

# Candida albicans biofilms are generally devoid of persister cells

Iryna Denega, Christophe d'Enfert, Sophie Bachellier-Bassi

► **To cite this version:**

Iryna Denega, Christophe d'Enfert, Sophie Bachellier-Bassi. Candida albicans biofilms are generally devoid of persister cells. 2018. pasteur-02076719

**HAL Id: pasteur-02076719**

**<https://hal-pasteur.archives-ouvertes.fr/pasteur-02076719>**

Preprint submitted on 22 Mar 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



1 ***Candida albicans* biofilms are generally devoid of persister cells**

2

3 Iryna Denega<sup>1,2</sup>, Christophe d'Enfert<sup>1</sup> and Sophie Bachellier-Bassi<sup>1,#</sup>

4

5 <sup>1</sup>Institut Pasteur, INRA, Unité Biologie et Pathogénicité Fongiques, 25 rue du Docteur  
6 Roux, Paris, France

7 <sup>2</sup>Univ. Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, rue du Docteur Roux,  
8 Paris, France

9

10

11 #Corresponding author: Sophie Bachellier-Bassi, Institut Pasteur, Unité Biologie et  
12 Pathogénicité Fongiques, Département Mycologie, 25 rue du Docteur Roux, F-75015  
13 Paris, France; Phone: +33 (1) 45 68 86 19; E-mail: [sophie.bachellier-](mailto:sophie.bachellier-bassi@pasteur.fr)

14 [bassi@pasteur.fr](mailto:bassi@pasteur.fr)

15

16

17

18 **RUNNING TITLE:** Lack of persister cells in *C. albicans* biofilms

19 **KEYWORDS:** *Candida albicans*, biofilms, antifungal tolerance, persistence

20

21 **ABSTRACT**

22 *Candida albicans* is known for its ability to form biofilms – communities of  
23 microorganisms embedded in an extracellular matrix developing on different  
24 surfaces. Biofilms are highly tolerant to antifungal therapy. This phenomenon has  
25 been partially explained by the appearance of so-called persister cells, phenotypic  
26 variants of wild-type cells, capable of surviving very high concentrations of  
27 antimicrobial agents. Persister cells in *C. albicans* were found exceptionally in  
28 biofilms while none were detected in planktonic cultures of this fungus. Yet, this topic  
29 remains controversial as others could not observe persister cells in biofilms formed  
30 by the *C. albicans* SC5314 laboratory strain. Due to ambiguous data in the literature,  
31 this work aimed to reevaluate the presence of persister cells in *C. albicans* biofilms.  
32 We demonstrated that isolation of *C. albicans* “persister cells” as described  
33 previously was likely to be the result of survival of biofilm cells that were not reached  
34 by the antifungal. We tested biofilms of SC5314 and its derivatives, as well as 95  
35 clinical isolates, using an improved protocol, demonstrating that persister cells are  
36 not a characteristic trait of *C. albicans* biofilms. Although some clinical isolates are  
37 able to yield survivors upon the antifungal treatment of biofilms, this phenomenon is  
38 rather stochastic and inconsistent.

39

40

## 41 INTRODUCTION

42 The yeast *Candida albicans* is a commensal of humans but also one of the most  
43 prevalent fungal pathogens, responsible for superficial infections as well as life-  
44 threatening systemic infections (1). *C. albicans* is recognized for its ability to form  
45 biofilms that are most frequently associated with nosocomial infections, particularly in  
46 immunocompromised patients.

47 *C. albicans* biofilms are communities of microorganisms with a complex structure  
48 composed of different cell types embedded in an extracellular matrix (2–4). They  
49 develop on different types of surfaces, either living or inert, and are characterized by  
50 their high tolerance to antifungals. The latter can result from the properties of the  
51 extracellular matrix that can serve as a trap for drug molecules (5–7). An additional  
52 source of antifungal tolerance has been proposed to result from the occurrence in  
53 biofilms of so-called persister cells, a subpopulation of phenotypic variants of wild-  
54 type cells, capable of surviving concentrations of antimicrobial agents well above the  
55 Minimal Inhibitory Concentration (MIC) (8). Persister cells are genetically identical to  
56 other biofilm cells. Upon removal of the antimicrobial agent they give rise to a new  
57 population comprised of the majority of susceptible cells and a new small  
58 subpopulation of persisters. Thus, persistence is a non-inherited trait (9–11).

