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

2

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

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

19

20 **ABSTRACT**

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

38

39

40 **INTRODUCTION**

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

46 *C. albicans* biofilms are communities of microorganisms with a complex structure
47 composed of different cell types embedded in an extracellular matrix (2–4). They
48 develop on different types of surfaces, either living or inert, and are characterized by
49 their high tolerance to antifungals. The latter can result from the properties of the
50 extracellular matrix that can serve as a trap for drug molecules (5–7). An additional
51 source of antifungal tolerance has been proposed to result from the occurrence in
52 biofilms of so-called persister cells, a subpopulation of phenotypic variants of wild-
53 type cells, capable of surviving concentrations of antimicrobial agents well above the
54 Minimal Inhibitory Concentration (MIC) (8). Persister cells were first described in
55 bacterial cultures as a drug tolerant subpopulation, that upon removal of the
56 antimicrobial agent gave rise to a new population of susceptible cells (9). Persisters
57 are known to be genetically identical to the rest of the population, thus persistence is
58 a non-inherited trait (10–12).

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 (13).

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 the
66 combination of both. This phenomenon is explained by the presence of a multidrug-
67 tolerant subpopulation of persister cells within a biofilm, while planktonic cultures of
68 *C. albicans* were found to be devoid of persisters. Notably, the experiments for this
69 study were performed using *in vitro* biofilm model of *C. albicans*, developed in
70 polystyrene 96-well plates. Following this work and relying on the protocol for
71 persister cells isolation described therein (8), persister cells in *C. albicans* biofilms
72 were described by a few other groups (14–16). However, later work by the Douglas
73 group showed that not all *Candida* species and strains were able to form persister
74 cells in laboratory-grown biofilms (17). This was in particular the case for *C. albicans*
75 strain SC5314 (18), the parental strain of almost all *C. albicans* strains used for
76 functional genomics and molecular genetics studies. Unlike in the previously
77 mentioned papers (8, 14–16), the protocol Al-Dhaheer and Douglas (17) used for
78 persisters isolation involved growing biofilms on silicone discs followed by their
79 immersion into an antifungal solution. As the topic of *C. albicans* persister cells
80 remains controversial, the main objective of this work was to re-evaluate their
81 occurrence in *in vitro*-grown *C. albicans* biofilms.

82

83 **METHODS**

84 **Strains and growth conditions**

85 In this study we used 3 reference strains (listed in Table 1) and a set of 95 *C.*
86 *albicans*, 3 *C. tropicalis* and 3 *C. parapsilosis* clinical isolates (Table S1).

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

89 Biofilms were grown either in RPMI 1640 medium with L-glutamine (buffered with 50
90 mM HEPES), as described in (8) and (20), or in GHAUM medium (SD supplemented
91 with 2% glucose and 1 mg/mL histidine, 1 mg/mL arginine, 0.02 mg/mL uridine and 2
92 mg/mL methionine (21)).

93 Resistance was checked on solid YNG (6,7 g/L yeast nitrogen base without amino
94 acids and with ammonium sulfate, 2% glucose and 2% agar) supplemented with 10
95 µg/mL AMB.

96

97 **Biofilm growth and persister cells isolation**

98 To assess persister cell appearance in biofilms we used two protocols adapted either
99 from (8) or (14). The first protocol uses 96-well plates and the biofilms are grown in
100 RPMI. In the second protocol the biofilms are grown in 24-well plates but using
101 GHAUM medium instead of YNB.

102 ***Biofilm growth***

103 Overnight cultures were washed in sterile 1x PBS and diluted in the corresponding
104 medium to OD₆₀₀ 0.3. Either 100 µL or 1 mL of cells in the 96-well plate or the 24-well
105 plate, respectively, were allowed to adhere for 1.5 h without agitation. The non-
106 adhered cells were then washed with 1X PBS, the same volume of fresh medium
107 was added, plates were covered with a breathable seal and biofilms were allowed to
108 form for 48 h at 37°C with agitation (110 rpm) with a medium change after 24 hours.

109 ***Antifungal treatment***

110 Media were carefully aspirated from the 48 h-old biofilms, without disrupting the
111 biofilm structure. Biofilms were washed once with either 100 µL or 1 mL of 1x PBS,
112 respectively, and treated with a 100 µg/mL AMB solution in either RPMI or GHAUM
113 for 24 hours at 37°C, statically. AMB solutions were prepared from an 8 mg/mL stock

114 in DMSO, so that the final concentration of DMSO in a working solution did not
115 exceed 1.25%. For control biofilms, corresponding amount of DMSO was added to
116 the medium instead of the antifungal solution.

