

ARTICLE

Investigation of the role of *Lip5* – a member of the secreted lipase gene family – in the virulence of *Candida albicans*

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ABSTRACT *Candida albicans* (Ca) is the most common species isolated from invasive candidiasis. It has been shown that secreted lipases of Ca contribute to the virulence of the fungus during infection. In this study, we generated heterozygous and homozygous lipase 5 (*LIP5*) mutants in Ca by the *caSAT1*-flipper technique. Notably, the Southern-hybridization results indicated a yet unknown allelic heterozygosity in *LIP5* in our laboratory strain. Quantitative reverse transcription-PCR experiments demonstrated the absence of *LIP5* expression in the homozygous knockout mutants. However, the knock out mutants showed no alteration in the rate of fungal growth, cell and colony morphology under temperature, pH or osmotic stress in comparison to wild type cells. *In vitro* infection studies using the J774.2 murine macrophage-like cell line revealed no alteration in the virulence of mutant cells. Thus, we conclude that the deletion of *LIP5*, somewhat surprisingly, has no effect on the physiology or virulence of Ca in our experimental setting.

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KEY WORDS

Candida
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Invasive candidiasis is a major global health problem and *Candida albicans* (Ca) is the most common cause of invasive candidiasis worldwide (Pfaller et al. 2010). Among others, secretion of hydrolytic enzymes has been identified as an important virulence factor of the fungus (Park et al. 2013). However, although the role of aspartic proteases and phospholipase B has been extensively investigated during Ca infection (Naglik et al. 2003),(Park et al. 2013), the role of other hydrolytic enzymes like lipases is less characterized. Secreted lipases of Ca are encoded by a gene family with at least 10 members (*LIP1-LIP10*) (Hube et al. 2000). Our previous findings illustrated that individual lipase genes are differentially regulated in a mouse model of systemic candidiasis as well as in an experimental model of oral infection or in human specimens (Stehr et al. 2004). *LIP5* and *LIP8*, situated on chromosome 7 of Ca, are two closely related, highly homologous genes of the lipase gene family (Stehr et al. 2004). Both were found to be expressed with constitutive or predominant transcript levels in *in vivo* experimental systems (Stehr et al. 2004). *LIP8* was selected for further study, as it has been shown to be the only lipase that is uniformly up-regulated 4 hours after infection in a systemic murine infection (Stehr et al. 2004). We have previously constructed *LIP8* knockout mutants, reconstituted strains and over-expression mutants to further

explore the role of lipases in Ca pathogenesis. *LIP8* knock-out mutants produced more mycelium, particularly at higher temperatures and $\text{pH} \geq 7$, and growth was reduced in lipid media (Gacsér et al. 2007). In contrast to wild type (WT), heterozygous or reconstituted strains, infection with *LIP8* homozygous deletion mutants was non-lethal in a murine intravenous infection model (Gacsér et al. 2007). The results show that lipases are major virulence factors in clinically important *Candida* species and therefore lipases are potential targets for drug development. In this study, we addressed the question whether the role of *LIP5* in the virulence of Ca is similar to that of *LIP8* and generated as well as subsequently characterized *LIP5* knock-out mutants in Ca.

Materials and Methods

Strains and growth conditions

Ca strains were maintained at -80°C in 35% glycerol. Ca wild type (WT) laboratory strain SC5314, $\Delta\text{lip5/LIP5::FRT}$ and $\text{LIP5}/\Delta\text{lip5::FRT}$ heterozygous deleted mutant strains and $\Delta\text{lip5}/\Delta\text{lip5::FRT}$ homozygous deleted mutant strain of SC5314 (developed by this work) were used. Strains were cultivated normally in YPD medium (0.5% yeast extract, 1% bacto-peptone, 1% glucose) at 37°C . For the purpose of elimination of the *caSAT1* cassette, transformants were cultivated in 1x yeast nitrogen base (YNB) supplemented with 2% maltose at 37°C . To examine the viability, strains