59 In the clinical setting, persisters are usually associated with relapse of infections and  
60 with the development of chronic infections. For bacterial persisters, several  
61 mechanisms and pathways involved in their development have been described (12).

62 In 2006, LaFleur et al. have presented the first report of persister cells in biofilms of  
63 *C. albicans*, which could contribute to biofilm tolerance to antifungals (8). In their  
64 paper the authors have reported that *C. albicans* exhibit a biphasic killing curve,  
65 when exposed to the antifungals such as amphotericin B (AMB), chlorhexidine or

66 the combination of both. This phenomenon is explained by the presence of a  
67 multidrug-tolerant subpopulation of persister cells within a biofilm. Notably, the  
68 experiments for this study were performed using in vitro biofilm model of *C. albicans*,  
69 developed in polystyrene 96-well plates. Following this work and relying on the  
70 protocol for persister cells isolation described therein (8), persister cells in *C. albicans*  
71 biofilms were described by a few other groups (13–15). However, later work by the  
72 Douglas group showed that not all *Candida* species and strains were able to form  
73 persister cells in laboratory-grown biofilms (16). This was in particular the case for *C.*  
74 *albicans* strain SC5314 (17), the parental strain of almost all *C. albicans* strains used  
75 for functional genomics and molecular genetics studies. Unlike in the previously  
76 mentioned papers (8, 13–15), the protocol Al Dhaheri and Douglas (16) used for  
77 persisters isolation involved growing biofilms on silicone discs followed by their  
78 immersion into an antifungal solution. As the topic of *C. albicans* persister cells  
79 remains controversial, the main objective of this work was to reevaluate their  
80 occurrence in *C. albicans* biofilms.

81

## 82 **METHODS**

### 83 **Strains and growth conditions**

84 In this study we used 3 reference strains (listed in Table 1) and a set of 95 clinical  
85 isolates (Table S1).

86 Yeast precultures were grown overnight in YPD (1% yeast extract, 2% peptone, 2%  
87 glucose) with shaking at 30°C.

88 Biofilms were grown either in RPMI 1640 medium with L-glutamine (buffered with 50  
89 mM HEPES), as described in (8) and (18), or in GHAUM medium (SD supplemented

90 with 2% glucose and 1 mg/mL histidine, 1 mg/mL arginine, 0.02 mg/mL uridine and 2  
91 mg/mL methionine (19)).

92

### 93 **Biofilm growth and persister cells isolation**

94 To assess persister cell appearance in biofilms we used two protocols adapted either  
95 from (8) or (13). The first protocol uses 96-well plates and the biofilms are grown in  
96 RPMI. In the second protocol the biofilms are grown in 24-well plates but using  
97 GHAUM medium instead of YNB.

#### 98 ***Biofilm growth***

99 Overnight cultures were washed in sterile 1x PBS and diluted in the corresponding  
100 medium to OD<sub>600</sub> 0.3. Either 100 µL or 1 mL of cells in the 96-well plate or the 24-well  
101 plate, respectively, were allowed to adhere for 1.5 h without agitation. The non-  
102 adhered cells were then washed with 1X PBS, the same volume of fresh medium  
103 was added, plates were covered with a breathable seal and biofilms were allowed to  
104 form for 24 h at 37°C with agitation (110 rpm). At this point the media were changed  
105 and biofilms were allowed to grow for 24 more hours.

#### 106 ***Antifungal treatment***

107 Old media were carefully aspirated, without disrupting the biofilm structure. Biofilms  
108 were washed once with either 100 µL or 1 mL of 1x PBS, respectively, and treated  
109 with a 100 µg/mL AMB solution in either RPMI or GHAUM for 24 hours at 37°C,  
110 statically. AMB solutions were prepared from a 8 mg/mL stock in DMSO, so that the  
111 final concentration of DMSO in a working solution did not exceed 1.25%. For control  
112 biofilms, corresponding amount of DMSO was added to the medium instead of the  
113 antifungal solution.

