TUP1-mediated filamentation in *Candida albicans* leads to inability to colonize the mouse gut

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Keywords

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Abbreviations

SD, standard deviation, aCT. Autoclaved chlortetracycline.

Abstract

Aims: To investigate the role of *Candida albicans TUP1*-mediated filamentation in the colonization of the mice gut. **Material & Methods:** We use molecular genetics to generate a strain where filamentation is regulated by altering the expression of the *TUP1* gene with tetracyclines. **Results:** The colonization rates reached with the $TUP1^{REP}$-RFP^{REP} strain were lower compared to wild type strain and completely absent after induction of filamentation. No differences in the susceptibility to bile salts nor in the adhesion to the mouse intestine epithelium was observed. **Conclusions**: Locking the blockage of *C. albicans* in a filamentous form impedes cells gut colonization in the mouse.

Introduction

Candida albicans is a pathogenic yeast that causes a wide range of infections, collectively called candidiasis. This yeast is part of the microbiota of most humans but depending on the immunological status of the host it may lead to systemic diseases on immune deficient individuals that are very severe and frequently life-threatening. This yeast is therefore considered an opportunistic pathogen, residing in the oral cavity and the gastrointestinal & urogenital tracts of immunocompetent individuals. *C. albicans* pathogenesis is considered to be multifactorial, as there is not a single factor responsible for the virulence of this fungus; rather, several components have been shown to be involved in its pathogenicity such as metabolic traits, signalling pathways, cell wall architecture and secreted products that make separate local and temporal contributions during pathogenesis [1, 2]. The relevance of most of these factors has been determined using either *in vitro* or *in vivo* models. Among the later, the intravenous systemic infection model has been the most widely used in the past for testing virulence determinants in this yeast [3] although superficial and mucosal models are clearly relevant considering the pathology of candidiasis [4]. The use of commensalism models developed long time ago [5, 6], but only popularized recently [7, 8], is enabling the analysis of the contribution of different genes to colonization [9].

Several *C. albicans* factors have been shown to play a role in the adaption to the commensal state during gastrointestinal colonization [9, 10]. They comprise epigenetic transitions [11], iron acquisition [12], signalling pathways [13] and metabolic traits [14] among others. One important feature of *C. albicans* is its dimorphic nature, the ability (under appropriate stimuli) to develop a yeast to hyphae transition. This feature has been related to its ability to cause infections: *C. albicans* mutants strains that grow only in the yeast form were found to be avirulent in the mouse systemic model [15], although mutant strains that only grew -or had an enhanced ability to grow- in the filamentous forms were also found to be attenuated in virulence [16, 17]. It is now generally accepted that neither of these forms are intrinsically virulent although they may play different specialized functions during infection within the host, both temporally and locally.

Although dimorphism has been mainly studied in the mouse systemic model [18], its role in the mouse commensalism model is less documented. An early study made use of an intrinsically filamentous mutant (*tup1*) that revealed reduced colonization in the gut [7]. Also, a recent study using strains locked in the filamentous form a similar behaviour [19]. However, both studies suffered from using intrinsically filamentous forms of *C. albicans*, which are difficult to handle in the laboratory due to its hyphal nature which does not easily allow standardization of inoculum administration. In addition, as the inoculum is administered as a complete hyphal form, and one could argue that hyphi are not deficient in long term colonization but only during the early stages that mediate adaptation to the gut cavity [20]. To circumvent these caveats and allow a temporal dissection of this process, we have generated a *C. albicans* mutant strain in which filamentation can be triggered by altering *TUP1* levels, as deletion of this transcriptional regulator lock cells in the filamentous form [21]. Using a filamentation doxycycline regulated strain we have determined that induction of filamentation once established in the mouse gut results in the inability to colonize the mouse gut and complete loss of the fungus. We have also validated the use of a heat inactivated chlortetracycline using functional bioassays, showing its usefulness in i*n vivo* analysis of gene expression in this fungus.

Strains and growth conditions

The strains used are described in Table 1. Cells were grown at 37ºC in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) unless otherwise stated. Chlortetracycline (Sigma-Aldrich) was heat inactivated

(121º C for 22 min) to obtain aCT (autoclaved ChlorTetracycline) that was used at 1 g/L or 20 mg/L for *in vivo* and *in vitro* assays respectively.

The susceptibility/resistance to different compounds was performed through drop test as follows. Cultures grown at 37ºC for 16 hours, in the presence or not of aCT, were adjusted to $2x10^7$ cells/mL, serially 10-fold diluted and deposited (5 µl) onto solid YPD plates supplemented (or not) with bile salts (Sigma-Aldrich). Plates were incubated at 37ºC for 24 hours before scanned.