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were cultured on solid YPD plates for 2 days at different temperatures (4°C, 20°C, 25°C, 30°C, 37°C). For pH sensitivity test, strains were grown on solid YPD plates at 37°C for 2 days. The pH of the media was adjusted to pH 4, pH 5, pH 6, pH 7 and pH 8. Growth tests were carried out using the following media: yeast carbon base (YCB) + 5% bovine serum albumin (BSA); YNB + 5% fetal bovine serum (FBS); S4D-agar (0.17% YNB, 0.5% (NH₄)₂SO₄, 4% glucose, 5 µg/ml Phloxine B, (Anderson and Soll 1987)), Spider medium (1% mannitol, 1% nutrient broth, 0.2% K₂HPO₄, (Liu et al. 1994)); YNB + 0.5% ammonium sulfate + 25 ml/liter Tween 80; YNB; YNB + olive oil; YNB + 25 ml/liter Tween 80; Lee's medium (Lee et al. 1975).

Generation of disruption construct pSFS2Lip5

The pSFS2Lip5 plasmid was constructed to disrupt the whole open reading frame of *LIP5* consisting of 1395 bp. In order to achieve homologous recombination events, a 782 bp region upstream to *LIP5* and a 671 bp region downstream to *LIP8* were amplified from genomic DNA of the Ca WT and cloned into *KpnI/XhoI* and *NotI/SacI* sites of pSFS2. Upstream region was amplified by primer pair 5'- ttttttgggtaccgatcctgaacgatatgtaccgattttgag -3' (*KpnI* site underlined) and 5'- ttttttctcgagggataagtaaacatattggaactattgagg -3' (*XhoI* site underlined) and downstream region was amplified by 5'- ttttttcgccgcgttactacggccatcagaagtctgctaag -3' (*NotI* site underlined) and 5'- ttttttgagctcattcgcaacattatcaattctatgtgttcag -3' (*SacI* site underlined) primers. The PCR products were digested and consecutively cloned into pSFS2.

Ca transformation and generation of heterozygous and homozygous *LIP5* deletion mutants from SC5314 strain

Ca WT cells were transformed by electroporation (Gacser et al. 2007). To generate heterozygous *LIP8* mutant strains, Ca SC5314 WT was transformed with 10 µg ethanol-precipitated *KpnI/SacI* digested DNA of deletion construction pSFS2-*LIP5*. Transformants were grown on selective YPD medium supplemented with 100 µg/ml nourseothricin (NAT). NAT resistant colonies were analyzed by Southern blot analysis and those transformants that carried single copy integration of selection marker cassette (*CaSAT1*) in the *LIP5* locus were further processed. To eliminate *CaSAT1* cassette, resistant mutants were grown overnight in YNB supplemented with 2% maltose to enable the expression of *CaSAT1 FLP* site-specific recombinase. NAT sensitive colonies were selected in the presence of permissive concentration of the antibiotics (10 µg/ml) in YPD medium as described previously (Gácser 2007). Selected NAT sensitive strains were checked by Southern hybridization to confirm the correct excision of the flipper cassette and validated sensitive strains were used to generate homozygous deleted mutant strains by the repetition of the whole transformation process.

DNA isolation and Southern blot analysis

For DNA isolation and Southern blotting, standard methods were used (Gacser et al. 2007). Total DNA from WT and transformed Ca cells was digested with *HindIII/BamHI* and Southern hybridization was performed by using the *LIP5* upstream region as DNA probe.

Growth tests

For growth tests on solid media, overnight cultures of Ca strains were adjusted to concentrations of 10⁴-, 10³-, 10²-, and 10 cells/ml and 5-5 µl from each suspension was inoculated in a spot onto the various media. Growth test in liquid YPD media was carried out by inoculating 5x10⁵ cells into 10ml of YPD and incubated at 37°C overnight with shaking (180 rpm). Concentration of cells was measured after 6, 12 and 24 hours of incubation time by photometric method at 620 nm wavelength.