114 This step was either performed using the same volumes of antifungal solution as for  
115 biofilm growth as described in (8) and (13). or increasing the volume of antifungal to  
116 fill the well up to the top (350  $\mu$ L or 3 mL for 96- and 24-well plates, respectively).  
117 Clinical isolates were first treated with 64  $\mu$ g/mL AMB solution. Strains giving rise to  
118 colonies were then tested 5 times with 100  $\mu$ g/mL AMB.

### 119 **Plating**

120 Upon 24 hours of antifungal treatment, AMB solution was aspirated and biofilms were  
121 washed twice with 1X PBS prior to plating on YPD-agar plates. Biofilms were  
122 resuspended in 1x PBS/0.05% Tween-20. For the AMB-treated samples, the whole  
123 biofilms were plated. For control biofilms, serial dilutions were performed to allow  
124 CFU counting. CFU were counted after incubating the plates at 30°C for 48 h.

125

## 126 **RESULTS AND DISCUSSION**

127 In this work, we aimed to study the occurrence of persister cells in *C. albicans*  
128 biofilms. We applied the protocol published by LaFleur and colleagues, growing the  
129 biofilms in RPMI and in a 96-well plate format (8). We set up the protocol with 3  
130 *C. albicans* prototroph strains, namely SC5314, CEC369 and CEC4664 - prototroph  
131 derivatives of BWP17 and SN76, respectively. BWP17 (20) and SN76 (21) are  
132 independent auxotroph derivatives of SC5314 and have been rendered prototroph  
133 with sequential transformation events.

134 We encountered a technical problem at the biofilm recovery step, usually performed  
135 by scraping the cells in 1x PBS and vortexing prior to plating (8, 13, 15, 22). In our  
136 hands, the cells could not be properly resuspended and plated, as clumps of the  
137 biofilms would usually remain stranded inside the tips. Consequently, the CFU

138 numbers obtained were highly variable for all samples, making any further analysis  
139 and comparison impossible (data not shown).

140 We decided to test alternative approaches to circumvent the stickiness of biofilms.  
141 Resuspending cells in 20% glycerol/1X PBS for plating helped reducing stickiness,  
142 but did not improve consistency (data not shown). We hypothesized that EDTA might  
143 reduce adherence of biofilms by binding bivalent cations that are required for the  
144 activity of cell surface adhesins (23). Thus, we attempted applying 20% glycerol with  
145 a range of EDTA concentrations (0, 50, 100 mM) for plating. 100  $\mu$ L of EDTA  
146 solutions of different concentrations were added to biofilms and left for 10 minutes at  
147 room temperature prior to biofilm disruption by scraping and vortexing. None of the  
148 applied EDTA solutions allowed abolishing stickiness. Additionally, colonies growing  
149 on YPD-agar exhibited a wrinkled morphology, most probably linked to the toxicity of  
150 EDTA (24). Finally, we tried adding Tween-20 (0.05%) to PBS. Tween-20 eradicated  
151 the problems of stickiness and poor disruption and improved recovery of cells from  
152 the biofilms (Fig. 1). The effect on cell viability was tested using a planktonic culture  
153 of SC5314 that was washed and plated on YPD-agar using PBS and PBS-Tween-20  
154 solutions. No impact on viability was observed (data not shown). Thus, in the  
155 experiments described below, biofilms were resuspended in a 0.05% Tween-20/1X  
156 PBS solution.

157 However, even after this modification, the ratio of cells that survived AMB treatment  
158 was still inconsistent between repeats. According to Lafleur and colleagues the ratios  
159 of *C. albicans* persister cells in biofilms vary from 0.1% to 2% for different strains,  
160 notably from 0.05 to 0.1% for strain CAI4 – a derivative of *C. albicans* SC5314 (8).  
161 Our values hardly ever exceeded 0.01% persisters per biofilm, even after improving  
162 the recovery protocol, thus bordering with statistical error. We reasoned that



163 increasing the surface of a biofilm and changing the growth media could improve  
164 persister yields and decided to test the protocol described in (13), applying the  
165 modifications that were mentioned previously. However, the problem of inconsistency  
166 and low ratios of persisters remained (Fig. 2).

