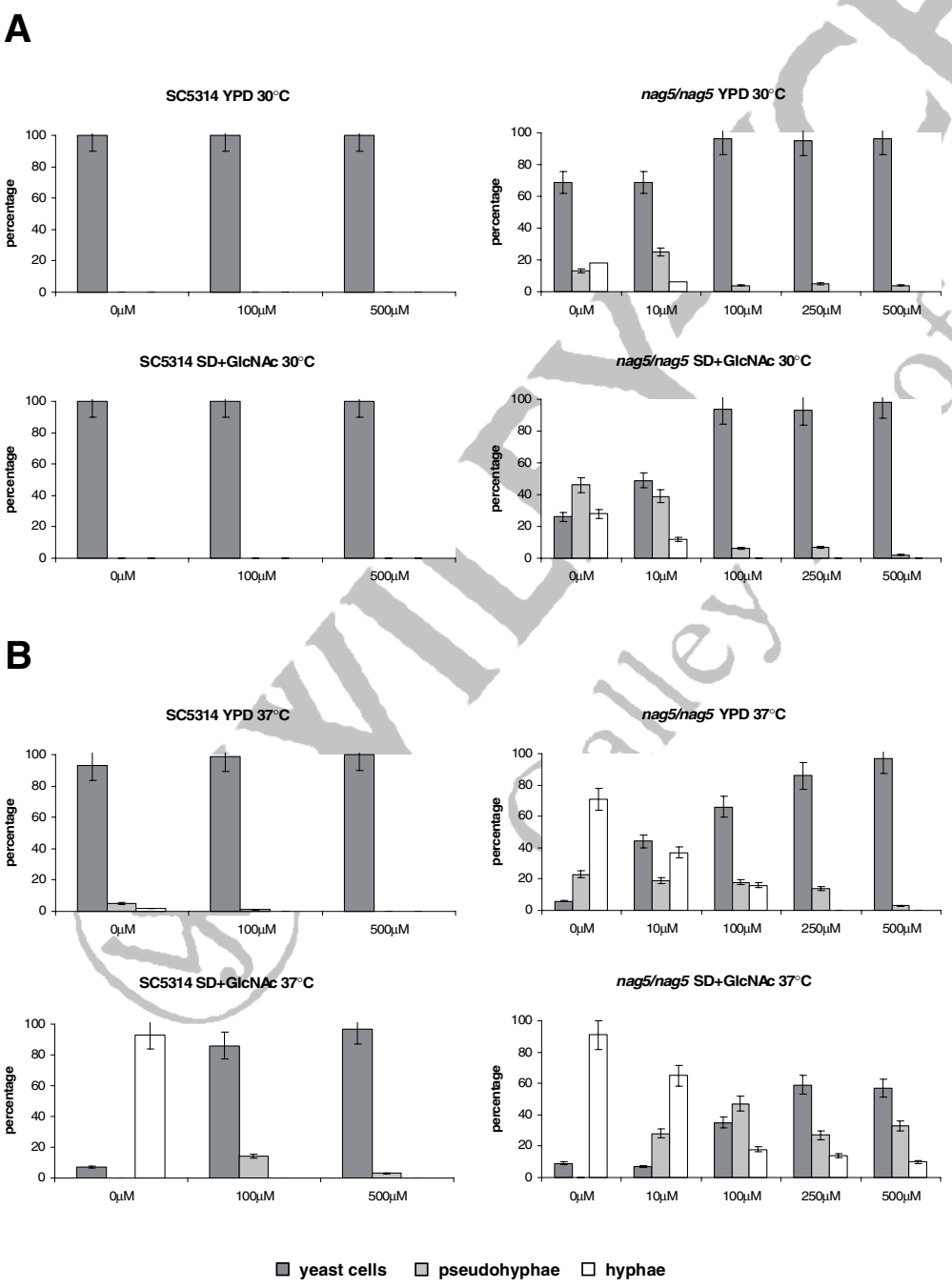

Fig. 5
Repression of filamentation using farnesol
The wild type (SC5314) and the homozygous *nag5* mutant strain were grown in either YPD or minimal SD medium containing the indicated amounts of farnesol. Cell morphologies were counted as percent of yeast, pseudohyphal or hyphal cells (150 to 300 cells were counted for each condition)

ample, the eight member *ALS*-family, which encodes agglutinin-like sequences has been shown to confer the ability to adhere to epithelial cells (ZHAO *et al.* 2004, 2005). On the other hand two transcriptional regulators, Efg1 and Cph1, are essential to promote the virulence of *C. albicans* cells by inducing hyphal specific gene expression, e.g. of *ALS*-genes (LO *et al.* 1997).

