Rifampicin induced virulence determinants increase *Candida albicans* biofilm formation [version 1; peer review: 3 approved with reservations]

Miriam Vogel¹*, Martin Köberle¹.²*, Holger Schäffler¹, Monika Treiber¹, Ingo B Autenrieth¹, Ulrike K Schumacher¹

¹Interfaculty Institute of Microbiology and Infection Medicine, Eberhard Karls University, Tübingen, 72076, Germany
²Dermatology, Eberhard Karls University, Tübingen, 72076, Germany

* Equal contributors

Abstract
Increased intravenous catheter use has been paralleled by increased bacterial and yeast bloodstream infection. Biofilm formation, which is associated with the cell surface hydrophobicity (CSH) phenotype, represents a major pathogenicity strategy of *Candida albicans*, becoming especially important in the colonization of intravascular medical devices. Increasing evidence shows the induction of virulence factors in *C. albicans* by diverse substances. Therefore, we investigated whether rifampicin, an antibiotic shown to be capable of inducing MDR1 expression in *C. albicans* may also promote the formation of a pathogenic biofilm. In response to 40 µg/mL rifampicin, an enhanced retention of *C. albicans* SC5314 cells on polystyrene culture plates was observed by measuring increased metabolic activity by XTT assay, indicating induction of biofilm formation. Rifampicin treatment also induced fibronectin binding, cell hydrophobicity and germ tube formation. Furthermore, increased RNA and protein expression of CSH1p, a major mediator of CSH, was demonstrated. We conclude that exposure to rifampicin may result in upregulation of key *Candida* virulence determinants, potentially boosting pathogenicity and supporting biofilm formation. This finding gains clinical significance from the increasing popularity of rifampicin-coated catheters, which might provide an advantageous gateway for *Candida* bloodstream infections.
Corresponding author: Ulrike K Schumacher (ulrike.schumacher@uk-halle.de)

Competing interests: UKS has participated as an investigator in multicenter studies for GSK, Pfizer and Wyeth Pharmaceuticals, and has received speaker's honoraria from BioMerieux, Becton Dickinson and Pfizer.

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Introduction

*Candida albicans* is an opportunistic yeast pathogen that has been established as a persistent and growing threat for critical ill patients over the last three decades. *Candida* species are the most common fungal species to cause invasive infections and at least one half of candidemia cases in non-neutropenic patients occur in an ICU or surgical ward. Invasive candidiasis is associated with poor prognosis, with mortality rates of up to 30%-53. Above all, *C. albicans* ranks as the fourth most common cause of bloodstream infection, carrying one of the worst prognoses3-5. The increase of *Candida* infections in the ICU comes at the same time as medical progress with the development of broad spectrum anti-bacterial therapy and immunosuppressive therapy6. There is also a close connection between the increased use of intravascular medical devices and the advent of bloodstream infections among the most frequent and potentially lethal nosocomial infections5,7. Catheter implantation frequently provides a gateway for the systemic entry of *Candida* and other pathogens from diverse sources. Additionally, hydrophobic catheter surfaces are a favorable habitat for pathogens that are able to form biofilms. They provide pathogens with a reservoir that is difficult to eradicate even by high dose antifungal therapy, as resistance to antifungals is increased 10 to 1000 times compared to planktonic cells8,9. Known mechanisms of antifungal resistance under biofilm conditions are the upregulation of drug efflux pumps, sequestration of antifungals by matrix glucans and the development of a persister cell subpopulation10-12. Therefore, hydrophobic attachment to tissues and artificial surfaces can be regarded as a key virulence determinant of *Candida* spp. Adhesion of *Candida* and other fungi to polymeric materials correlates with cell surface hydrophobicity (CSH) phenotype13, though tissue and plastic binding are also mediated by adhesins14. The *CSH1* gene product has been shown to be one of the mediators of CSH phenotype, localizing on the yeast cell surface and enhancing cell hydrophobicity as well as fibronectin binding15-17. It is thought to be induced by germ tube formation of single attaching yeast cells, subsequently evolving into a dense network of hyphae and pseudohyphae18. Quorum sensing of the densely packed cells regulates transcription of glucans that form a polymeric intercellular matrix19,20. We and others have recently shown that substances of diverse structure and origin may induce Candida pathogenicity factors. 17-β-oestradiol increased growth and germ tube formation mediated increased temperature resistance by induction of the *Hsp90* chaperon and elevated expression of the multidrug transporters *CDR1* and *CDR2*. As coumarin and phenol also upregulated *Hsp90* and *CDR1*, the authors of this study concluded that the response to estrogen might be rather unspecified21,22. Cigarette smoke induced the expression of histolytic enzymes and increased candidal adhesion in vitro23. Rifampicin, a common antibiotic, induces MDR-1 expression by *Candida albicans* that in turn can lead to modestly elevated minimal inhibitory concentrations (MIC) for fluconazole by some isolates24,25.