167 In all protocols described previously, the volumes of the media and solutions used for  
168 biofilm growth, washing, and AMB treatment were identical. Upon a careful  
169 observation, we noticed that *C. albicans* cells form a dense rim at the border of the  
170 air and liquid phases, as a result of agitation during growth. Treating a biofilm with the  
171 exact same volume of antifungal and growth medium in static conditions thus could  
172 result in cells from the rim escaping treatment. We decided to increase the volume of  
173 the applied antifungal solution (filling wells to the top) and, to our surprise, this  
174 change in the protocol led to a complete eradication of persisters for the laboratory  
175 strain SC5314 and its derivatives. Reproducibly, we did not get any persisters after  
176 applying this change for all strains for both RPMI- and GHAUM-grown biofilms. Thus,  
177 the volume of the antifungal applied in the original protocols for persister isolation  
178 was skewing the results. Increasing the volume of antifungal eliminated this bias,  
179 resulting in a complete eradication of any survivors after the antifungal treatment.

180 In our work we used a modified protocol for persister cells isolation with a starting cell  
181 suspension of OD<sub>600</sub> 0.3 used for biofilm growth instead of 0.1 as described in the  
182 original protocols (8, 13). To assess the impact of the initial cell number used for  
183 seeding biofilms on persister cells' appearance, we tested our protocol for SC5314  
184 using cell suspensions of OD<sub>600</sub> 0.1, 0.3 and 0.5 for seeding. Regardless of the initial  
185 biomass, persister cells did not form in SC5314 biofilms grown either in RPMI or  
186 GHAUM (data not shown).

187 These results made us question the very existence of persister cells in *C. albicans*  
188 biofilms. Previously, Al-Dhaheri and Douglas showed that not all strains of  
189 *C. albicans* can form persister cells (16). Particularly, in their hands, SC5314 biofilms  
190 lost all viability after exposure to 30 µg/mL AMB. However, biofilms of another clinical  
191 isolate, GDH2346, appeared to contain a small proportion (0.01%) of cells that  
192 survived 100 µg/mL AMB treatment. These authors used a different *in vitro* model for  
193 assessing persistence, as they grew biofilms on silicone disks that were transferred  
194 to a new well filled with an antifungal solution. This prevented an escape of any cells  
195 from the antifungal treatment. Thus, our modified protocol for treatment of biofilms  
196 formed in 96-well or 24-well plates corroborated the results obtained by the Douglas  
197 group for *C. albicans* strain SC5314 (16).

198 Since the clinical isolate GDH2346 could give rise to survivors (16), we could not  
199 exclude that persisters could emerge in biofilms of different *C. albicans* isolates.  
200 Additionally in 2010, LaFleur and colleagues isolated and described *C. albicans*  
201 strains from patients with long-term oral infection, that gave yield to increased levels  
202 of persisters (up to 8.9%) (23). These were called *hip*-mutants, by analogy with the  
203 high persister strains previously described for bacteria (26, 27). Although *hip*-mutants  
204 were identified using a protocol that showed limitations in our hands, we  
205 hypothesized that some *C. albicans* clinical isolates could generally be more prone to  
206 form persisters than others (namely SC5314). To test this assumption, we tested 96  
207 clinical isolates (Table S1) for their ability to form biofilms and the occurrence of  
208 persister cells following AMB treatment. In a first round of experiments, biofilms were  
209 treated with a 64 µg/mL AMB solution. Only 38 isolates (39.6%) displayed survivors  
210 (notably, never exceeding a rate of 0.02%). According to the generally accepted  
211 concept of persistence (9), the frequency of persisters' appearance is independent of

