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1 **N-Acetylglucosamine Induces White-to-Opaque Switching and Mating in**
2 ***Candida tropicalis* Providing New Insights into Adaptation and Fungal**
3 **Sexual Evolution**

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24 **ABSTRACT**

25 Pathogenic fungi are capable of switching between different phenotypes, each
26 of which has a different biological advantage. In the most prevalent human
27 fungal pathogen *Candida albicans*, phenotypic transitions not only improve its
28 adaptation to a continuously changing host microenvironment, but also
29 regulate sexual mating. In this study, we report that *Candida tropicalis*, another
30 important human opportunistic pathogen, undergoes a reversible and heritable
31 phenotypic switching, referred to as "white-opaque" transition. Herein we show
32 that N-acetylglucosamine (GlcNAc), an inducer of white-to-opaque switching in
33 *C. albicans*, promotes opaque cell formation and mating and also inhibits
34 filamentation in a number of natural *C. tropicalis* strains. Our results suggest
35 that host chemical signals may facilitate this phenotypic switching and mating
36 of *C. tropicalis*, which has been previously thought to reproduce asexually.
37 Overexpression of *C. tropicalis WOR1* gene in *C. albicans* induces opaque cell
38 formation. Additionally, an intermediate phase between white and opaque was
39 obsrved in *C. tropicalis,* indicating that the switching could be tristable.

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41 **INTRODUCTION**

42 Fungal infections caused by *Candida* species have increased dramatically
43 over the past decades. Although *Candida albicans* remains the single most
44 important causative agent, non-*albicans* species are increasingly isolated in
45 nosocomial samples (25, 31). *Candida tropicalis*, which is frequently
46 associated with nosocomial candidemias, appears to be more prominent
47 among patients with hematologic malignancies (31).

48 Phenotypic transitions and sexual mating play a critical role in the adaptation
49 of fungi to host environments and promote the evolution of fungal pathogenic
50 traits (16, 39). The ability to switch between different morphologies is believed
51 to be associated with virulence (43). Sexual reproduction is pervasive in
52 eukaryotes and has many advantages over asexual reproduction (9). Firstly, it
53 produces recombinant types that can be more environmentally adaptable.
54 Secondly, it provides an efficient way to eliminate harmful mutations. Of note,
55 white-opaque switching and sexual mating are two tightly linked biological
56 processes in *C. albicans* , *C. dubliniensis* and *C. tropicalis* (26, 33, 34).

57 The white-opaque transition in *C. albicans* was first identified in the clinically
58 isolated strain WO-1 in 1987(38). The switching between white and opaque
59 cells is heritable and reversible (3). White and opaque cells have different
60 cellular morphologies and form two distinct colony appearances on solid media.
61 Microscopically, white cells are small, round to ovoid and bud like
62 *Saccharomyces cerevisiae* cells, while opaque cells are twice as large,

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63 elongated and bean-shaped. Macroscopically, white cells form white, shiny
64 and dome-shaped colonies, while opaque cells form relatively darker and
65 flatter colonies on agar (3, 40). On special phloxine B containing media,
66 opaque cells are selectively stained red (3).

67 Opaque cells also differ from white cells with respect to their vulnerability to
68 immune cells, virulence and mating competence (39). For example, opaque
69 cells are more virulent in cutaneous infections whereas white cells are better at
70 tissue colonization as shown in a murine systemic virulence model (18).
71 Interestingly, only opaque cells are mating-competent. Specifically, opaque
72 cells mate ~$10^6$ times more frequently than white cells (26). In order to mate, *C.*
73 *albicans* white cells first have to undergo homozygosis at the mating type locus
74 (*MTL*) and then switch to the opaque phenotype. Of note is that the *MTL* locus
75 controls mating as well as white-to-opaque switching in *C. albicans* (26). The
76 MTL**a**/α complex inhibits the expression of the master regulator gene *WOR1*
77 (white-opaque regulator 1), which activates white-to-opaque switching via a
78 positive feedback loop (14, 41, 44). Wor1 is a conserved transcription factor in
79 fungi, with two DNA-binding domains at its N-terminal end (24).