Genetic procedures

To develop a regulated filamentous strain, the ORF of *TUP1* was amplified by PCR from the clinical isolate SC5314 strain using the primers up-TUP1-myc (GATCTCGAGTATGTATCCCCAACGCACCC) and rev-TUP1-myc (ATCGCGGCCGCATTTTTTGGTCCATTTCCAAATTC). The 1536 bp PCR product was cloned in the intermediate pGEMT plasmid (Promega), digested with *Xho* I and *Not* I and accommodated in pNRU-RFP [22], a doxycycline repressible plasmid, previously digested with *Sal* I and *Not* I to generate pNRU-TUP1, were *TUP1* is tagged with the myc epitope. Homologous recombination occurs at the *ADH1* locus by means of *Kpn* I-*Sac* II digestion. Transformation of *C. albicans* in a *tup1* background [21] generated a $TUP1^{REP}$ strain. For RFP labeling, we used the pDS2-dTOM2 plasmid, which expresses, together with pNRU, express RFP under the control of the *TET* promoter and recombines at the *ARD1* locus by *Kpn* I digestion. It was constructed as follows: the *TET* promoter (*tet*O+*OP4*) was amplified by PCR from pNIM1 plasmid using the primers TET^{PR} up (CTAGTCGACTTTACCACTCCCTATCAGTGATAGAGAA) and TET^{PR} rev (GTAGCTAGCTATTTATATTTGTATGTGTGTAGGAGTTAACG), digested with *Sal* I and *Nhe* I and introduced in the plasmid pDUM0-L [23], previously digested with *Xho* I and *Nhe* I, to generate the pDUM2-L plasmid. The dTOM2 ORF was amplified with the primers dTOM2 up (ACTGCTAGCGAGATGGTTTCTAAAGGTGAAGAATCG) and dTOM2 rev (AAGGATCCTCGAGCCCATATTATTATCTTCAGAAGAAGCAGTACC) from pNIM1R-dTOM2 [13], digested with *Nhe* I and *BamH* I and introduced in pDUM2-L by replacing the CbLUC gene to generate pDUM2-dTOM2. Finally, the *SAT1* marker was amplified from pNIM1 with the primers SAT1_up (ACTTCTAGATGTCTGCAGGACCACCTTTGATTG) and SAT1_rev (CTGCCGCGGAGCGTCAAAACTAGAGAATAATAAAGAAAACG), digested with *Xba* I and *Sac* II and ligated in pDUM2-dTOM2, digested with the same pair of enzymes, replacing the *URA3* marker, to generate the final pDS2-dTOM2 plasmid.

Protein extracts and immunoblot analysis

All procedures involving cell lysis, protein extraction, gel electrophoresis and transfer to nitrocellulose membranes were made as previously described [24]. Protein extracts were measured at A_{280nm} to equalize the amount of protein loaded for western blot analysis and probed with anti-myc, clone 4A6 (Millipore). Western blots were developed according to the manufacturer´s conditions using the Hybond ECL kit (Amersham Pharmacia Biotech).

In vivo fitness assays

The murine gut colonization assays were performed following the protocol previously described [13]. Briefly, after 4 days of antibiotic pre-treatment (2 mg/mL streptomycin, 1 mg/mL bacitracin, and 0.1 mg/mL gentamycin) with or without aCT, 10^7 *C. albicans* cells were intragastrically inoculated in a single gavage dose. Stool samples were obtained on different days (indicated in the accompanying figures) and homogenized in PBS prior to be cultured in SD plates to determine CFUs per gram. 4 mice were used in

each experiment and repeated at least twice; only a representative experiment is shown. To analyze *C. albicans* loads in the gastrointestinal tract, mice were sacrificed and intestinal content from stomach, cecum, small and large intestine were aseptically obtained, homogenized in sterile PBS and cultured in SD plates to count CFUs. Female mice C57BL/6 were purchased from Harlan Laboratories, Inc. (Italy) and used within an age of 7 to 10 weeks-old. Mice housing and other non-invasive procedures took place in the animal housing facility from the Medical School of the Universidad Complutense de Madrid.