117 This step was either performed using the same volumes of antifungal solution as for
118 biofilm growth as described in (8) and (14) or increasing the volume of antifungal to
119 fill the well up to the top (350 μ L or 3 mL for 96- and 24-well plates, respectively).

120 Clinical isolates were first treated with 64 μ g/mL AMB solution. Strains giving rise to
121 colonies were then tested 5 times with 100 μ g/mL AMB.

122 **Plating**

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

128

129 **RESULTS AND DISCUSSION**

130 In this work, we aimed to study the occurrence of persister cells in *C. albicans*
131 biofilms. We applied the protocol published by LaFleur and colleagues, growing the
132 biofilms in RPMI and in a 96-well plate format (8). We set up the protocol with 3
133 *C. albicans* prototroph strains, namely SC5314, CEC369 and CEC4664 - prototroph
134 derivatives of BWP17 (22) and SN76 (23), respectively. BWP17 and SN76 are
135 independent auxotroph derivatives of SC5314.

136 We encountered a technical problem at the biofilm recovery step, usually performed
137 by scraping the cells in 1x PBS and vortexing prior to plating (8, 14, 16, 24). In our
138 hands, the cells could not be properly resuspended and plated, as clumps of the

139 biofilms would usually remain stranded inside the tips. Consequently, the CFU
140 numbers obtained were highly variable for all samples, making any further analysis
141 and comparison impossible (data not shown).

142 We decided to test alternative approaches to circumvent the stickiness of biofilms.
143 Resuspending cells in 20% glycerol/1X PBS for plating helped reducing stickiness,
144 but did not improve consistency (data not shown). We hypothesized that EDTA might
145 reduce adherence of biofilms by binding bivalent cations that are required for the
146 activity of cell surface adhesins (25). Thus, we attempted applying 20% glycerol with
147 a range of EDTA concentrations (0, 50, 100 mM) for plating. 100 μ L of EDTA
148 solutions of different concentrations were added to biofilms and left for 10 minutes at
149 room temperature prior to biofilm disruption by scraping and vortexing. None of the
150 applied EDTA solutions allowed abolishing stickiness. Additionally, colonies growing
151 on YPD-agar exhibited a wrinkled morphology, most probably linked to the toxicity of
152 EDTA (26). Finally, we tried adding Tween-20 (0.05%) to PBS. Tween-20 eradicated
153 the problems of stickiness and poor disruption and improved recovery of cells from
154 the biofilms (Fig. 1). The effect on cell viability was tested using a planktonic culture
155 of SC5314 that was washed and plated on YPD-agar using PBS and PBS-Tween-20
156 solutions. No impact on viability was observed (data not shown). Thus, in the
157 experiments described below, biofilms were resuspended in a 0.05% Tween-20/1X
158 PBS solution.

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

164 the recovery protocol, thus bordering with statistical error. We reasoned that
165 increasing the surface of a biofilm and changing the growth media could improve
166 persister yields and decided to test the protocol described in (14), applying the
167 modifications that were mentioned previously. However, the problem of inconsistency
168 and low ratios of persisters remained (Fig. 2).

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

182 In our work we used a modified protocol for persister cells isolation with a starting cell
183 suspension of OD_{600} 0.3 used for biofilm growth instead of 0.1 as described in the
184 original protocols (8, 14). To assess the impact of the initial cell number used for
185 seeding biofilms on persister cells' appearance, we tested our protocol for SC5314
186 using cell suspensions of OD_{600} 0.1, 0.3 and 0.5 for seeding. Regardless of the initial
187 biomass, persister cells did not form in SC5314 biofilms grown either in RPMI or
188 GHAUM (data not shown).

189 These results made us question the very existence of persister cells in *C. albicans*
190 biofilms. Previously, Al-Dhaheeri and Douglas showed that not all strains of
191 *C. albicans* can form persister cells (17). Particularly, in their hands, SC5314 biofilms
192 lost all viability after exposure to 30 µg/mL AMB. However, biofilms of another clinical
193 isolate, GDH2346, appeared to contain a small proportion (0.01%) of cells that
194 survived 100 µg/mL AMB treatment. These authors used a different *in vitro* model for
195 assessing persistence, as they grew biofilms on silicone disks that were transferred
196 to a new well filled with an antifungal solution. This prevented an escape of any cells
197 from the antifungal treatment. Thus, our modified protocol for treatment of biofilms
198 formed in 96-well or 24-well plates corroborated the results obtained by the Douglas
199 group for *C. albicans* strain SC5314 (17).