80 A variety of host microenvironmental cues including N-acetylglucosamine
81 (GlcNAc) and $CO_2$ induce white-to-opaque switching in *C. albicans* (1,13, 15,
82 23, 28, 39). GlcNAc functions through the Ras-cAMP/PKA pathway and finally
83 activates Wor1 to promote switching (15). Inactivation of the *RAS1* or *CDC35*,
84 encoding a small GTPase and adenylyl cyclase, respectively, remarkably

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85 reduces GlcNAc induced switching (15).

86 Several *Candida* species including *C. albicans* and *C. tropicalis* can also grow
87 in filamentous forms in response to environmental changes (29, 43). The
88 underlying mechanism that regulates filamentous growth in *C. albicans* has
89 been intensively investigated. For example a plethora of environmental factors,
90 including serum, high temperature (37°C), GlcNAc and $CO_2$, induce
91 filamentation (4, 8). GlcNAc also promotes filamentous growth also via the
92 Ras-cAMP/PKA pathway (4, 6). The relationship between white-opaque
93 switching and yeast-filamentous growth transition is not clear, although a
94 variety of environmental inducers and genes have been found to regulate both
95 phenotypic transition systems (39). Of note is that we have recently proposed
96 that white-opaque switching is a newly evolved developmental process (15).

97 In this study, we investigated whether other *Candida* species could also
98 undergo similar white-opaque switching, and thus to uncover the evolutionary
99 trajectory of opaque phenotype and the relationship between switching and
100 mating. Here, we demonstrate the white-to-opaque switching and sexual
101 mating of *C. tropicalis*. GlcNAc induces opaque cell formation and thus
102 promotes mating in *C. tropicalis*. By using a similar assay, we tested any
103 potential white-to-opaque switching ability of other *Candida* species including
104 *Candida parapsilosis*, *Lodderomyces elongisporus*, *Candida guilliermondii*,
105 *Candida lusitaniae* and *Debaryomyces hansenii,* however we did not observe
106 any opaque or sectored colony formations. Bennett and colleagues have

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107 recently reported a similar discovery of white-opaque switching and mating in
108 *C. tropicalis* (33). They have found that two *MTL***a/a** and $\alpha/\alpha$ strains
109 engineered from a natural *MTL***a**/$\alpha$ strain can switch and mate. Our study not
110 only validates their discoveries, but also provides some new findings as
111 described in the following sections.
112

113 **MATERIALS AND METHODS**

114 **Strains and growth conditions**

115 The strains used in this study are listed in **Table S1**. YPD (20 g/L glucose, 20
116 g/L peptone. 10 g/L yeast extract) was used for routine growth. Lee's + glucose
117 and Lee's + GlcNAc media were used for mating and white-opaque switching
118 assays (15, 20).

119 **Construction of plasmids and *C. tropicalis ura3/ura3* mutant**

120 The primers for construction of pNIM-ctWOR1 (TETp-ctWOR1) were
121 ctWOR1F (5-aatcttgtcgacATGTCGGCGTCTAGATTATCA-3) and ctWOR1R
122 (5-aatcttAGATCTTCAATTTGCAGTGGTGTAATATGG-3). The PCR product of
123 ctWOR1 was digested with BglII and SalI and subcloned into the BglII-SalI site
124 of pNIM1 (30), generating pNIM-ctWOR1. To construct the plasmid
125 pSFS2A-ctURA3 for *URA3* gene disruption in *C. tropicalis*, two PCR fragments
126 of 5' and 3' of *ctURA3* genes were subcloned into pSFS2A. The primers used
127 for PCR were:

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128 ctURA3apaF (5-aatacaGGGCCCAGATGAAGAGGTTACAAGTTTTG-3),

129 ctURA3xhoR (5-aatacaCTCGAGTGATACCTTTGGGTCTTCCTC-3) and

130 ctURA3sacIIF (5-aatacaCCGcGGCACCAATAATGCAATAGAAGTAG-3),

131 ctURA3sacIR (5-aatacaGAGCTCATGGGATGATGATCAAGTTGATG-3).

132 The first copy of *URA3* gene was deleted by using the plasmid

133 pSFS2A-ctURA3. Then, the *URA3/ura3::SAT1* heterozygous transformants

134 were streaked onto 5-FOA containing plates to screen auxotrophic isolates for

135 uridine.