All experiments involving animals performed in this work were carried out in strict accordance with the regulations in the "Real Decreto 1201/2005, BOE 252" for the Care and Use of Laboratory Animals of the "Ministerio de la Presidencia," Spain. The protocol used in the commensalism model was approved by the Animal Experimentation Committee of the University Complutense of Madrid (CEA 33-2015) and Comunidad de Madrid according to Artículo 34 del RD 53/2013 (PROEX 226/15). The treatments did not result in noticeable disease in the animals; nevertheless, all procedures were conducted minimizing any suffering. The number of animals per experiment was adjusted to a minimum for ethical reasons. Experiments were done at least twice and only one representative experiment is shown in the Figures.

Adhesion assays

Adhesion to the intestinal mucosa was assessed as previously described [13]. Briefly, a 1 cm-piece of the large intestine of a non-colonized euthanized mouse was opened, washed gently with PBS and placed in a 4 mm-diameter methacrylate chamber made of two different sheets of methacrylate joined by clamps. The chamber was immediately filled with RPMI medium pre-warmed at 37°C. Then, two *C. albicans* strains (from overnight YPD cultures) to be tested were mixed (1:1 ratio) and adjusted to $2x10^6$ cells/mL concentration in serum-free RPMI medium. 10^5 yeast cells from this suspension were placed facing the original lumen of the colonic tissue in the chamber and incubated for 150 min at 37°C. After this period, the piece of tissue was carefully washed twice with PBS and mechanically disaggregated. Serial dilutions of this last fraction was spread on SD media for CFUs determination. Three similar (size, region) pieces of colonic tissue were used for each competitive determination.

An internal control (CAF2-GFP [13]) was introduced in all adhesion assays; therefore, adherence is quantified by the adhesion relative index (ARI). This index is calculated by dividing the relative amounts of the strain under analysis in adhered cells relative to the value of that same strain in the inoculum and therefore reflects the adhesion relative to the parental CAF2-GFP strain.

Statistical analysis

Statistical differences were assessed with the SPSS Software (UCM, Madrid, Spain). A $p < 0.05$ was considered significant using ANOVA tests (followed by Dunett's multiple comparisons test).

Results

Development of a filamentation - regulatable strain by tetracyclines

In order to analyse the role of the yeast-to-hypha transition, we constructed a strain in which the *TUP1* gene is under the control of the tetracycline repressible version (TET-OFF). For this purpose, the *TUP1* coding region was cloned in the pNRU plasmid (see Material and Methods), which also tagged the protein with a

c-terminal myc epitope, and the gene reporter RFP (dTOM2) in the pDS2 plasmid. The constructs were integrated in a *tup1* mutant generating the TUP1^{REP}-RFP^{REP} strain. To confirm the conditional expression system, we analysed *TUP1* function by observation of the expected filamentous characteristic phenotype of *tup1* mutants in the TUP1^{REP} -RFP^{REP} strain both in liquid and solid media. Under normal conditions (YPD without antibiotic), only yeast cells were observed (Figure 1A). However, the addition of doxycycline to the medium induced the filamentation due to the repression of *TUP1* and wrinkled colonies appeared on solid agar plates. In both cases, the wild type CAF2 strain showed the corresponding yeast phenotype while a homozygous *tup1* mutant remained permanently blocked in the filament form.

Due to the broad antibacterial spectrum of doxycycline and its potential effect on mice microbiota during *in vivo* experiments, which could interfere with the results, we tested a heat inactivated variant of tetracycline (autoclaved chlortetracycline, aCT) [13, 25]. To validate the use of aCT in colonization experiments, we determined *in vitro* its antimicrobial activity (as determined by CMI measures) against *E. coli* DH5α strain compared to the non-inactivated doxycycline. aCT resulted in a 30x fold reduction in its CMI compared to non-inactivated doxycycline (1.5 mg/L vs 50 mg/L) (data not shown). On the other hand, aCT efficiently repressed *TUP1* expression in TUP1^{REP}-RFP^{REP} strain similarly to doxycycline, since both compounds showed the same efficiency in inducing filamentation (via *TUP1* repression) when concentration reaches 0.05 mg/L where hyphae and pseudohyphae were detected (Figure 1B).

We also analysed if this effect was due to the repression of *TUP1.* We studied the ability of aCT and doxycycline to repress *TUP1* by western blot (Figure 1C). In the absence of antibiotic, a clear band of Tup1-myc with the expected molecular weight of 59 kDa appeared while no band was observed when antibiotic (> 0.05 mg/L) was added to the medium indicating the tight regulation of both compounds.