200 Since the clinical isolate GDH2346 could give rise to survivors (17), we could not
201 exclude that persisters could emerge in biofilms of different *C. albicans* isolates.
202 Additionally in 2010, LaFleur and colleagues isolated and described *C. albicans*
203 strains from patients with long-term oral infection, that gave yield to increased levels
204 of persisters (up to 8.9%) (24). These were called *hip*-mutants, by analogy with the
205 high persister strains previously described for bacteria (27, 28). Although *hip*-mutants
206 were identified using a protocol that showed limitations in our hands, we
207 hypothesized that some *C. albicans* clinical isolates could generally be more prone to
208 form persisters than others (namely SC5314). To test this assumption, we tested 95
209 clinical isolates (Table S1) for their ability to form biofilms and the occurrence of
210 persister cells following AMB treatment. In a first round of experiments, biofilms were
211 treated with a 64 µg/mL AMB solution. Only 38 isolates (39.6%) displayed survivors
212 (notably, never exceeding a rate of 0.02%). According to the generally accepted
213 concept of persistence (10), the frequency of persisters' appearance is independent

214 of the increase in antibiotic concentration. Thus in a second round of experiments,
215 biofilms were developed for these 38 isolates and treated with a 100 µg/mL AMB
216 solution. Notably, only 7 isolates out of these 38 displayed survivors when grown with
217 100 µg/mL AMB (CEC3622, CEC3668, CEC3669, CEC4514, CEC4521, CEC5317,
218 CEC5318). These 7 strains, together with 4 other isolates randomly picked in the
219 remaining 31 strains (CEC712, CEC3708, CEC3711, CEC5316), were tested seven
220 more times with 100 µg/mL of AMB. In most cases these strains did not yield
221 persister cells (Fig. 3); however, 7 strains (CEC3622, CEC3669, CEC4514,
222 CEC4521, CEC5316, CEC5317 and CEC5318) gave rise to small numbers of
223 survivors in one to four of the experiments (Fig. 3), with the survival rate never
224 exceeding $9.1 \times 10^{-4}\%$ per biofilm (for CEC3622). This could be explained either by the
225 stochastic nature of persistence as a phenomenon or by technical errors during the
226 experiment.

227 We tested up to 30 randomly picked colonies for three isolates (CEC3622, CEC4514
228 and CEC5316) on YNG medium containing 10 µg/mL of AMB. None of the tested
229 colonies was able to grow in presence of amphotericin B (data not shown), proving
230 that their survival was not a result of AMB resistance development.

231 With an improved protocol in our hands, we decided to test other *Candida* species for
232 their ability to form persister cells in biofilms. Previously, Al Dhaheri and Douglas (17)
233 reported that clinical isolates of *C. krusei* (Glasgow strain) and *C. parapsilosis* (AAHB
234 4479) developed persister cells in biofilms (approximately 0.001% and 0.07%,
235 respectively) upon treatment with 100 µg/mL AMB. We selected 3 clinical isolates of
236 *C. tropicalis* (CEC5296, CEC5297, CEC5298) and 3 of *C. parapsilosis* (CEC5299,
237 CEC5300, CEC5301) from our lab collection to test with our protocol. One of the *C.*
238 *tropicalis* strains (CEC5298), as well as the 3 selected *C. parapsilosis* strains were

239 unable to grow as biofilms, and were excluded from the study. *C. tropicalis* CEC5296
240 and CEC5297 formed proper biofilms, with a small fraction of persisters varying
241 between 2×10^{-5} - $6.4 \times 10^{-3}\%$ and 2.3×10^{-7} - $2.6 \times 10^{-4}\%$ respectively (data not shown).
242 Such low values are comparable to the survival rates we observed for some of the *C.*
243 *albicans* clinical isolates tested in this study. As before, we cannot exclude that these
244 survivors are persister cells arising within *C. tropicalis* biofilms, or that they are the
245 consequence of a technical error during the experiment.

246

247 **CONCLUSION**

248 Since 1944, when Bigger first described persister cells in *Staphylococcus* (9), many
249 advances have been made in exploring this phenomenon, especially in bacteria. It is
250 known that microbial cultures growing *in vivo* can sometimes be very difficult to
251 eradicate completely by an antibiotic treatment, causing relapses or development of
252 chronic infections in patients. From an evolutionary point of view, a small pool of cells
253 with the same genotype as the rest of the population but differing in their ability to
254 tolerate stress – including drug treatment – provides a form of insurance to the
255 population.