136 **White-opaque switching and mating assays**

137 White-opaque switching was analyzed as described previously (15). Cells

138 were patched or plated onto Lee's + glucose and Lee's + GlcNAc agar and

139 incubated at 25 or 37°C for 4 to 7 days as indicated in the main text. Mating

140 experiments were performed according to previous reports with slight

141 modification (14, 26). Briefly, a mixture of $10^6$ cells of two *MTL* opposite strains

142 were dropped onto Lee's + glucose and Lee's + GlcNAc agar and incubated

143 for 4 days. The resulting mating mixture was then used for further analysis.

144 Primers used for *C. tropicalis MTL***a***1, α2* and *MDR1*PCR were:

145 MTLa1F: 5-GCTCAAAAGGAAGAGGAGGAA-3,

146 MTLa1R: 5-TCAATTCTCTTTCCCGTCTGTT-3,

147 MTLal2F: 5-CCGGTTTTCGACTCAAAGAG-3,

148 MTLal2R: 5-AGCAAGTTCCGGAGACACCT-3,

149 MDR1F: 5-TGTTGGCATTCACCCTTCCT-3,

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150 MDR1R: 5-TGGAGCACCAAACAATGGGA-3.

151 **Scanning electron microscopy (SEM) assay**

152 The SEM assay was performed according to our previous publication (11).
153 Briefly, the samples were gently washed with 1 x PBS and fixed with 2.5%
154 glutaraldehyde. Then, the samples were washed with 0.1 M $Na_3PO_4$ buffer (pH
155 7.2), dehydrated with increasing concentrations of ethanol and coated with
156 gold. The images were obtained with a scanning electron microscopy (FEI
157 QUANTA 200).

158 **Invasive and filamentous growth assays**

159 Lee's + glucose and Lee's + GlcNAc media plates were used for invasive and
160 filamentous growth. 3 μl of liquid medium containing ~2 x $10^4$ cells was
161 dropped onto the agar for 4 days (at 37 ºC) or 7 days (at 25 ºC) of incubation.
162 Images of plates were taken before and after washing with $ddH_2O$.

163

164 **RESULTS**

165 **Comparative analysis of *MTL* locus genes.**

166 Our analysis of its genomic sequence indicated that *C. tropicalis* contains a
167 conserved *MTL* locus similar to *C. albicans* and *C. dubliniensis* (5, 34). The
168 *MTL***a** locus of *C. tropicalis* contains the *MTL***a**1, **a**2, *OBP***a**, *PIK***a** and *PAP***a**
169 genes, while the *MTL*α locus contains the *MTL*α1, α2, *OBP*α, *PIK*α and *PAP*α
170 genes. All four *MAT* genes (**a**1, **a**2, α*1* and α*2*) remain intact in the *C. tropicalis*

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171 genome, although their identity and similarity of protein sequences is worse
172 between *C. tropicalis* and *C. albicans* (identity 46 to 82%, similarity 69 to 92%)
173 compared to *C. dubliniensis* and *C. albicans* (identity 83 to 95%, similarity 91
174 to 98%) (**Fig. S1**). We also searched and analyzed the sequences of a
175 number of *C. tropicalis* genes whose homologues are involved in
176 white-opaque switching or mating in *C. albicans*. These genes are conserved
177 in *C. albicans*, *C. dubliniensis* and *C. tropicalis*, indicating that *C. tropicalis* can
178 possibly undergo a phenotypic switching and mating similar to *C. albicans* and
179 *C. dubliniensis*.
180

181 **Overexpression of *C. tropicalis WOR1* gene in *C. albicans* induces**
182 **opaque cell formation.**

183 We found the homologue of *C. albicans WOR1* gene, a master regulator of
184 white-opaque switching, in *C. tropicalis* by blast search in the *Candida* genome
185 database (Broad Institute). Sequence analysis demonstrated that the two DNA
186 binding domains of Wor1 are highly conserved between *C. albicans* Wor1 and
187 *C. tropicalis* Wor1 (ctWor1) (24). To overexpress *ctWOR1*, we replaced the
188 native *ctWOR1* promoter with a *ctACT1* promoter in a *C. tropicalis MTL***a**/**a**
189 strain. However, no obvious effects on the induction of opaque cell formation
190 were observed. To test whether ctWor1 plays a similar role in white-opaque
191 switching, we then turned to examine the inducing effect of opaque cell
192 formation in *C. albicans* by constructing an overexpression strain containing a