Role of *TUP1*-mediated filamentation in mice gut colonization

The ability of aCT to regulate cellular morphology via *TUP1* enabled us to determine the contribution of the filamentous form in the colonization of mice gut. To validate aCT as a tool to regulate gene expression *in vivo* without alteration in the gut fitness, we analyse aCT bioavailability and activity along mouse intestine. aCT 1 g/L was incorporated in the drinking water of mice for 7 days; the animals were then sacrificed and intestinal samples were used in a bioassay for aCT quantification *in vitro* (Figure 2A). Intestinal samples from aCT-treated or not mice were homogenized in PBS and 10 fold diluted in YPD, media that was used to grow the TUP1^{REP}-RFP^{REP} strain for 16 hours at 37 ºC. We observed that filaments were present only in samples from aCT-treated mice, while this morphology was absent in standard-antibiotic-treated mice (non-aCT-treated), suggesting that the addition of 1 g/L aCT in the drinking water is enough to induce filamentation of the TUP1^{REP} -RFP^{REP} strain in the mouse intestine.

We next analysed the role of filamentation during colonization. After four days of antibiotic treatment (2 g/L streptomycin, 1 g/L bacitracin and 0.1 g/L gentamicin) in drinking water, 10^7 *C. albicans* cells of the TUP1^{REP}-RFP^{REP} strain were inoculated in a single dose by gavage and fecal samples were analysed. As we can observed in Figure 2B, in the first 7 days TUP1^{REP}-RFP^{REP} strain colonized similarly to the wt CAF2 strain (≈10^7 CFUs/g), as previously described [13]. After this period, the TUP1^{REP}-RFP^{REP} colonization levels dropped to ≈10^5 CFUs/g and were maintained until the end of the experiment (≈20 days). At day 20, aCT was administered in drinking water which led to a significant drop in colonization levels (≈10^3 CFU/g, Figure 2B). Colonization was lost in 5-10 days after the administration of aCT. No CFUs were detected in post-mortem analysis of mice, indicating the complete disappearance of the cells in the luminal cavity. These results suggest that the filamentous form is less efficient in gut colonization. When aCT was administered from the beginning of the colonization experiment and before *C. albicans* inoculation, the TUP1^{REP}-RFP^{REP} strain showed a clear decrease and the colonization ratios began to decrease from day 1 until its complete

disappearance (Figure 2C), and no candida cells were detected in post-mortem analysis of the mice. Collectively, we conclude that the blockage in a filamentous growth due to *TUP1* repression impairs *C. albicans* to colonize and maintains in the mice gut.

Sensitivity to bile salts and adhesion to the intestine

Different reasons have been proposed to be the cause for the impairment of gut colonization, such as susceptibility to bile salts or adhesion to the gut mucosa [13]. Sensitivity to bile salts, present in the small intestine, has been proposed as a potential cause to the defects in fitness of candida strains within the gut. For this reason, we performed a drop susceptibility assay with exponentially growing cells of the $TUP1^{REP}$-RFP^{REP} growing in the presence or absence of aCT to allow yeast and filament forms respectively. However, no significant differences were found compared to the wild type CAF2 strain nor to the filamentation status due to the presence or absent of aCT (Figure 3A).

We also tested the adhesion to the gut mucosa in a competitive *ex vivo* assay, as previously described [13]. Similar proportions of $TUP1^{REP}$-RFP^{REP}, growing in the absence or presence of aCT, and a CAF2-GFP wt strain growing in the same conditions (used as internal control) were mixed and incubated with intestinal mucosa. After 150 minutes, fungal cells were recovered and the relative proportions of both cells type relative to the inoculum was determined as described in Material and Methods. We observed, that the induction of filamentation (+aCT) reduced adhesion from 1.25 ± 0.12 (mean) to 1.05 ± 0.18, although these differences were not statistically significant (Figure 3B).

These results suggest that neither sensibility to bile salt nor adhesion to the gut epithelium explain the defects in colonization associated to *TUP1*-mediated filamentation.

Discussion

The controversy on the role of morphology in the virulence of *C. albicans* sustained in the past an intense debate in the mycology field [26-28]. While mutants unable to filament were frequently avirulent in certain animal models [15], it soon became evident that certain hyperfilamentous mutants or strains locked in the filamentous form were also avirulent [16, 21]. The development of genetics soon enabled the construction of strains where hyphal formation could be either triggered or repressed *in vivo*. These tools demonstrated that hyphal conversion is important for virulence but, more importantly, that ability to alternate between different morphologies was indeed essential for the fungus to cause disease [18]; differences were appreciated between both morphologies and yeast forms were found to colonize more abundantly target organs in the standard mouse systemic model of infection [29]. As the gastrointestinal location may be an important source of endogenous infections [30, 31], understanding the mechanisms underlying colonization is essential for the design of efficient prophylactic and therapeutic strategies [11]. In fact, those factors impeding colonization may be relevant strategies to prevent candidiasis.