Rifampicin-impregnated central-venous catheters have been increasingly used in clinical trials and one of these showed significantly increased *Candida* colonization26. To investigate whether rifampicin used in antibacterial catheter coatings to control bacterial catheter-related bloodstream infections may boost *Candida* virulence on these devices, we examined the possible induction of *C. albicans* SC5314 virulence factors involved in surface adhesion and biofilm formation by this drug.

Methods

*C. albicans* strains and growth conditions

*C. albicans* wild-type strain SC531427 was kindly provided by J. Morschhäuser, Institute for Molecular Infection Biology, Würzburg, Germany. The strain was kept as a frozen stock in glycerol at -80°C. For experiments, frozen cells were streaked on yeast-dextrose agar plates (5 g of yeast extract, 10 g of peptone, 20 g of dextrose, 15 g of agar, 40 mg gentamicin per litre), incubated at 30°C overnight and sub-cultured in YNB liquid medium (0.67 % Yeast Nitrogen Base, 0.5 % dextrose) at 30°C. Cultures were diluted in 25 mL YNB medium to an optical density at 600 nm (OD600) of 1 and were incubated at 30°C for 4 h with gentle shaking. For induction of the rifampicin response, 40 µg/mL rifampicin was used if not indicated otherwise.

Reagents

Unless stated otherwise, all reagents have been purchased at Sigma-Aldrich (Taufkirchen, Germany). Compounds were dissolved in acetone (menadione), DMSO (rifampicin) or water (all others).

Metabolic activity assay

Biofilm formation of SC5314 was quantified by the metabolic activity retained on a 96-well polystyrene plate (Falcon; BD Labware, Franklin Lakes, NJ) as described by Rammage and co-workers28. In brief, 10^4 cells/well were allowed to adhere to the plate for 48 h. After three times washing thoroughly with phosphate-buffered saline (PBS) (Gibco; Invitrogen, Carlsbad, CA), 2 h incubation with 2,3- bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) substrate (0.5 mg/mL + 1 µM menadione in Ringer’s solution) was performed in the dark. Absorbance of reduced XTT was measured in a microtiter plate reader (Tecan, Maennedorf, Switzerland) at 490 nm.

Microsphere adherence

To assay Candida cell hydrophobicity, the number of adherent micro particles (Serva, Heidelberg, Germany) per cell was quantified as described by Hazen & Hazen29. 100 µL of a 2x10^4 cells/mL SC5314 suspension in ice cold PBS were mixed with 100 µL microsphere solution (~ 8.4x10^3 particles/µL) in acid-washed glass vessels. Two minutes of incubation at room temperature was followed by 30 s of rigorous mixing. Samples of
this mixture were subjected to phase contrast microscopy (Axio- 
lab / Axiovert 200 + AxioCam HRc microscope; Carl Zeiss 
Microimaging, Esslingen, Germany). Relative hydrophobicity 
was determined as the fraction size of cells with three or more 
adhering particles.

**Hydrocarbon extraction**
Extraction of *Candida* cells from an aqueous suspension with 
xylene was performed by a protocol adapted from Rosenberg 
and co-workers. In brief, 4 mL of SC5314 suspension were 
vortexed with 1 mL of xylene for 2 min in glass tubes. After 
15 min incubation at 37°C, tubes were cooled to room tem-
perature and the OD_{400} of the aqueous phase was determined 
(Biophotometer, Eppendorf, Hamburg, Germany). Samples 
without xylene treatment served as controls. Relative hydro-
phobicity was calculated as the ratio of control samples to xy-
lene treated samples.

**Fibronectin binding**
For the detection of fibronectin binding, *Candida* cells were 
sub-cultured in medium supplemented with 0.001% or 
0.0001% human fibronectin (Sigma-Aldrich) for 1 h. Subse-
quently, cells were examined microscopically or washed 3x with 
PBS and subjected to immunoblot analysis.