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193 plasmid with an inducible *TETp* promoter controlled *ctWOR1* gene (30).
194 Overexpression of *ctWOR1* in a *C. albicans MTL***a/a** strain obviously induced
195 opaque sector formation (switching frequency = 100%) in the presence of 50
196 μg/ml doxycycline (inducing condition), while the switching frequency
197 remained very low (<1%) in the absence of doxycycline (non-inducing
198 condition). The switching frequencies of the WT+vector control were also very
199 low (<1%) under both inducing and non-inducing conditions (**Fig. 1**). The
200 above findings, coupled with the observation that the deletion of *ctWOR1* locks
201 *C. tropicalis* cells in the white phase (33), indicate that *ctWOR1* could play a
202 similar role in promoting white-to-opaque switching.
203
204 **White-opaque switching in *C. tropicalis***
205 Since only *MTL* homozygous strains undergo white-to-opaque switching in *C.*
206 *albicans* we hypothesized that this could be the same case in *C. tropicalis*.
207 Therefore, we first assessed the *MTL* zygosity (**a/a**, $\alpha/\alpha$, or **a**/$\alpha$) of 150 natural
208 *C. tropicalis* strains. By testing these strains with polymerase chain reaction
209 (PCR), 2 were *MTL***a** homozygous (**a/a**), 3 were *MTL*$\alpha$ ($\alpha/\alpha$) and 145 were
210 *MTL* heterozygous (**a**/$\alpha$).
211 To test whether the natural *C. tropicalis* strains underwent white-to-opaque
212 switching, we patched and plated 2 **a/a** and 2 $\alpha/\alpha$ strains onto Lee's + glucose
213 plates and cultured them for 7 days at 25°C. *C. albicans* WO-1($\alpha/\alpha$) and 2 *C.*
214 *tropicalis* **a**/$\alpha$ strains served as controls. We observed that *C. tropicalis* **a/a** and

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215 $\alpha/\alpha$ strains indeed underwent white-to-opaque switching and formed sectored
216 colonies, although the switching frequency was extremely low (~0.1%). On the
217 other hand we did not observe opaque sector formation in the tested *C.*
218 *tropicalis* **a**/$\alpha$ strains on Lee's + glucose plates. As expected, the reference
219 strain *C. albicans* WO-1 exhibited a higher white-to-opaque switching
220 frequency (~5.0%).
221 Since we have previously showed that GlcNAc is a potent inducer of
222 white-to-opaque cell formation in *C. albicans* (15), we tested whether GlcNAc
223 could also regulated white-to-opaque switching in *C. tropicalis*. More
224 specifically, we patched or plated several natural *C. tropicalis* strains onto
225 Lee's + glucose as well as Lee's + GlcNAc plates and cultured them both at
226 25°C. The observed switching frequency of the GlcNAc-containing plates was
227 much higher than that of the glucose-containing ones (**Fig. 2**). Also, the
228 switching frequency of the *MTL***a**/**a** strain (JX1374) was over 50.0% when
229 cultured on GlcNAc medium. For a more detailed review of the images of
230 switching in natural strains please refer to **Fig. 3A**. Surprisingly, we found that
231 in the GlcNAc medium the *MTL* heterozygous strain (JX1016) underwent
232 white-to-opaque switching whereas white-to-opaque switching on glucose
233 medium was absent. Of note, *C. albicans* opaque cells undergo mass
234 conversion to white at 37°C (the host temperature) (3). Here we showed that *C.*
235 *tropicalis* could undergo white-to-opaque transition at 37°C on GlcNAc medium
236 as well as on glucose medium (**Fig. 3B, Fig. S2**). Similar results of switching at