Detection of hypha production

To examine hypha formation, Ca strains were cultured in DMEM medium supplemented with 10% FBS and 1% 100 x Penicillin-Streptomycin solution. Hypha formation was examined after 0.5, 1, 6, 12, 24 and 48 hours by light microscopy.

Phagocytosis assay

J774.2 cells were cultured in DMEM medium (Lonza) supplemented with 10% heat-inactivated FCS (Lonza) and 1% 100 x Penicillin-Streptomycin solution. Macrophages were co-incubated with the different Ca strains at an effector/target ratio of 1:5. Following the incubation period, phagocytic events were detected by light microscopy.

LDH (lactate dehydrogenase) assay

LDH in medium from cultures containing uninfected or infected macrophages was measured by CytoTox-ONE kit (Promega) according to the manufacturer's instructions. *Candida* cells alone incubated under identical conditions were included as negative controls.

Killing assay

J774.2 macrophages were co-incubated in plastic cell culture plates with the different Ca strains at an effector/target ratio of 1:5. As a control, the same number of yeast cells was incubated in the appropriate cell culture medium without macrophages. After 3 hours of incubation, macrophages were lysed by forcibly pulling the culture through a 26-gauge needle 5 times. The lysates were serially diluted and plated on Sabouraud dextrose agar at 37°C. CFU determinations were made after 72 hours. The killing efficiency was calculated as follows: (number of live *Candida* cells in control wells –