212 the increase in antibiotic concentration. Thus in a second round of experiments,  
213 biofilms were developed for these 38 isolates and treated with a 100 µg/mL AMB  
214 solution. Notably, only 7 isolates out of these 38 displayed survivors when grown with  
215 100 µg/mL AMB (CEC3668, CEC4514, CEC4525, CEC3554, CEC3634, CEC3669,  
216 CEC4521). These 7 strains, together with 4 other isolates randomly picked in the  
217 remaining 31 strains (CEC4512, CEC3706, CEC712, CEC3708), were tested five  
218 more times with 100 µg/mL of AMB. In most cases these strains did not yield  
219 persister cells (Fig. 3); however, 6 strains gave rise to survivors in one (CEC4525,  
220 CEC3634, CEC3669) or two (CEC5414, CEC3554, CEC4521) of the experiments  
221 (Fig. 3), which could be explained either by the stochastic nature of persistence as a  
222 phenomenon or by technical errors during the experiment.

223

## 224 **CONCLUSION**

225 Since 1944, when Bigger first described persister cells in *Staphylococcus* (28), many  
226 advances have been made in exploring this phenomenon, especially in bacteria. It is  
227 known that microbial cultures growing *in vivo* can sometimes be very difficult to  
228 eradicate completely by an antibiotic treatment, causing relapses or development of  
229 chronic infections in patients. A small pool of cells with the same genotype as the rest  
230 of the population but differing in their ability to tolerate stress – including drug  
231 treatment – provides a form of insurance to the population from an evolutionary point  
232 of view.

233 The phenomenon of persistence has been described not only for bacteria, but other  
234 types of pathogens, and it has been proposed that persister cells significantly  
235 contributed to the recalcitrance of *C. albicans* biofilms to antifungal treatments (29–  
236 31).

237 *C. albicans* persister cells were first described in 2006 (8), and since then just a  
238 handful of reports, sometimes contradictory, have been presented. In our study, we  
239 explored standard protocols to obtain persisters, and showed that their proportion in  
240 biofilms formed by different *C. albicans* strains has been overestimated. Our results  
241 show that the detected “persister cells” were likely the result of survival of cells that  
242 were not reached by the antifungal. Notably, Al-Dhaheri and Douglas (16) were able  
243 to detect some persisters in biofilms of a clinical isolate, but the ratio they obtained  
244 was much lower (0.01%) than the numbers published by other authors (8, 13).  
245 Although some of the tested clinical isolates of our study were occasionally able to  
246 yield survivors after the treatment of biofilms with AMB, this phenomenon was rather  
247 inconsistent, pointing either to the stochastic nature of persistence itself, or another  
248 skew in the protocol while carrying out particular experiments.  
249 At that point we cannot completely exclude the possibility of persistence in all existing  
250 *C. albicans* strains, though with our protocol we managed to disprove their presence  
251 for 91 analysed strains out of 98.

252

## 253 **ACKNOWLEDGEMENTS**

254 Iryna Denega is part of the Pasteur - Paris University (PPU) International PhD  
255 Program. This project has received funding from the European Union's Horizon 2020  
256 research and innovation programme under the Marie Skłodowska-Curie grant  
257 agreement No 665807, and from the Institut Carnot Pasteur Microbes & Santé. This  
258 work has been supported by grants from the French Government's Investissement  
259 d'Avenir program (Laboratoire d'Excellence Integrative Biology of Emerging  
260 Infectious Diseases, ANR-10-LABX-62-IBEID) to C.d'E.