We have analysed the role of filamentation in commensalism using a *TUP1*-regulated strain where the addition of doxycycline efficiently leads to filamentation. We first validated the use of this drug in *in vivo* studies, as doxycycline, a broad spectrum antibacterial used in *C. albicans* genetics [32] could significantly alter the microbiota of colonized mice. Our data indicate that heat inactivated chlortetracycline (aCT) is as efficient as doxycycline in repressing *TUP1* expression. We demonstrate that the $TUP1^{REP}$-RFP^{REP} strain was indeed able to colonize the mouse gut: initial fungal loads in the gut were $\approx 10^7$ CFU/g but dropped to $\approx 10^5$ after one week and remained constant onwards.

In this model, induction of filamentation due to *TUP1* repression led to a complete loss of *C. albicans* cells in the gut. This result is in agreement with recent data that indicate that filamentous locked strains (*nrg1*Δ) colonized less efficiently the mouse [19]. However, one important feature of our results is that we introduce cells in the mouse in the yeast form and trigger filamentation inside the host, thus discarding the possibility (not addressed in the mentioned work) that that filamentous forms would be able to colonize once in the intestine but unable to initiate it. This may be relevant as different adaptation phases can be detected when colonizing the mouse gut in this particular commensal model [20]. We still have no explanation for this behaviour; susceptibility to bile salts influences initial colonization loads achieved [33] but no major changes were detected between wt and $TUP1^{REP}$-RFP^{REP} cells. Transcriptomic analysis of *tup1* cells reveals changes in several metabolic traits that could be relevant during adaptation to the gut environment, mainly anaerobic [34] but they are obtained from *in vitro* cultured cells. Adhesion to the gut epithelium may be also relevant in the context of colonization, as several adhesins are either present or absent in *tup1* mutants. It may be as well that the filamentous form is intrinsically less efficient in colonizing due to a mere mechanical reasons (and more efficient removal natural flow of the aliments in the lumen). Our results are in agreement with recent results obtained using germ free mice [35]. These authors identified different *C. albicans* genes (*ZFY2*, *ZCF8*, *ZFU2* and *TRY4*) whose deletion resulted in enhanced filamentation and reduced colonization. In addition, expression of the *UME6* filamentation regulator had a similar effect. As these experiments were obtained with germ free mice, mice microbiota does not seem to influence the preference of yeast versus filaments in the mouse lumen. The reasons for this may be speculative at this stage but a different immune response, resistance to innate antifungal mechanisms, adherence to the lumen or even simple mechanical features may be responsible for this effect. Therefore, our work supports that the *C. albicans* yeast form is more prone to colonize than hyphae. Importantly, our results reinforce the concept that induction of filamentation of *C. albicans* in the human gut via drugs or food products may be an alternative approach to reduce colonization and minimize systemic fungal infections caused by this yeast.

Conclusions

The work presented here show that induction of filamentation via *TUP1* repression results in a loss of *C. albicans* cells within the mouse gut, while the corresponding yeast forms efficiently colonizes at lower levels. These effects are not due to a defect in adhesion to the gut epithelium nor to an increased susceptibility to bile salt suggesting that filamentous forms are intrinsically less adapted to grow in the mammalian intestines.

Summary Points

TUP1 repression led to a filament locked phenotype both in liquid and solid media

Autoclaved chlortetracycline, aCT, has a reduced antimicrobial effect but is still able to regulate the TETON/TETOFF regulatable system in *Candida albicans*

TUP1 repression renders *candida* filament cells that are unable to establish in the murine gut

The induction of filamentation by repressing *TUP1* induced the loss of candida cells once stablished in the murine gut

No differences in susceptibility to bile salt nor to the ability to adhere to the gut mucosa is due to the repression of *TUP1*

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. All experiments involving animals performed in this work were carried out in strict accordance with the regulations in the “Real Decreto 1201/2005, BOE 252” for the Care and Use of Laboratory Animals of the “Ministerio de la Presidencia,” Spain. The protocol used in the commensalism model was approved by the Animal Experimentation Committee of the University Complutense of Madrid (CEA 33-2015) and Comunidad de Madrid according to Artículo 34 del RD 53/2013 (PROEX 226/15). The treatments did not result in noticeable disease in the animals; nevertheless, all procedures were conducted minimizing any suffering. The number of animals per experiment was adjusted to a minimum for ethical reasons.