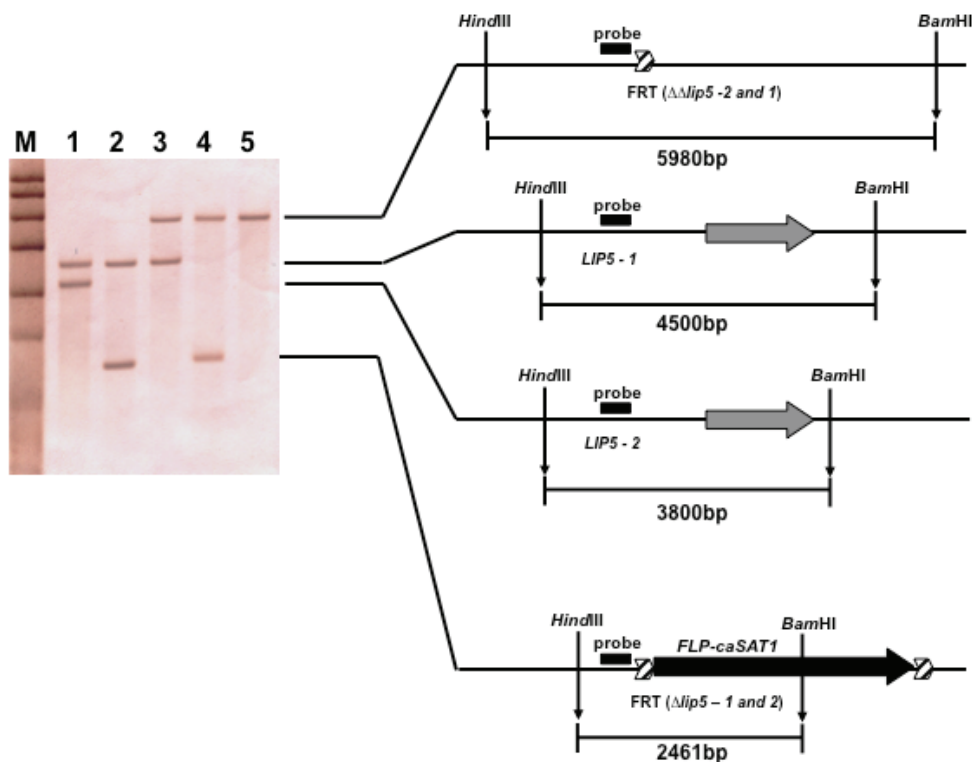


Figure 1. Deletion of *LIP5* in *C. albicans* SC5314. (A) Southern blot hybridization analysis of genomic DNA (*Hind*III, *Bam*HI double digested) isolated from WT *C. albicans* (lane 1), the heterozygous mutant *CaLIP5-1/Δcalip5-2:SAT1-FLP* before FLP activation (heterozygous resistant strain, lane 2), the heterozygous mutant *CaLIP5-1/Δcalip5-2:FRT* (HE) after excision of *SAT1* flipper cassette (heterozygous sensitive strain, lane 3), the homozygous mutant *Δcalip5-2:FRT/Δcalip5-1:SAT1-FLP* before FLP activation (homozygous resistant strain, lane 4), the homozygous mutant *Δcalip5-2:FRT/Δcalip5-1:FRT* (KO) after excision of *SAT1* flipper cassette (homozygous sensitive strain, lane 5). Diagrams of the structures and size of the hybridization fragments are shown at right. (B) Expression of *LIP5* in WT, heterozygous and homozygous *LIP5* mutant *C. albicans*. Strains were incubated in YPD + olive oil or YNB + olive oil medium for 6 hours before total RNA isolation. Relative gene expression was determined by qRT-PCR. Actin (*ACT1*) was used as an endogenous control; relative gene expression was calculated by the $\Delta\Delta C_T$ method. Wt, wild type; LIP5/ Δ lip5, heterozygous mutant; Δ lip5/ Δ lip5, homozygous mutant; nd, not detected.

number of live *Candida* cells in co-cultures) / number of live *Candida* cells in control wells x 100. All tests were performed in triplicate.

Quantitative Real-Time PCR (qRT-PCR)

Ca strains were cultured overnight at 37°C in 10 ml DMEM with or without J774.2 macrophage cells. Total RNA isolation and single-stranded complementary DNA synthesis were performed as described previously (Nguyen et al. 2011). As endogenous control, actin primers qAct1F (5'-GACCGAAGCTCCAATGAATC-3') and qAct1R (5'-TGGAACGCTAGAAAGCTGGA-3') were used. qRT-PCR was performed using the following LIP8 specific primers: qLIP8F (5'-TTCAAAGTTGCTTTGGACAC-3') qLIP8R (5'-AAAACCTTCTAAGGTCGTCTGG-3') and LIP 5 specific primers: qLIP5F (5'-TGTTCTCCATCTCGGACTTG-3') qLIP5R (5'-CCACCTCATTTC AATTGATCC-3'). Expression levels of *LIP8* were normalized to the actin gene, and the fold change values were calculated using the $\Delta\Delta C_T$ method.

qPCR was carried out with Maxima SYBR Green/Fluorescein qPCR Master Mix (Fermentas) in 20 μ l final volume in CFX96™ Real-Time PCR Detection System in three parallels for each sample.

Results

Disruption of the *LIP5* gene in *Ca*

Both alleles of the *LIP5* gene were successfully deleted in *Ca* SC5314 by using the *CaSAT1-Flipper* method (Reuss et al. 2004). Mutant strains were analyzed by Southern blot hybridization to monitor for single-copy integrated transformants and follow the success of excision of *CaSAT1-Flipper* cassette from the genome of NAT resistant transformants upon maltose induction. Figure 1 A summarizes the results of the Southern blot analyses of a sequential series of heterozygous and homozygous *LIP5* mutant strains. The absence of *LIP5* mRNA in *LIP5* KO cells was confirmed by qRT-PCR (Figure 1 B). Our results indicate a yet not reported allelic heterozygosity in *LIP5* gene.