**Germ tube induction**
To induce germ tube formation, 1x10^6 *C. albicans* yeasts/mL 
were transferred into the cell culture medium RPMI 1640 
(Biochrom, Berlin, Germany) supplemented with 10% (v/v) 
(Sigma-Aldrich) FCS, 2 mM L-glutamine, penicillin (100 U/ 
ml) and streptomycin (100 µg/mL) (Biochrom KG) and seed-
ed into a 24-well plate. After 1 h, pictures were taken by phase 
contrast microscopy and germ tube length was determined 
with the Photoshop 6 measure tool (Adobe, San Jose, CA).

**RNA preparation and reverse transcription-PCR (RT-PCR)**
Total RNAs were extracted from *C. albicans* cells by use of the MasterPure™ Yeast RNA Purification Kit (EPICENTRE, 
Madison, Wisconsin). Contaminating DNA was removed by 
the TURBO DNA-free™ Kit (Ambion Inc, Austin, Texas). 
Briefly, RNA was incubated at 37°C for 30 min with 2 U TUR-
BO DNase per 10 µg of RNA. DNase was inactivated by add-
ing DNase inactivation reagent for 2 min at room temperature. 
Total RNA concentrations were spectrophotometrically quan-
tified. RNA samples were stored at -80°C or were used imme-
diately. cDNA was synthesized with oligo(dT)-primers (New 
England Biolabs, Frankfurt, Germany) and Superscript™ III 
reverse transcriptase (Invitrogen, Karlsruhe, Germany), using 1 
µg of total RNA. Controls examined *C. albicans* elongation fac-
tor 1 (*EFB1*) housekeeping gene, which contains an intron of 
365 bp. Absence of genomic DNA was verified by a single in-
tronless PCR product of *EFB1*. PCR were performed according 
standard protocols with 0.5 U *Taq* DNA polymerase (Roche, 
Mannheim, Germany). To amplify *CSH1* or *EFB1* the prim-
ers AGT AGA AAG CAT ATC TTA GCC G (fwd) and GCT 
TGT TCT TCT AGA AGC ACT GC (rev) or AGT CAT TGA ACG 
AAT TCT TGG C (fwd) and ATC AAC TTC ATC AGA ACC G (rev) were used. Cycling conditions were 94°C for 30 
s, 63°C for 30 s and 72°C for 60 s for *CSH1* and 94°C for 30 s, 
60°C for 30 s and 72°C for 60 s for *EFB1*. Only samples from 
the exponential phase of PCR amplification were examined. 
Equivalent volumes of PCR product were analysed on 1.8% 
agarose gels stained with ethidium bromide.

**Analysis of protein expression**
Proteins were isolated by the method of Hiller et al. Equal protein 
amounts as determined by Bradford assay were separated on a 10% SDS gel and transferred to nitrocellulose membranes 
by wet-blot. Further membrane protein binding was blocked 
by incubation in 5% skimmed milk powder containing wash 
buffer. CSH1p or fibronectin were detected by subsequent in-
cubation with mouse anti- CSH1 (1:10,000; kindly provided 
by D. Singleton) or rabbit anti-human fibronectin (1:4000; 
Sigma-Aldrich) primary antibodies and horseradish peroxidase 
(HRP) labeled rabbit anti-mouse or swine anti-rabbit IgG sec-
ondary antibodies (both 1:1000; DAKO, Glostrup, Denmark), 
respectively. Chemiluminescence of Amersham ECL reagent 
(GE Healthcare, Waukesha, WI), was detected on CL-XPosure 
Film (Thermo, Rockford, IL).

**Statistical analysis**
Unless indicated otherwise, data shown are representative of at 
least two independent experiments. Differences between mean 
values were analyzed using two tailed Student's *t* test. *P* < 0.05 
was considered statistically significant.