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237 37°C have been reported by the Bennett group (33). Notably, on a phloxine
238 B-containing GlcNAc medium (5 μg/ml), aged opaque sectors were stained red
239 (**Fig. 2A, B; Fig. 3**), while they could hardly be stained on glucose medium
240 (**Fig. S2**). As we expected, the *C. albicans* reference strain WO-1 showed a
241 high switching frequency on GlcNAc medium as well. These data suggest that
242 GlcNAc can induce opaque cell formation not only in *C. albicans* but also in *C.*
243 *tropicalis*.
244
245 **White and opaque cell features of *C. tropicalis.***
246 To confirm that the sectors formed by *C. tropicalis* contained true opaque cells,
247 we used microscopy and scanning electron microscopy (SEM) assays. Our
248 findings revealed that the cellular shape of *C. tropicalis* was similar to that of *C.*
249 *albicans*. For example the white cells of *C. tropicalis* were round and small and
250 showed a strong tendency to undergo filamentous growth. On the other hand,
251 the opaque cells of *C. tropicalis* were large and elongated and contained one
252 or more vacuoles (**Fig. 2B**). Interestingly, the cell wall surface of few opaque *C.*
253 *tropicalis* cells was pimpled, while it was smooth in others. Of note is that the
254 smooth surface is a prominent feature of white cells. Consistent with one
255 previous report (3), the surface of most *C. albicans* opaque cells was pimpled,
256 whereas white cells were smooth (**Fig. 2C, D**). The observation of a cellular
257 form, which shares both white and opaque *C. tropicalis* cell features, probably
258 suggests the existence of a transitional cellular state between the two forms.

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259 We tried to verify this hypothesis by culturing *C. tropicalis* cells for 4 instead of
260 7 days. In this experiment some colonies contained a majority of opaque-like
261 cells (large and elongated, but with small or no obvious vacuoles) and could
262 not be stained red on phloxine B-containing plates. Whether these cells were
263 the intermediate state remains to be further investigated.
264

265 **Mating in *C. tropicalis*.**

266 It is well known that the white-to-opaque transition serves as a mating
267 prerequisite in *C. albicans* (26, 39). Thus our next step was to investigate
268 whether *C. tropicalis* cells had to switch from white-to-opaque in order to mate
269 similarly to *C. albicans*. To achieve that we used natural *C. tropicalis* strains
270 sensitive to nourseothricin (ClonNAT). The strategy of quantitative mating is
271 shown in more detail in **Fig. 4A**. In brief, we first deleted a copy of the *URA3*
272 gene in a *C. tropicalis MTL***a/a** strain by using a plasmid containing a dominant
273 ClonNAT resistance marker *SAT1* (35). The *URA3/ura3::SAT1* heterozygous
274 transformants were then streaked onto 5-fluoroorotic acid (5-FOA) containing
275 plates in order to screen auxotrophic isolates for uridine. The resulting isolates
276 (JX1374u) could not grow in uridine-depleted media and remained ClonNAT
277 resistant because of the introduction of a *SAT1* gene. The natural *MTL*$\alpha/\alpha$
278 strain (JX1003) was prototrophic for uridine and sensitive to ClonNAT.
279 Therefore, quantitative mating of the **a/a** and $\alpha/\alpha$ strains could be monitored by
280 selection for $Uri^+$ and ClonNAT resistance. A mixture containing $10^6$ cells of **a/a**

281 (JX1374u) and $10^6$ cells of α/α (JX1003) was dropped onto Lee's + glucose or
282 GlcNAc plates and cultured at 25°C for 4 days. $10^7$ cells of the mating mixture
283 were plated onto the selectable uridine-depleted plates (containing 150 μg/ml
284 ClonNAT). After 2 days of incubation at 37°C, the colonies formed by the
285 conjugants arose from the selectable plates. To confirm that the resulting
286 colonies were the actual mating products, colonies were re-streaked onto new
287 selectable plates. The cells were then used for analysis in a
288 fluorescence-activated cell sorter (FACS). As shown in **Fig. 4B**, the DNA
289 content of the fusant was about twice as much as the diploid mating partners
290 (JX1374u and JX1003), indicating that the fusant was tetraploid. To confirm
291 that the mating products contained DNA from both JX1374u and JX1003
292 strains, we tested for the existence of the *MTL***a**1 and α2 genes by PCR (**Fig.
293 4C**). We also found a single nucleotide polymorphism (SNP) at the *MDR1*
294 gene locus of the parental strains JX1374u (5'-CATCATT**T**CAG-3') and
295 JX1003 (5'-CATCATT**C**CAG-3'). This sequence of the multidrug resistance
296 protein *MDR1* gene in the mating products was 5'-CATCATT**Y**CAG-3' (Y=T, C),
297 suggesting that the genome contained the *MDR1* gene sequences of both
298 parents. Mating zygotes formed by fusion were observed in the mating mixture
299 on both glucose and GlcNAc-containing media by light microscopy (**Fig. 4D**).
300 An SEM analysis of fusant cells showed the formation of conjugation tubes
301 between opaque **a/a** and α/α cells (**Fig. 5**). These results indicate that the
302 mating process of *C. tropicalis* was highly similar to that of *C. albicans* (21). Of