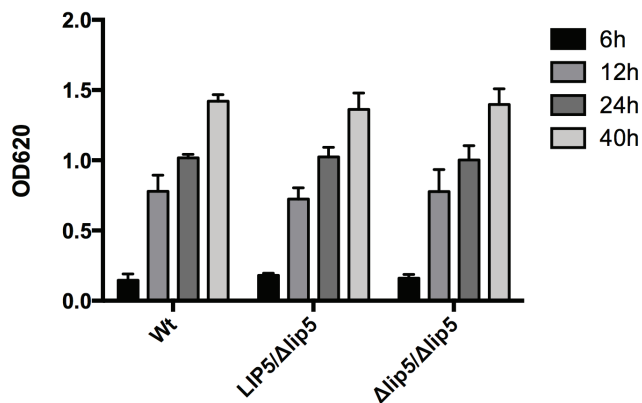


Figure 2. Growth capabilities of *LIP5* mutant *Ca* strains in liquid medium. *Ca* strains were incubated in liquid YPD medium and growth rates were determined by measuring the optical density of cultures at 620 nm after 6, 12, 24 and 40 hours. Wt, wild type; *LIP5/Δlip5*, heterozygous mutant; *Δlip5/Δlip5*, homozygous mutant.

Phenotypic characterization of the *Ca LIP5* deleted strains

(i) Growth capabilities in liquid medium

Growth test in liquid YPD medium was carried out to compare the growth rates of WT cells as well as heterozygous and homozygous *LIP5* mutants. As Figure 2 shows, there was no difference between the growth capability of the mutants and that of the wt strain.

(ii) pH and temperature dependence on solid YPD medium

To test the pH-sensitivity of *LIP5* mutants, *Ca* strains were cultured on solid YPD medium at pH 4, pH 5, pH 6, pH 7 and pH 8. There was no difference between the growth properties of the examined strains. Temperature-dependent growth ability of WT and *LIP5* mutants was compared on YPD solid medium at 4°C, 20°C, 25°C, 30°C or 37°C. All the tested strains had similar growth abilities (data not shown).

(iii) Test of growth ability in the presence of different nitrogen- and carbon sources

Growth abilities of WT and *LIP5* mutants were also compared on different media with different nitrogen- and carbon sources: BSA (YCB + 5% BSA), FBS (YNB + 5% FBS), Tween80 (YNB + Tween 80) and olive oil (YNB + olive oil). Growth capabilities were also tested on S4D agar (glucose-Phloxine B, a special medium for the induction of temperature-dependent phenotype switching between the white and opaque forms of *Ca* WO-1 (Anderson and Soll 1987)); Spider medium and Lee’s medium (media capable of inducing mycelial growth (Liu et al. 1994, 9]). However, there was no difference between the growth capabilities of the studied strains, as it is shown by Figure 3.

(iii) Hypha-forming ability of WT and *LIP5* mutant *Ca*

Hypha production was monitored in DMEM medium that triggers the phenotype switching. Neither the ratio of hyphae to yeast cells, nor the rate of hypha production was different in *LIP5* mutants (data not shown).