**Results**
*Rifampicin induces SC5314 cell surface hydrophobicity* 
The cell surface hydrophobicity (CSH) phenotype was identi-
fied as a major factor conferring virulence to *Candida* species 
by many means, first of all by enhancing attachment to tissues 
and foreign materials, thus being a prerequisite for efficient 
biofilm formation. To examine whether rifampicin might 
increase *C. albicans* CSH phenotype expression, we quantified 
the adhesion of microspheres to SC5314 with or without ri-
fampicin treatment. Microsphere adherence to SC5314 cells 
robustly doubled in response to rifampicin treatment, show-
ing increased hydrophobicity and suggesting CSH phenotype 
induction (Figure 1A and 1B). Another method established for 
the measurement of microbial surface hydrophobicity is the 
measurement of extraction from an aqueous suspension with 
an organic solvent. After rifampicin treatment, extraction of 
SC5314 was significantly increased, providing additional 
support for increased hydrophobicity (Figure 1C).
40 µg/mL rifampicin. We observed a 1.6-fold significant increase in metabolic activity retained after thoroughly washing on the culture plate induced by rifampicin treatment, indicating that rifampicin enhances biofilm formation by *C. albicans* (Figure 2A).

Rifampicin promotes germ tube formation

The morphological differentiation ability of *C. albicans* plays an important role in biofilm maturation that follows hydrophobic attachment. Mutants deficient in the yeast phenotype show reduced adhesion to tissues and polymers and are more easily removed under high-salt assay conditions, indicating improved anchoring of the biofilm by yeast phenotype cells. However, the dimorphic phenotype enables the formation of thicker biofilms with higher cell numbers than yeast-only strains that may display superior resistance to chemical and mechanical stress, respectively. In our experiments, rifampicin treatment turned out to profoundly induce germ tube formation, accelerating germ tube growth leading to increased hyphae to yeast cells ratio. Average germ tube length was doubled 4 h after induction (Figure 2B and 2C). Interestingly, our data are paralleled by the

Enhanced fibronectin binding after rifampicin treatment

The affinity of *Candida* for fibronectin has been known for a long time and has been regarded as a virulence property enabling tissue adhesion by extracellular matrix binding, thus promoting initiation as well as dissemination of candidiasis\(^\text{33}\). Strikingly, microscopic observation revealed dense clustering to long, bottle brush like aggregates (Figure 1D). Western blot analysis of SC5314 cultured in medium supplemented with human plasma fibronectin in the presence or absence of rifampicin proved strongly enhanced fibronectin retention of rifampicin treated cells after thoroughly washing (Figure 1E).

Rifampicin promotes SC5314 biofilm formation

Biofilm formation by *Candida* spp. on tissues or implanted materials is a process with high clinical impact, as it mediates increased resistance to antifungal agents and protection from host defenses. Additionally, biofilms act as pathogen reservoirs, boosting persistent infection\(^\text{9}\). To get a quantitative idea of how rifampicin affects *Candida* retention on plastic materials, we assayed XTT reduction of adhering *C. albicans* SC5314 after culture in polystyrene wells for 48 h, untreated or treated with 40 µg/mL rifampicin. We observed an 1.6-fold significant increase in metabolic activity retained after thorough washing on the culture plate induced by rifampicin treatment, indicating that rifampicin enhances biofilm formation by *C. albicans* (Figure 2A).
In this work we addressed the question of whether rifampicin might promote *Candida albicans* biofilm formation, thus potentially aggravating the threat of catheter-related and other infections by this pathogen. We could show increased SC5314 cell hydrophobicity, indicating a shift to a CSH phenotype. It has to be taken into account that a recent report failed to correlate the hydrophobicity of 50 clinical *C. albicans* isolates with their adhesiveness to polystyrene. Other reports however, have shown hydrophobicity not only to correlate with *Candida* adhesiveness but to be part of concerted pathogenicity factor expression. Fibronectin binding was also strongly increased after rifampicin treatment, showing the induction of an additional trait contributing to increased adherence. In line with these findings we could demonstrate that rifampicin treatment induces enhanced *Candida* retention on plastic by metabolic activity assay. Additionally, rifampicin enhanced germ tube formation, known to promote an exploratory and invasive lifestyle. Reports showing germ tube formation to be induced a few hours after CSH increase or surface attachment strongly argue it to be part of the biofilm program. Germ tubes also enhance biofilm compression strength and mediate phagocyte observations of other authors who report enhanced filamentous growth induced by tetracycline and 17-β-estradiol.

CSH phenotype is mediated by CSH1p expression

*CSH1* gene expression may be a mediator of the CSH phenotype. Upon rifampicin treatment, an immediate increase in *CSH1* transcription could be detected in SC5314, as well as sustained high level expression of *CSH1* mRNA until 4 h after induction (Figure 3A). Induction of *CSH1* transcription is matched by elevated CSH1p protein levels in SC5314 lysates (Figure 3B).