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303 note, the white-opaque transition regulated sexual mating has also been
304 observed by Porman et al. (33).
305
306 **GlcNAc increases *C. tropicalis* mating efficiency.**
307 In *C. albicans*, efficient mating requires the conversion of white cells to opaque
308 (26). Interestingly we found that this is also the case in *C. tropicalis*. More
309 specifically we observed that only opaque cells of *C. tropicalis* formed shmoos
310 and mating conjugations in a mixture of opposite *MTL* cells (**Fig. 4D and Fig.**
311 **5**). On the other hand white cells retained their cellular shape without any
312 change.
313 Since GlcNAc could induce white-to-opaque cell switching in *C. albicans* as
314 well as in *C. tropicalis*, we then tested whether GlcNAc promoted mating in *C.*
315 *tropicalis*. Our quantitative mating assay revealed that the mating efficiency on
316 GlcNAc medium was over 100 times higher than that on glucose medium both
317 at 25°C and at 37°C (**Fig. 6**). Surprisingly, on the medium that contained the
318 same carbon source, the mating efficiencies were similar at both temperatures.
319 These results were consistent with our previous observation of
320 white-to-opaque switching at 37°C, suggesting that opaque cells of *C.*
321 *tropicalis* were not sensitive to high temperatures. However, in *C. albicans*,
322 an increase of the temperature to 37°C remarkably reduced its mating
323 efficiency. A likely explanation is that in host temperatures (37°C) opaque
324 cells are unstable and undergo mass conversion to the white state which is

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325 largely incompetent to mate (3, 26).

326

327 **GlcNAc inhibits filamentous and invasive growth in *C. tropicalis*.**

328 Interestingly, GlcNAc has been previously found to be a potent inducer of
329 filamentous development in *C. albicans* (37). Other studies have shown that
330 the conserved Ras-cAMP/PKA pathway likely mediates GlcNAc induced
331 filamentous growth as well as white-to-opaque transition in *C. albicans* (6, 15).
332 Although GlcNAc played a similar role in the induction of opaque cell formation
333 in *C. tropicalis* as well as in *C. albicans*, we found that GlcNAc had an opposite
334 effect on the filamentous growth of *C. tropicalis*. On Lee's + glucose medium,
335 some *C. tropicalis* strains developed robust filamentous colonies even at 25°C
336 (**Fig. 2B; Fig. 3A**), a temperature unfavorable for filamentous growth in *C.*
337 *albicans.* However, when we replaced glucose with GlcNAc we observed an
338 inhibition of filamentous growth in a number of natural *C. tropicalis* strains
339 including all the three mating types (**a/a**, $\alpha/\alpha$ and **a**/$\alpha$) at both 25°C and 37°C
340 (**Fig. 2B; Fig. 3**). By examining all 6 natural strains (2 **a/a**, 2 $\alpha/\alpha$ and 2 **a**/$\alpha$), we
341 found that GlcNAc robustly reduced their invasive growth ability at 25°C as
342 well as 37°C (**Fig. 7**). These results suggest that GlcNAc plays a negative role
343 in the filamentous growth of *C. tropicalis*.