Figure 1. TUP1-myc regulation in regulated strains under the control of the TET-OFF system.

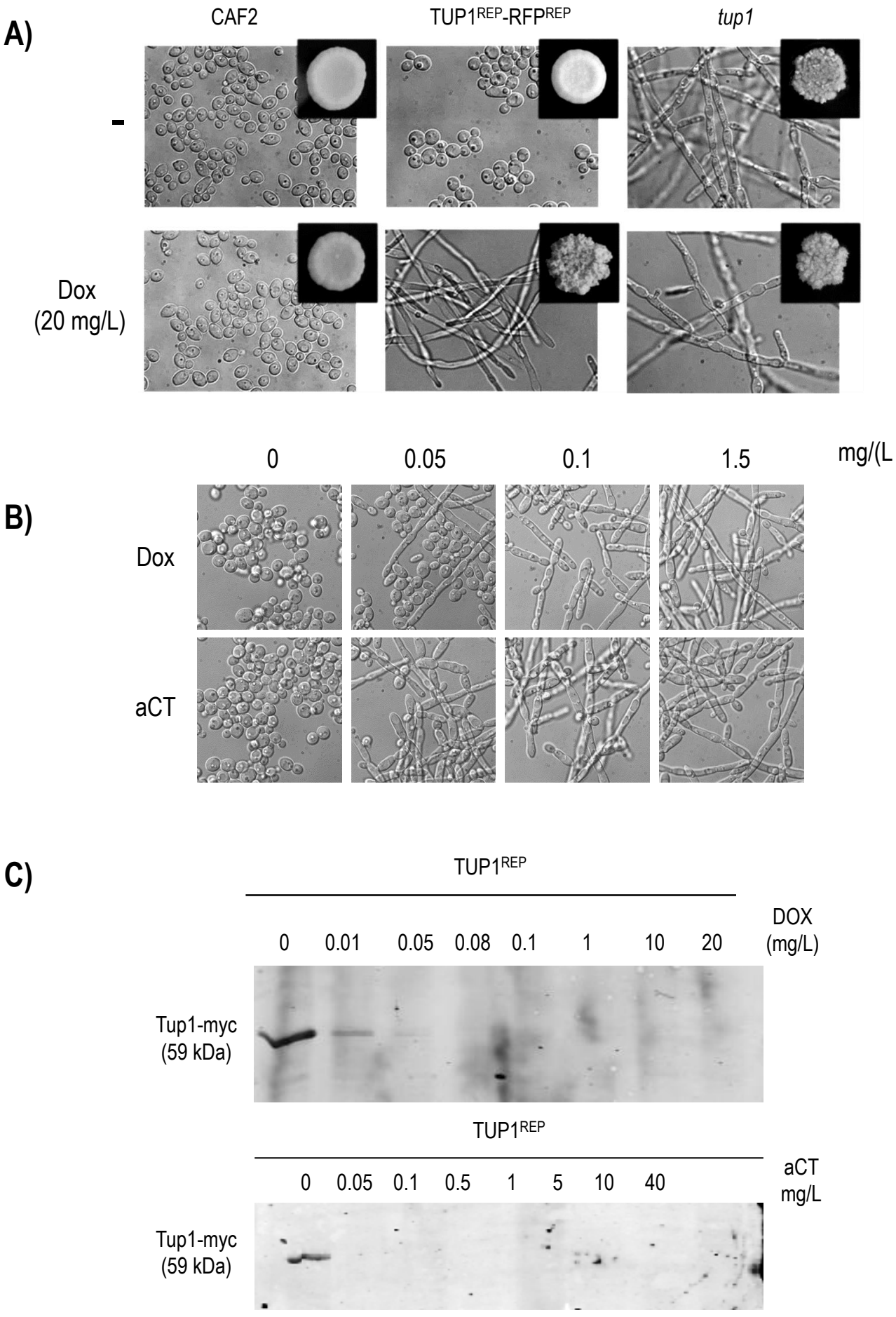
A) Cellular morphology after 16 hours at 37ºC of growth in the absence (-) or presence of 20 mg/L doxycycline. An image from a drop assay on solid media with or without dox after 48 h growth is shown (inset); B) Cellular morphology of the TUP1^{REP}-RFP^{REP} strain in YPD supplemented with increasing concentration of dox after 16 hours of growth at 37ºC. C) Tup1-myc detection under different doxycycline (upper panel) or aCT (lower panel) concentrations in YEPD at 37ºC