256 The phenomenon of persistence has not only been described for bacteria, but also in
257 other types of pathogens, and it has been proposed that persister cells significantly
258 contributed to the recalcitrance of *C. albicans* biofilms to antifungal treatments (29–
259 31).

260 *C. albicans* persister cells were first described in 2006 (8), and since then just a
261 handful of reports, sometimes contradictory, have been presented. In our study, we
262 explored standard protocols to obtain persisters, and showed that their proportion in
263 biofilms formed by different *C. albicans* strains has been overestimated. Only Al-

264 Dhaheri and Douglas did not detect persisters in SC5314 biofilms (17). In their study,
265 biofilms were grown on silicon discs that were transferred in antifungal solutions for
266 treatment. In contrast, the other published experiments were performed using 96-well
267 plates and RPMI medium, or 24-well plates and SD-based medium, while keeping
268 the incubation volumes constant throughout the experiment (8, 14–16). In this study,
269 we modified the latter protocols (8, 14–16) by increasing the volume of antifungal.
270 This change led to the eradication of biofilms, indicating that previously detected
271 “persisters” were likely the result of survival of cells that were not reached by the
272 antifungal. Our results corroborate the findings of Al-Dhaheri and Douglas (17).
273 Notably, these authors were able to detect some persisters in biofilms of a clinical
274 isolate (17), but the ratio obtained was much lower (0.01%) than the numbers
275 published by others (8, 14). Although some of the clinical isolates of *C. albicans* and
276 *C. tropicalis* tested in our study were occasionally able to yield survivors after the
277 treatment of biofilms with AMB, this phenomenon was rather inconsistent, pointing
278 either to the stochastic nature of persistence itself, or another skew in the protocol
279 while carrying out particular experiments.
280 At this time, we cannot completely exclude the possibility of persistence in all *C.*
281 *albicans* strains, though with the described protocol we managed to disprove their
282 presence for 92 *C. albicans* strains out of 98. It is important to stress that our results
283 reflect only the behaviour of *C. albicans* biofilms grown *in vitro*; we cannot rule out
284 that in the context of the host, persister cells could appear and contribute to the
285 general resistance and dissemination of *C. albicans*.

286

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295

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424

425

426

427 **FIGURE LEGENDS**

428

429 **Fig. 1 Effect of Tween 20 on the recovery of CFUs from *C. albicans* SC5314**

430 **biofilms.** *C. albicans* SC5314 was allowed to form biofilms in 100 µL RPMI in a 96-

431 well plate according to the protocol adapted from (8). Error bars: standard deviation

432 (SD) of 6 biological replicates generated from 2 independent experiments. # - non-

433 significant difference, *** - significant difference, p=0,0007 (unpaired t-test was

434 applied to compare datasets).

435

436 **Fig. 2. Schemes of the protocols (A) and levels of persisters (B) obtained**
437 **from biofilms grown using modified protocol from (14).** Biofilms were grown in 1
438 mL of GHAUM medium in 24-well plates before application of either 1 mL of AMB
439 solution (on the left) or 3 mL of AMB solution (on the right). Ratios of surviving cells
440 are as follow: SC5314 – $5.6 \cdot 10^{-4}\%$, CEC369 – $2.6 \cdot 10^{-5}\%$, CEC4664 – $9.4 \cdot 10^{-5}\%$.
441 Error bars: SD of 6 biological replicates generated from 2 independent experiments.

442

443 **Fig. 3. Analysis of persister cell formation in 11 clinical isolates.** Biofilms were
444 grown in 1 mL of GHAUM medium in 24-well plates, and treated with 3 mL of AMB
445 solution (modified protocol from (14)). The values obtained from 7 biofilms were used
446 to draw the graph.
447

448

449 **TABLE 1. *C. albicans* reference strains used in this study**

STRAIN	GENOTYPE	REFERENCE
SC5314		(18)
CEC369	<i>ura3::λimm434/ura3::λimm434 ARG4/arg4::hisG HIS1/his1Δ::hisG RPS1/RPS1::Clp10</i>	(19)
CEC4664	<i>ura3Δ::λimm434/ura3Δ::λimm434 iro1Δ::λimm434/iro1Δ::λimm434 ADH1/adh1::P_{TDH3}- carTA::SAT1 arg4Δ/ARG4 his1Δ::hisG/HIS1 RPS1/RPS1::Clp10</i>	Lab's collection