261

262       **REFERENCES**

- 263       1. Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJS.  
264 2013. *Candida* species: current epidemiology, pathogenicity, biofilm formation,  
265 natural antifungal products and new therapeutic options. *J Med Microbiol* 62:10–24.
- 266       2. Ramage G, Saville SP, Thomas DP, López-Ribot JL. 2005. *Candida biofilms*:  
267 an update. *Eukaryot Cell* 4:633–638.
- 268       3. Nobile CJ, Johnson AD. 2015. *Candida albicans* biofilms and human disease.  
269 *Annu Rev Microbiol* 69:71–92.
- 270       4. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum  
271 MA. 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development,  
272 architecture, and drug resistance. *J Bacteriol* 183:5385–5394.
- 273       5. Nett JE, Crawford K, Marchillo K, Andes DR. 2010. Role of Fks1p and matrix  
274 glucan in *Candida albicans* biofilm resistance to an echinocandin, pyrimidine, and  
275 polyene. *Antimicrob Agents Chemother* 54:3505–3508.
- 276       6. Nett JE, Sanchez H, Cain MT, Andes DR. 2010. Genetic basis of *Candida*  
277 biofilm resistance due to drug sequestering matrix glucan. *J Infect Dis* 202:171–175.
- 278       7. Nett JE, Sanchez H, Cain MT, Ross KM, Andes DR. 2011. Interface of  
279 *Candida albicans* biofilm matrix-associated drug resistance and cell wall integrity  
280 regulation. *Eukaryot Cell* 10:1660–1669.
- 281       8. LaFleur M. 2006. *Candida albicans* biofilms produce antifungal-tolerant  
282 persister cells. *Antimicrob Agents Chemother* 50:3839–3846.
- 283       9. Brauner A, Fridman O, Gefen O, Balaban NQ. 2016. Distinguishing between  
284 resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol*  
285 14:320–330.

- 286 10. Lewis K. 2007. Persister cells, dormancy and infectious disease. *Nat Rev*  
287 *Microbiol* 5:48–56.
- 288 11. Lewis K. 2010. Persister cells. *Annu Rev Microbiol* 64:357–372.
- 289 12. Harms A, Maisonneuve E, Gerdes K. 2016. Mechanisms of bacterial  
290 persistence during stress and antibiotic exposure. *Science* 354:aaf4268.
- 291 13. Li P, Seneviratne CJ, Alpi E, Vizcaino JA, Jin L. 2015. Delicate metabolic  
292 control and coordinated stress response critically determine antifungal tolerance of  
293 *Candida albicans* biofilm persists. *Antimicrob Agents Chemother* 59:6101–6112.
- 294 14. Truong T, Zeng G, Qingsong L, Kwang LT, Tong C, Chan FY, Wang Y,  
295 Seneviratne CJ. 2016. Comparative ploidy proteomics of *Candida albicans* biofilms  
296 unraveled the role of the *AHP1* gene in the biofilm persistence against amphotericin  
297 B. *Mol Cell Proteomics* 15:3488–3500.
- 298 15. Sun J, Li Z, Chu H, Guo J, Jiang G, Qi Q. 2016. *Candida albicans*  
299 amphotericin B-tolerant persister formation is closely related to surface adhesion.  
300 *Mycopathologia* 181:41–49.
- 301 16. Al-Dhaheri RS, Douglas LJ. 2008. Absence of amphotericin B-tolerant  
302 persister cells in biofilms of some *Candida* species. *Antimicrob Agents Chemother*  
303 52:1884–1887.
- 304 17. Gillum AM, Tsay EY, Kirsch DR. 1984. Isolation of the *Candida albicans* gene  
305 for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3*  
306 and *E. coli* *pyrF* mutations. *Mol Gen Genet* 198:179–182.
- 307 18. Enjalbert B, Rachini A, Vedyappan G, Pietrella D, Spaccapelo R, Vecchiarelli  
308 A, Brown AJP, d'Enfert C. 2009. A Multifunctional, synthetic *Gaussia princeps*  
309 luciferase reporter for live imaging of *Candida albicans* infections. *Infect Immun*  
310 77:4847–4858.