**Figure 2** Rifampicin increased biofilm formation on polystyrene and induced germ tube formation. (A) Biofilm formation of SC5314 after treatment with 40 µg/mL rifampicin. Mean and SD of XTT absorption normalized on untreated controls of three independent experiments; *difference is statistically significant (p < 0.05).* (B) Rifampicin-induced germ tube formation, representative phase-contrast microscopy. (C) Germ tube length in µm; mean + SD of three independent experiments. *Significant difference (p < 0.005).*

Rifampicin-induced virulence determinants increase *Candida albicans* biofilm formation: germ tube length.

1 Data File
http://dx.doi.org/10.6084/m9.figshare.656872

Rifampicin-induced virulence determinants increase *Candida albicans* biofilm formation: hydrocarbon extraction.

1 Data File
http://dx.doi.org/10.6084/m9.figshare.656873

Rifampicin-induced virulence determinants increase *Candida albicans* biofilm formation: microsphere assay.

1 Data File
http://dx.doi.org/10.6084/m9.figshare.656874

**Discussion**

In this work we addressed the question of whether rifampicin might promote *Candida albicans* biofilm formation, thus potentially aggravating the threat of catheter-related and other infections by this pathogen. We could show increased SC5314 cell hydrophobicity, indicating a shift to a CSH phenotype. It has to be taken into account that a recent report failed to correlate the hydrophobicity of 50 clinical *C. albicans* isolates with their adhesiveness to polystyrene. Other reports however, have shown hydrophobicity not only to correlate with *Candida* adhesiveness but to be part of concerted pathogenicity factor expression. Fibronectin binding was also strongly increased after rifampicin treatment, showing the induction of an additional trait contributing to increased adherence. In line with these findings we could demonstrate that rifampicin treatment induces enhanced *Candida* retention on plastic by metabolic activity assay. Additionally, rifampicin enhanced germ tube formation, known to promote an exploratory and invasive lifestyle. Reports showing germ tube formation to be induced a few hours after CSH increase or surface attachment strongly argue it to be part of the biofilm program. Germ tubes also enhance biofilm compression strength and mediate phagocyte...
escape, suggesting a key role in biofilm pathogenesis\textsuperscript{18,37}. Csh1p is an aldo-keto reductase and is homologous to aryl-alcohol dehydrogenases in \textit{Saccharomyces cerevisiae}\textsuperscript{17}. \textit{CSH1} transcription is activated by Zap1, a negative regulator of the matrix component soluble \(\beta\)-1,3 glucan and thought to mediate the Zap1 effect by intercellular signalling\textsuperscript{20}. To answer the question of whether \textit{CSH1} expression might be part of the CSH phenotype induced by rifampicin, we analysed \textit{CSH1} mRNA and protein levels in \textit{C. albicans} SC5314. We found \textit{CSH1} mRNA upregulation and an increase in CSH1p protein levels.

Interestingly, CSH1p deficiency has been shown to drastically reduce the fibronectin binding properties of \textit{C. albicans}, thus opening the possibility that increased fibronectin binding results from CSH1p upregulation, though host fibronectin binding seems to be mediated by multiple \textit{Candida} surface proteins\textsuperscript{15,16}. Previously we demonstrated that rifampicin upregulated \textit{C. albicans} \textit{MDR1} expression\textsuperscript{24}. Co-induction of \textit{CSH1} and \textit{MDR1} has also been shown for a fluconazole-resistant \textit{C. albicans} patient isolate\textsuperscript{38}. Moreover, Mdr1 has been shown to be upregulated immediately after adhesion\textsuperscript{39}, and both \textit{CSH1} (orf19.4477) and \textit{MDR1} (orf19.5604) transcription is mediated by the multidrug resistance regulator Mrr1p\textsuperscript{40}. This raises the possibility that Mrr1p may be a target of rifampicin in \textit{C. albicans}. Taken together, the results of this study show a significant upregulation of \textit{Candida} virulence determinants that promote pathogenic biofilm behaviour by the antibiotic rifampicin. Thus, antibacterial rifampicin coatings of intravascular medical devices could potentially oppose efforts to diminish their microbial colonization. This effect could also be suspected to interfere with the promising effects of some recent antimycotics in inhibiting biofilm growth\textsuperscript{41}. Furthermore, the data shown here contribute to the growing evidence showing that miscellaneous structurally unrelated substances that are xenobiotic to yeasts are capable of inducing mediators of \textit{Candida} virulence and drug resistance. Eventually, enhanced induction by cooperation of several of these substances cannot be excluded (e.g. tetracycline + rifampicin coated catheter in combination with estrogen)\textsuperscript{21,26,34}.