Recently, reconstituted human tissues have been used to monitor adhesion and invasion of *C. albicans* (KORTING *et al.* 1998, DIETERICH *et al.* 2002, SCHALLER *et al.* 2003). It was shown that secreted aspartyl proteases encoded by the *SAP1* and *SAP2* genes are involved in causing tissue damage (SCHALLER *et al.* 2003). In a model of reconstructed intestinal epithelium a *C. albicans* strain bearing deletions of *CPH1* and *EFG1* was shown to be unable to adhere or penetrate (DIETERICH *et al.* 2002). In this report we demonstrate the use of porcine intestinal epithelium in an assay to analyze the individual contribution of several mutant strains during the very early stages of infection, namely adhesion and filamentation. The system we describe here enjoys several advantages (i) reconstituted epithelia consist of only a limited number of cell types and cell layers in contrast to *ex vivo* porcine intestinal epithelium. (ii) The PIE-assay is easy to handle and prepare and closely mimics the *in vivo* situation. The PIE-assay could therefore be a simple alternative model to analyze adhesion and colonization properties of *C. albicans* mutants, particularly during the analysis of a larger collection of mutant strains. An additional advantage of using the PIE assay will be the analysis of tissue destruction and invasion which requires the preparation of thin sections of embedded epithelium that can be used for histological analyses to monitor deep tissue penetration. This may aid in answering whether as a commensal *C. albicans* is a benign member of the microbiota (MARTINEZ and LJUNGDAHL 2005) or if *C. albicans* is simply a pathogen that is constantly being kept in check by the immune system. Other members of the microbiota do not seem to influence the morphogenetic programme of *C. albicans*. Particularly a co-cultivation of *C. albicans* with *E. coli* did not result in filament formation. During prolonged incubation periods the PIE will eventually start to deteriorate. However, the filamentation response of *C. albicans* on the PIE is rather an immediate answer. This indicates that *C. albicans* is ready to adhere to the PIE to avoid clearance from the gut by mechanical forces. Using the PIE-assay it will be of interest in the future to analyze the amount of tissue damage that *C. albicans* brings about and if there is a preferred route for deep penetration. At this stage we did not analyze the host situation in the PIE concerning apototitc or necrotic processes that occur due to the preparation of the PIE or during the assay itself.

In our hands, the *efg1/cph1* and *wal1* mutant strains showed severely reduced adhesion and were non-filamenting. Our results with the *cph1/efg1* mutant are in accord with previous experiments using reconstituted epithelia (DIETERICH *et al.* 2002). The functional analysis of *WAL1* revealed defects in hyphal morphogenesis and vacuolar morphology (WALTHER and WENDLAND 2004). We show here that the *wal1* mutant was found to be attenuated in virulence in a mouse model of systemic infection. The lack of adhesion and filamentation of *wal1* cells as assayed on the PIE may thus contribute to this reduced virulence. The PIE-assay could therefore be a simple alternative model to analyze adhesion and colonization properties of *C. albicans* mutants, particularly during the analysis of a larger collection of mutant strains.

In this report we also used several mutant strains defective in either amino acid transporters, *SAP*-genes, or in *NAG5*, which encodes a GlcNAc-kinase. All of these mutants strains analysed that were able to filament *in vitro* were also found to be able to adhere to the PIE. Specifically, the *nag5* mutant strain showed vigorous filamentation in this assay. This adds to the controversy whether filamentation is essential for the virulence of *C. albicans* since *nag5* strains were reported to be avirulent in a mouse model of systemic infection (YAMADA-OKABE *et al.* 2001). On the other hand, we found that the *nag5* mutant strain showed a reduced growth rate, which may also contribute to its weak performance in the mouse

model. Interestingly, deletions of *NAG5* and the chitinase encoding gene *CHT2* stimulate hyphal morphogenesis suggesting an influence of chitin degradation on hyphal morphogenesis (DUNKLER *et al.* 2005). At high cell densities quorum sensing via the production of the extracellular molecule farnesol was shown to inhibit filamentation (HORNBY *et al.* 2001, MOSEL *et al.* 2005). Interestingly, the *nag5* mutant was at least partially "blind" against farnesol as a large fraction of cells was still able to filament in the presence of farnesol concentrations (>100 μM) that shut down wild type filamentation.

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