Figure 2. Filamentous forms are less efficient to colonize de GI tract

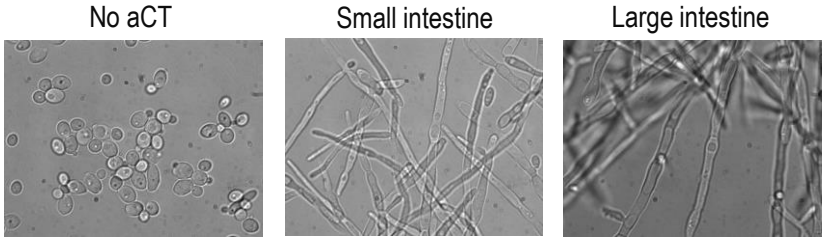
A) Bioassay to detect the aCT presence in mice intestine. Cellular morphology of the TUP1^{REP}-RFP^{REP} strain in the presence of the intestine content from mice treated or not with aCT after 16 hours of growth at 37 ºC., B) TUP1^{REP}-RFP^{REP} colonization rates expressed as logarithmic values of the CFUs per gram of stool along the time in mice treated with standard antibiotic treatment. At day 20 aCT 1 g/L was added to the drinking water; C) TUP1^{REP}-RFP^{REP} colonization rates in mice treated with standard antibiotic cocktail plus aCT 1 g/L from the beginning of the experiment.

Figure 3. Analysis of bile salt sensitivity and adhesion to mouse mucosa

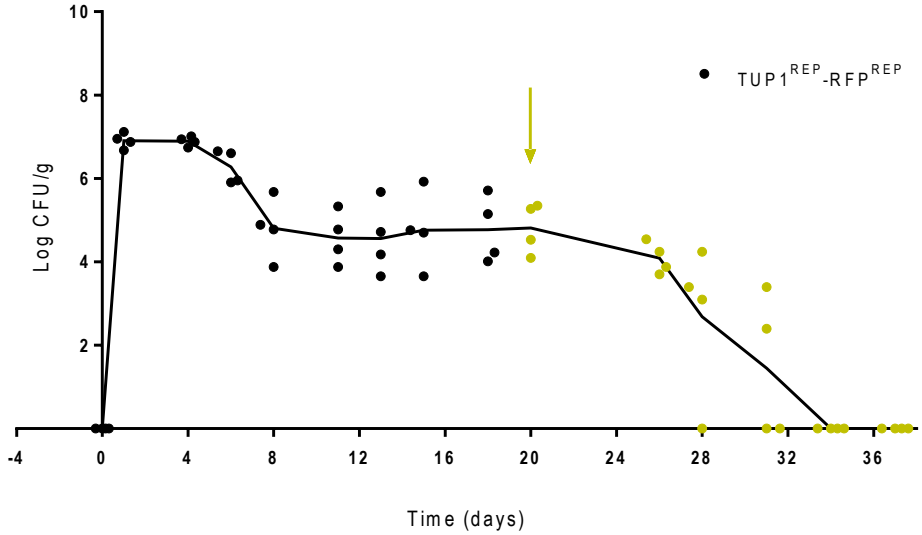
A) Tenfold serial dilutions of overnight growing cultures from the indicated strains were spotted in YPD plates supplemented or not with bile salts in the presence or not of aCT 20 mg/L; B) The adhesion relative index (ARI) was determined for the TUP1^{REP}-RFP^{REP} in the large intestine in the presence or absence of aCT. Individual values are shown with the mean ± SEM.


A)
CAF2
TUP1REP-RFPREP
tup1
-
Dox
(20 mg/L)
B)
0
0.05
0.1
1.5
mg/(L
Dox
aCT
C)
TUP1REP
DOX
(mg/L)
0
0.01
0.05
0.08
0.1
1
10
20
Tup1-myc
(59 kDa)
TUP1REP
aCT
mg/L
0
0.05
0.1
0.5
1
5
10
40
Tup1-myc
(59 kDa)