- 311 19. De Brucker K, De Cremer K, Cammue BPA, Thevissen K. 2016. Protocol for  
312 determination of the persister subpopulation in *Candida albicans* biofilms. *Methods*  
313 *Mol Biol* 1333:67–72.
- 314 20. Cabral V, Znaidi S, Walker LA, Martin-Yken H, Dague E, Legrand M, Lee K,  
315 Chauvel M, Firon A, Rossignol T, Richard ML, Munro CA, Bachellier-Bassi S,  
316 d'Enfert C. 2014. Targeted changes of the cell wall proteome influence *Candida*  
317 *albicans* ability to form single- and multi-strain biofilms. *PLoS Pathog* 10:e1004542.
- 318 21. Wilson RB, Davis D, Mitchell AP. 1999. Rapid hypothesis testing with *Candida*  
319 *albicans* through gene disruption with short homology regions. *J Bacteriol* 181:1868–  
320 1874.
- 321 22. Noble SM, Johnson AD. 2005. Strains and strategies for large-scale gene  
322 deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot*  
323 *Cell* 4:298–309.
- 324 23. LaFleur MD, Qi Q, Lewis K. 2010. Patients with long-term oral carriage harbor  
325 high-persister mutants of *Candida albicans*. *Antimicrob Agents Chemother* 54:39–44.
- 326 24. Klotz SA, Rutten MJ, Smith RL, Babcock SR, Cunningham MD. 1993.  
327 Adherence of *Candida albicans* to immobilized extracellular matrix proteins is  
328 mediated by calcium-dependent surface glycoproteins. *Microb Pathog* 14:133–147.
- 329 25. Chudzik B, Malm A, Rautar B, Polz-Dacewicz M. 2007. *In vitro* inhibitory  
330 activity of EDTA against planktonic and adherent cells of *Candida* sp. *Ann Microbiol*  
331 57:115.
- 332 26. Moyed HS, Bertrand KP. 1983. *hipA*, a newly recognized gene of *Escherichia*  
333 *coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J*  
334 *Bacteriol* 155:768–775.

335 27. Wolfson JS, Hooper DC, McHugh GL, Bozza MA, Swartz MN. 1990. Mutants  
336 of *Escherichia coli* K-12 exhibiting reduced killing by both quinolone and beta-lactam  
337 antimicrobial agents. *Antimicrob Agents Chemother* 34:1938–1943.

338 28. Bigger J. 1944. Treatment of staphylococcal infections with penicillin by  
339 intermittent sterilisation. *The Lancet* 244:497–500.

340 29. Borghi E, Borgo F, Morace G. 2016. Fungal Biofilms: update on resistance, p.  
341 37–47. In *Fungal Biofilms and related infections*. Springer, Cham.

342 30. Ramage G, Rajendran R, Sherry L, Williams C. 2012. Fungal biofilm  
343 resistance. *Int J Microbiol* 2012: 1-14.

344 31. Mathé L, Dijck PV. 2013. Recent insights into *Candida albicans* biofilm  
345 resistance mechanisms. *Curr Genet* 59:251–264.

346

347

## 348 **FIGURE LEGENDS**

349

350 **Fig. 1 Effect of Tween 20 on the recovery of CFUs from *C. albicans* SC5314**  
351 **biofilms.** *C. albicans* SC5314 was allowed to form biofilms in 100 µL RPMI in a 96-  
352 well plate according to the protocol adapted from (8). Error bars: standard deviation  
353 (SD) of 6 biological replicates generated from 2 independent experiments.

354

355 **Fig. 2. Schemes of the protocols (A) and levels of persisters (B) obtained**  
356 **from biofilms grown using modified protocol from (13).** Biofilms were grown in 1  
357 mL of GHAUM medium in 24-well plates before application of either 1 mL of AMB  
358 solution (on the left) or 3 mL of AMB solution (on the right). Ratios of surviving cells



359 are as follow: SC5314 –  $5.6 \cdot 10^{-4}\%$ , CEC369 –  $2.6 \cdot 10^{-5}\%$ , CEC4664 –  $9.4 \cdot 10^{-5}\%$ .

360 Error bars: SD of 6 biological replicates generated from 2 independent experiments.

361

362

363 **Fig. 3. Analysis of persister cell formation in 11 clinical isolates.** Biofilms were

364 grown in 1 mL of GHAUM medium in 24-well plates, and treated with 1 mL of AMB

365 solution (modified protocol from (13)). The values obtained from 5 biofilms were used

366 to draw the graph.