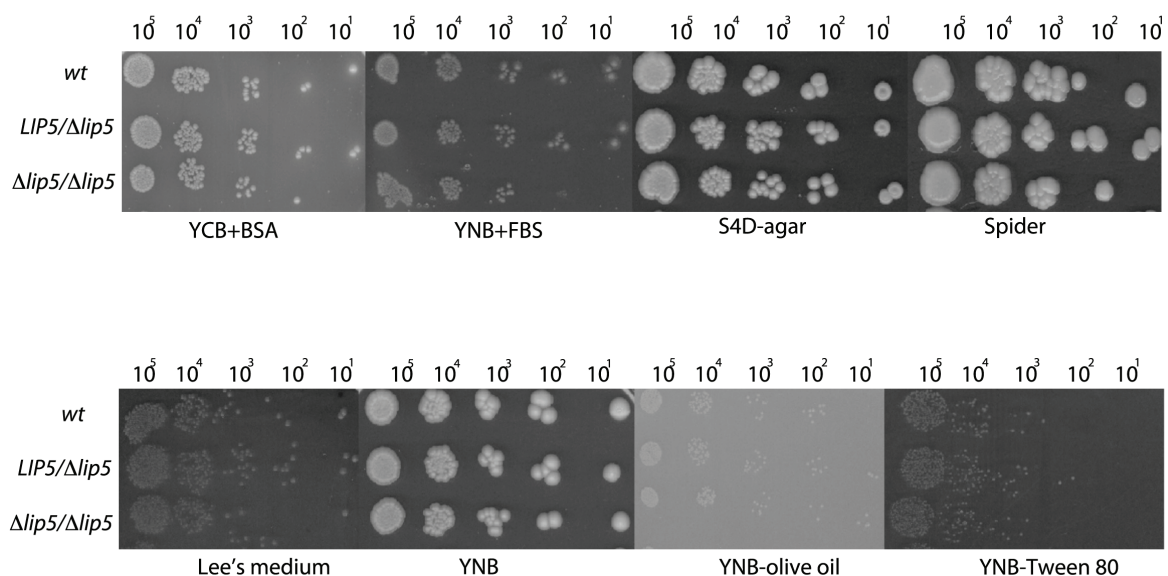


Figure 3. Growth abilities of WT and *LIP5* mutants on different media. *Ca* strains were cultured on different media: BSA, FBS, S4D agar, Spider medium, Lee’s medium, YNB, olive oil or Tween 80 (see materials and methods for details). Wt, wild type; *LIP5/Δlip5*, heterozygous mutant; *Δlip5/Δlip5*, homozygous mutant.

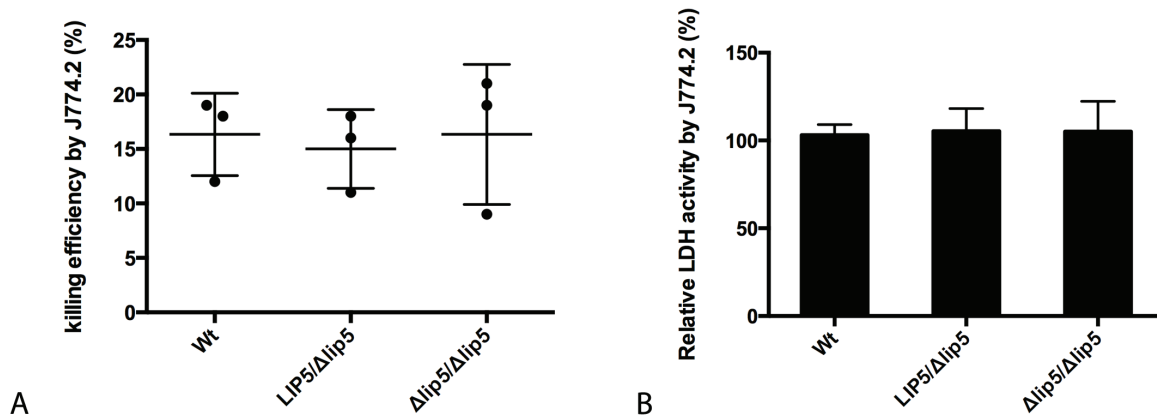


Figure 4. Interactions of LIP5 mutant Ca strains with J774.2 macrophages. A: Killing of LIP5 mutant Ca strains by J774.2 macrophages. Co-cultures were incubated for 3 hours and the killing efficiency was calculated by CFU-determinations. Experiments were performed in triplicate. Data represent killing efficiency in % \pm SEM. B: Host-cell damaging capacity of LIP5 mutant Ca strains. J774.2 cells were co-incubated with LIP5 mutant Ca strains for 24 hours, and the activity of LDH was measured in cell culture supernatants. Data represent relative LDH activity (relative to the LDH activity released by the J774.2 cells infected with wt cells in %) \pm SEM. Wt, wild type; LIP5/Δlip5, heterozygous mutant; Δlip5/Δlip5, homozygous mutant.

Analysis of the virulence of LIP5 mutants in in vitro infection models

To examine whether LIP5 mutants have altered virulence during infection, we assessed the killing of WT and heterozygous as well as homozygous LIP5 mutant Ca strains by murine J774.2 macrophage-like cells, as well as the macrophage-damaging capacity of the Ca strains. We found that WT and LIP5 mutants were eliminated by macrophages with similar efficiency, as it is shown in Figure 4 panel A. Monitoring the release of LDH from macrophages upon infection with the Ca strains showed that there was no difference in the macrophage-damaging capacity of the strains either (Figure 4 panel B). We also examined the phagocytosis of mutant strains by J774.2 macrophages; however, all strains were internalized with similar efficiency (data not shown).