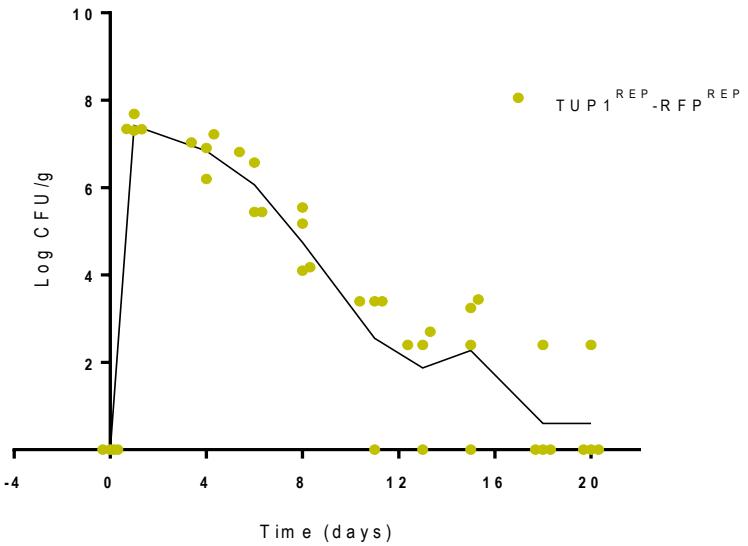
A)


No aCT
Small intestine
Large intestine

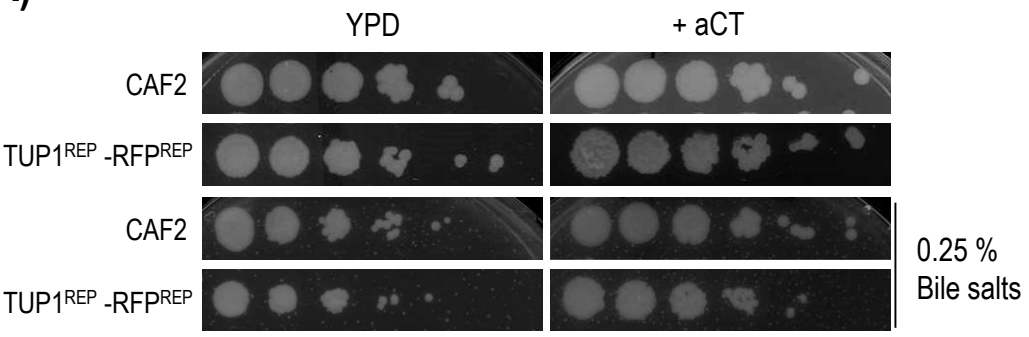
B)


Log CFU/g
10
8
6
4
2
-4
0
4
8
12
16
20
24
28
32
36
Time (days)
TUP1REP-RFPREP

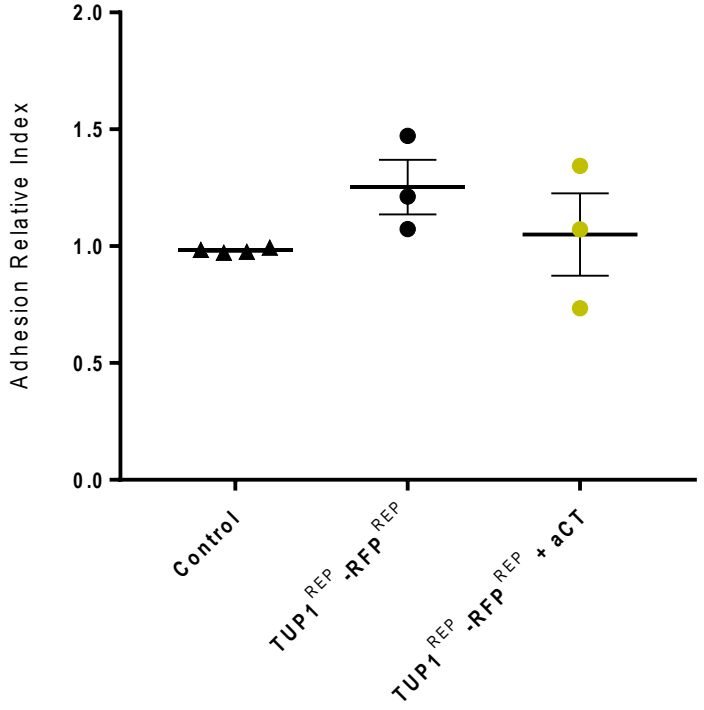
C)


Log CFU/g
10
8
6
4
2
-4
0
4
8
12
16
20
Time (days)
TUP1REP-RFPREP

A)


YPD
+ aCT
CAF2
TUP1REP -RFPREP
CAF2
TUP1REP -RFPREP
0.25 %
Bile salts

B)


Adhesion Relative Index
2.0
1.5
1.0
0.5
0.0
Control
TUP1 REP -RFP REP
TUP1 REP -RFP REP + aCT