344

345 **Analysis of white-to-opaque switching of other *Candida* species in the**
346 **CTG clade.**

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347 It has been previously shown that the *MTL* locus controls white-opaque
348 switching and mating in *C. albicans* (26). The organization of *MTL* loci in the
349 CTG clade (the species in this clade translate CUG codons as serine instead
350 of leucine (17) is very similar and conserved among *Candida* species (5).
351 Therefore, we examined whether other species in the *Candida* clade could
352 also undergo white-opaque switching. Three to 5 natural strains of each
353 species were examined on Lee's + glucose medium and Lee's + GlcNAc
354 medium at 25°C and 37°C. Notably, we observed white-to-opaque transition in
355 *C. albicans*, *C. dubliniensis* and *C. tropicalis* in both media, but not in *Candida*
356 *parapsilosis*, *Lodderomyces elongisporus*, *Candida guilliermondii*, *Candida*
357 *lusitaniae* and *Debaryomyces hansenii* in any conditions tested (**Table 1**).
358
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360 **DISCUSSION**

361 In the present study, we show that *C. tropicalis* has the ability to undergo
362 white-opaque switching and mating. More importantly, we have found that
363 GlcNAc induces white-opaque switching as well as mating in *C. tropicalis*.
364 Although these two biological processes are highly similar in both *C. tropicalis*
365 and *C. albicans, C. tropicalis* exhibits some unique features. For instance, we
366 show that *C. tropicalis* undergoes white-to-opaque switching and mating at
367 both 37°C and 25°C, while *C. albicans* opaque cells become extremely
368 unstable and mate poorly at 37°C.

369 A comparative analysis between *C. tropicalis* and *C. albicans* indicates that
370 genes involved in mating and white-to-opaque switching are generally
371 conserved. Notably, *C. tropicalis* ctWor1 is homologous and highly similar to
372 the master regulator Wor1 of *C. albicans*. For example, the overexpression of
373 Wor1 in a wild-type (WT) *C. albicans* strain promotes opaque cell formation, a
374 finding that pinpoints Wor1 as a key regulator of white-to-opaque switching in
375 *C. albicans* through a positive feedback loop (14, 41, 44). In this study, we
376 demonstrate that the overexpression of *ctWOR1* in *C. albicans* induces
377 white-to-opaque switching (**Fig. 1**), suggesting that Wor1 may have similar
378 functions in both species. We also tried to overexpress *ctWOR1* in a *C.*
379 *tropicalis MTL***a**/**a** strain by replacing the native *ctWOR1* promoter with a
380 *ctACT1* promoter. We did not observe any obvious effects on the induction of
381 opaque cell formation in the overexpressing *C. tropicalis* strain. A possible

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382 reason could be that ctWor1 does not activate its own expression in *C.*
383 *tropicalis*, while Wor1 does via a self feedback loop in *C. albicans*. This may
384 explain why the white-to-opaque switching frequency in *C. tropicalis* is
385 relatively low. Porman et al. have reported that deletion of ctWOR1 locked *C.*
386 *tropicalis* cells in white phase (33). These findings suggest that ctWor1 is
387 indeed involved in the regulation of white-opaque switching in *C. tropicalis*,
388 while the underlying regulatory mechanisms could be different.
389 We subsequently showed that a number of natural *C. tropicalis* strains
390 including all the three mating types (**a**/$\alpha$, **a**/**a** and $\alpha/\alpha$) have the ability to
391 undergo white-to-opaque switching and mating at 25°C and 37°C. The *MTL***a**1
392 and $\alpha$2 genes were not lost in the opaque cells of **a**/$\alpha$ strains with PCR
393 confirmation. Neither *MTL***a**/**a** nor $\alpha/\alpha$ opaque cells could mate with the opaque
394 cells formed by *MTL***a**/$\alpha$ strains (data not shown). These results exclude the
395 possibility that the *MTL***a**/$\alpha$ strains underwent a spontaneous conversion to
396 *MTL* homozygous before switching to opaque. We also showed that the
397 phenotypes of *C. tropicalis* white and opaque cells are similar to those of *C.*
398 *albicans*. For instance, most *C. tropicalis* opaque cells are elongated and have
399 a big vacuole just like in *C. albicans*. Also the cell wall surface of white cells in
400 both species is smooth. A small percentage of *C. tropicalis* opaque cells exhibit
401 pimpled cell wall surfaces, whereas most *C. albicans* opaque cells are pimpled
402 (21). Although aged *C. tropicalis* opaque sectors could be stained red by
403 phloxine B, the newly-formed opaque colonies remained white or slightly pink.