\textbf{Acknowledgements}

We thank David Singleton for kindly providing the CSH1p antibody and Tarun Mehra for proofreading the manuscript. Parts of this work were presented at the 8th ASM conference on Candida and Candidiasis, Denver, USA 2006.
Authors' contributions

MV performed the experiments and analyzed the data. HS, MT and MK participated in performing experiments and data analysis. MK drafted the manuscript. UKS designed and coordinated the study, analyzed the data, supervised MV, HS and MT and together with MV participated in finalizing the manuscript. IBA participated in study design and executed critical readings of the manuscript. All authors read and approved the final manuscript.

Grant information

This work has been funded by a research grant from Landesstiftung Baden-Württemberg to UKS.

Competing interests

UKS has participated as an investigator in multicenter studies for GSK, Pfizer and Wyeth Pharmaceuticals and has received speaker’s honoraria from BioMérieux, Becton Dickinson and Pfizer.

References

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Version 1

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Dipshikha Chakravortty
Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India

This article by Vogel et al. raises the very interesting and crucial problem of rifampicin coated catheters posing a threat due to Candida growth. Rifampicin by itself is not active against Candida, however various studies have shown that its combination with amphotericin B may enhance the anti-Candida activity.

Comments

- Fig. 1D: Detection of fibronectin retention - This is very difficult to conclude from the given photograph. The photograph depicts crowded accumulation of Candida. Where are the “Fibronectin fibres”? Please provide high resolution photos, and provide evidence for the fibronectic fibres.

- Fig. 1E: “Western blot of SC5314” - this statement is not correct. Western blot or immunoblot should be detecting the protein, though in this figure the detection is of fibronectin - the readers will get confused as the sentence is not correct. Please provide the fibronectin addition as μg/ml rather than the percentage. It would be beneficial if the authors look into the status of fibronectic binding proteins in Candida’s response to rifampicin.

- Fig. 3A: Densitometric analysis is required to appreciate the induction of CSH1 gene in response to rifampicin.

- Fig. 3B: For the western blot, housekeeping genes from Candida should be used as a loading control, and the densitometric analysis should be provided. The dose dependent response to rifampicin is required.

- How does rifampicin induce the CSH expression? What is the mechanism? The discussion should be strengthened by providing the probable mechanism.

- Is this phenomenon specific to rifampicin?

Competing Interests: No competing interests were disclosed.
I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 14 May 2013
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? Guilhem Janbon
Institut Pasteur, Paris, France

This paper describes the effect of rifampicin on adhesion and hydrophobicity of Candida albicans. This is an important issue because (1) this antibiotic is commonly used and (2) Candida albicans is prone to develop biofilms that are resistant to a number of antifungal drugs. The authors convincingly show that cells treated with rifampicin make more germ tubes, change their hydrophobicity and become more adherent to fibronectin. They also demonstrate that these phenotypic changes are associated with an increased expression of the gene CSH1.

However, it remains to be proved that these phenotypes and the increased expression are mechanistically linked. The analysis of CSH1Δ strain phenotype in presence of rifampicin could provide information concerning this matter.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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? Malcolm Whiteway
Department of Biology, Concordia University, Montreal, Quebec, Canada

The logic of this paper is reasonable – if rifampicin enhances biofilm formation in C. albicans, then treating medical implants with rifampicin could have unexpected negative consequences. The experiments themselves are straightforward and in general support the idea that rifampicin influences surface
hydrophobicity in *C. albicans*, and ultimately has a moderate effect on biofilm formation assayed through essentially XTT detected activity on a plastic surface. Considerably more sophisticated assays of biofilms are possible, and a number of genes have been identified that are critical for standard biofilm formation. To really probe the impact of the drug on Candida biofilms, the authors could assess the impact of mutants on the process under study, do ultrastructural assessment of the biofilms, and do analysis of the effect of rifampicin on a variety of biofilm models. Thus, the impact of the paper would be considerably enhanced if the sophistication of the biofilm assay was greater.

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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