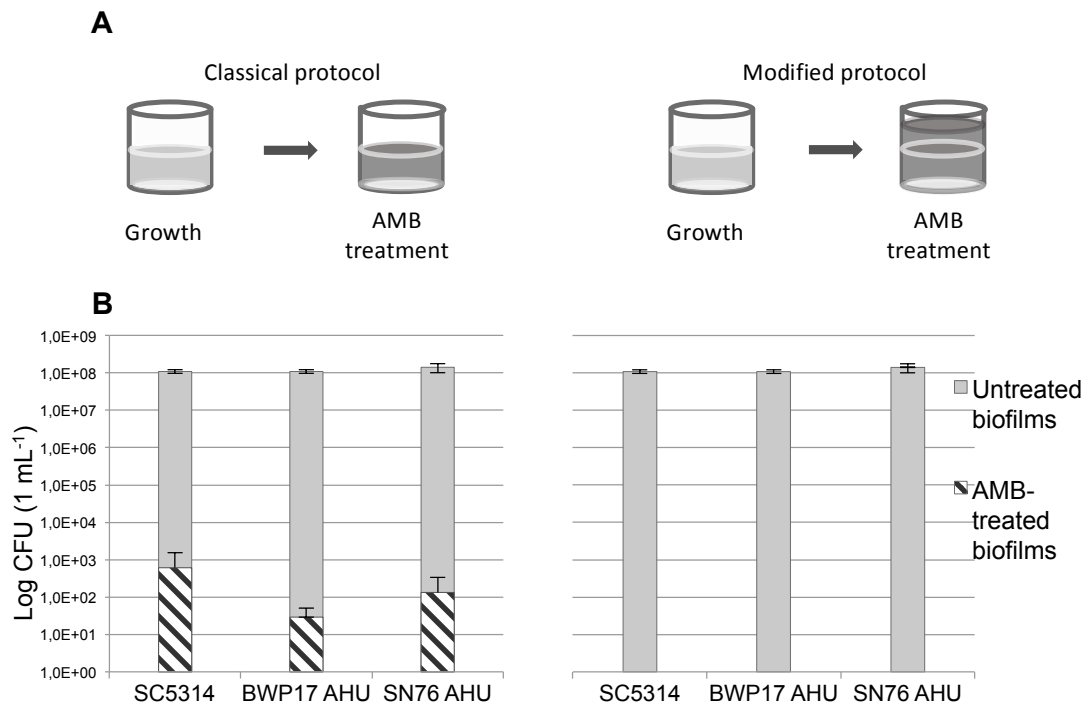
367

368 **TABLE 1. *C. albicans* strains used in this study**

STRAIN	GENOTYPE	REFERENCE
SC5314		(17)
CEC369	<i>ura3::λimm434/ura3::λimm434 ARG4/arg4::hisG</i> <i>HIS1/his1Δ::hisG RPS1/RPS1::Clp10</i>	(18)
CEC4664	<i>ura3Δ::λimm434/ura3Δ::λimm434</i> <i>iro1Δ::λimm434/iro1Δ::λimm434 ADH1/adh1::P<sub>TDH3</sub>-</i> <i>carTA::SAT1 arg4Δ/ARG4 his1Δ::hisG/HIS1</i> <i>RPS1/RPS1::Clp10</i>	Lab's collection

369

**Fig. 1 Effect of Tween 20 on the recovery of CFUs from *C. albicans* SC5314 biofilms.** *C. albicans* SC5314 was allowed to form biofilms in 100  $\mu$ L RPMI in a 96-well plate according to the protocol adapted from (8). Error bars: standard deviation (SD) of 6 biological replicates generated from 2 independent experiments.



**Fig. 2. Schemes of the protocols (A) and levels of persisters (B) obtained from biofilms grown using modified protocol from (13).** Biofilms were grown in 1 mL of GHAUM medium in 24-well plates before application of either 1 mL of AMB solution (on the left) or 3 mL of AMB solution (on the right). Ratios of surviving cells are as follow: SC5314 –  $5.6 \cdot 10^{-4}\%$ , CEC369 –  $2.6 \cdot 10^{-5}\%$ , CEC4664 –  $9.4 \cdot 10^{-5}\%$ . Error bars: SD of 6 biological replicates generated from 2 independent experiments.



**Fig. 3. Analysis of persister cell formation in 11 clinical isolates.** Biofilms were grown in 1 mL of GHAUM medium in 24-well plates, and treated with 1 mL of AMB solution (modified protocol from (13)). The values obtained from 5 biofilms were used to draw the graph.