Monitoring the expression of LIP8 gene in WT and LIP5 mutants

To assess whether there is a compensatory mechanism upon the lack of LIP5 expression, we analyzed the expression of the LIP8 gene in WT and LIP5 KO Ca in the presence or absence of J774.2 macrophages. LIP8 gene was selected on the basis of results of previous studies (Stehr et al. 2004), where both LIP8 and LIP5 showed strong up-regulation upon infection. However, we found that LIP8 expression was not affected by the absence of LIP5 alleles (Fig. 5).

Discussion

In this study, we used a targeted gene disruption technique to generate LIP5 KO mutants in Ca. We constructed a cassette that, in addition to the caSAT1 selection marker, contained an

inducible *MAL2P-FLP* fusion, direct repeats of the minimal FLP recognition site (*FRT*) and flanked by DNA regions upstream and downstream to the target Ca LIP5 gene. To restore the targeted gene's activity, a linear DNA fragment containing the open reading frame of the LIP5 gene as well as required upstream and downstream sequences were constructed and was transformed into the knock-out mutants. The generated homozygous mutants and the reconstituted strains were analyzed by Southern blotting and by RT-PCR. Notably, the hybridization results indicated a yet unknown

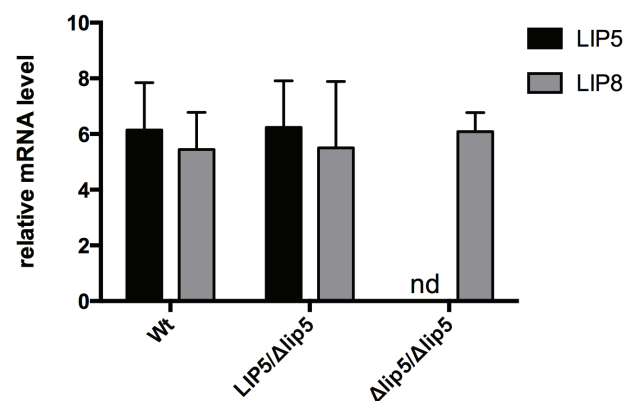


Figure 5. Expression of LIP8 in LIP5 mutant Ca strains. Strains were incubated in medium with or without J774.2 macrophages for 3 hours and relative LIP8 expression was determined by qRT-PCR using Actin (ACT1) as an endogenous control. Data represent relative gene expression (relative to the „medium alone“ control, calculated by the $\Delta\Delta$ CT method) \pm SEM. Wt, wild type; LIP5/Δlip5, heterozygous mutant; Δlip5/Δlip5, homozygous mutant.

allelic heterozygosity in *LIP5*. Both the heterozygous and homozygous mutant strains were analyzed to evaluate the effects of gene deletion on the rate of fungal growth, cell and colony morphology under various growth conditions (different media, pH, temperature) and virulence capacity. However, the analysis of phenotypic properties showed no significant differences in mutant strains in comparison to the wild type strain. Similarly, the capacity of hypha formation was identical both in the mutants and the wild type strains. *In vitro* infection studies using the J774.2 murine macrophage-like cell line revealed no alteration in the virulence of mutant cells. An explanation of this finding could have been a potential compensatory effect by the highly homolog *LIP8* gene product that was still present in our system; however, qRT-PCR experiments clearly showed that *LIP8* mRNA level was unaffected by the absence of *LIP5* either in normal culture or in yeast-macrophage co-cultures. Thus, there is no compensatory mechanism working that was seen for example in case of *SAPP* genes in *Candida parapsilosis* (Horvath et al. 2012). Taken together, we conclude that - at least in our experimental setting - the deletion of the *LIP5* alone does not affect the physiology or the virulence of Ca.

Acknowledgements

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