450

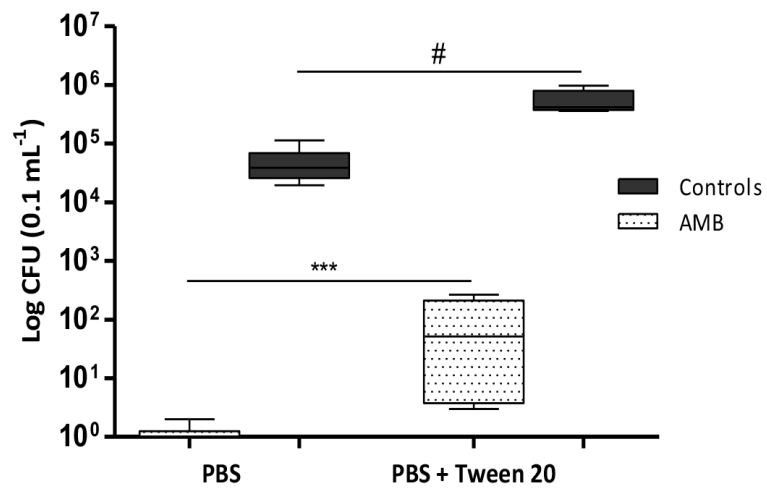
451

452 **TABLE 2. Clinical isolates used in this study**

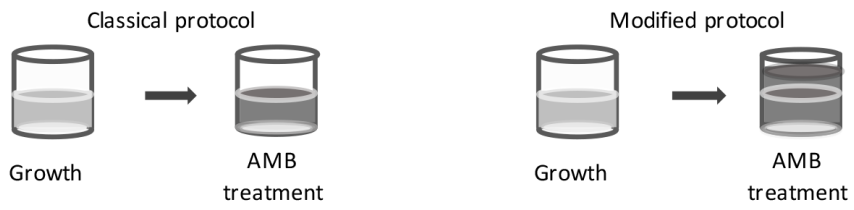
NAME	REFERENCE
CEC704	(32)
CEC712	(32)
CEC718	(32)
CEC723	(32)
CEC1289	(33)
CEC1424	(34)
CEC2018	(35)
CEC2019	(34)
CEC2020	(36)
CEC2021	(35)
CEC2022	(37)
CEC2871	(38)
CEC2876	(38)
CEC3494	(39)
CEC3533	(37)
CEC3534	(36)
CEC3535	(36)
CEC3536	(36)
CEC3540	(32)
CEC3541	(33)
CEC3544	(39)
CEC3547	(39)
CEC3548	(39)
CEC3549	(39)
CEC3550	(40)
CEC3553	(39)
CEC3555	(33)
CEC3556	(36)
CEC3560	(34)
CEC3561	(39)
CEC3596	(33)
CEC3611	(36)
CEC3614	(36)
CEC3615	(36)
CEC3621	(32)
CEC3622	(36)
CEC3623	(32)

CEC3626	(39)
CEC3627	(36)
CEC3634	(36)
CEC3637	(34)
CEC3659	(35)
CEC3662	(35)
CEC3663	(32)
CEC3664	(35)
CEC3665	(35)
CEC3668	(35)
CEC3669	(35)
CEC3672	(35)
CEC3675	(35)
CEC3681	(35)
CEC3682	(35)
CEC3685	(35)
CEC3706	(35)
CEC3708	(35)
CEC3711	(35)
CEC4035	(32)
CEC4039	(32)
CEC4103	(41)
CEC4104	(41)
CEC4106	(41)
CEC4108	(41)
CEC4256	(42)
CEC4259	(42)
CEC4481	(32)
CEC4482	(32)
CEC4485	(32)
CEC4486	(32)
CEC4487	(32)
CEC4488	(32)
CEC4489	(32)
CEC4492	(32)
CEC4494	(32)
CEC4495	Lab's collection
CEC4496	(32)
CEC4501	Lab's collection
CEC4504	Lab's collection
CEC4505	Lab's collection
CEC4511	Lab's collection
CEC4514	Lab's collection
CEC4515	Lab's collection
CEC4517	Lab's collection
CEC4521	Lab's collection
CEC4524	Lab's collection
CEC4526	(32)
CEC4527	Lab's collection
CEC4547	Lab's collection
CEC4548	Lab's collection
CEC4549	Lab's collection
CEC4550	Lab's collection
CEC4552	(32)
CEC5029	(32)

453 CEC5316 Lab's collection
CEC5317 Lab's collection
CEC5318 Lab's collection



A



B