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404 These findings suggest that an intermediate form between white and opaque
405 phase may exist. Whether the elongated cells with smooth cell wall surfaces
406 and the non-stained cells are in the intermediate state remains to be
407 investigated. As in *C. albicans*, only opaque cells can mate efficiently in *C.*
408 *tropicalis*. Since the mating efficiency of *C. tropicalis* opaque cells remain
409 robust at low (25°C) and high (37°C) temperatures we can assume that the
410 opaque cells of *C. tropicalis* can remain stable at both temperatures (**Fig. 6**).
411 GlcNAc is a well-known component of the bacterial cell wall and it is also found
412 in the mucus of the human gastrointestinal tract (GI tract), the ultimate human
413 reservoir for *Candida* infections (12, 29). Notably, *Candida* species are
414 abundant in the GI tract where they can be found in more than 21% of humans
415 (7, 42). In one previous study, we reported that GlcNAc induced
416 white-to-opaque transition in *C. albicans* (15). In the present study, we have
417 found that GlcNAc can also induce opaque cell formation and promote mating
418 in *C. tropicalis*. Our sequence analysis demonstrated that the *C. tropicalis*
419 genome contains most genes required for GlcNAc sensing and metabolism
420 including the Ras-cAMP/PKA pathway, the transporter *NGT1* and
421 glucosamine-6-phosphate deaminase *NAG1* genes. These results indicate
422 that the two *Candida* species, as successful commensals and pathogens in the
423 human body, have evolved to respond to the signals produced by the gut
424 microbiota and by the GI epithelial cells.
425 In *C. albicans*, white cells can differentiate into filamentous or opaque cells in

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426 response to different stimuli. A variety of environmental cues including GlcNAc
427 and $CO_2$ not only can induce opaque cell formation but also promote
428 filamentous development in *C. albicans* (8, 13, 15). Filamentous cells are
429 genetically white and express a set of white-specific genes. Once the white
430 cells switch to opaque, they lose the ability to form filaments under regular
431 conditions (2). To undergo filamentous growth, opaque cells have to switch
432 back to white. Interestingly, compared to its effect on *C. albicans* GlcNAc plays
433 an opposite role in the filamentation of *C. tropicalis*. More specifically we found
434 that it inhibited the filamentation ability of all tested natural *C. tropicalis* strains.
435 We propose that the opaque cellular state and filamentation represent two
436 directions of white cell differentiation. To differentiate into opaque, the white
437 cells have to first block the expression of the regulatory machinery of initiating
438 filamentation, and then increase the expression of opaque-promoting genes
439 like the master regulator *WOR1*. This hypothesis has been confirmed in *C.*
440 *albicans* (data not shown). Induction and maintenance of the opaque phase
441 needs a Wor1 involved positive feedback loop. The *C. albicans wor1/wor1*
442 mutant carrying a copy of an *ACT1* promoter that controlls *WOR1* gene can
443 not switch to the opaque phase, and, surprisingly, also loses the ability to grow
444 into the filamentous form even under inducing conditions (data not shown).
445 These results suggest that moderate expression of *WOR1* under the control of
446 *ACT1* promoter is not sufficient to induce opaque phenotype, but is sufficient to
447 inhibit filamentous growth.

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448 Given that UDP-GlcNAc is a constituent of the GI tract as well as the blood (32,
449 36), inhibition of *C. tropicalis* filamentation by GlcNAc may also have a
450 potential clinical significance. Compared to *C. albicans*, natural *C. tropicalis*
451 shows stronger tendency to undergo filamentous growth especially at the host
452 temperature (37°C). Although filamentous cells are better at the initial phase of
453 tissue penetration, yeast cells can be more easily disseminated through the
454 bloodstream. Therefore, the inhibiting effect of GlcNAc on filamentous growth
455 in *C. tropicalis* may facilitate dissemination in the host and play an important
456 role in infections.

457 Since GlcNAc facilitates the phenotypic switching of *C. albicans* and *C.*
458 *tropicalis*, we aimed to figure out whether this was possible with other *Candida*
459 species in the CTG clade. Although we have not observed white-to-opaque
460 switching in the natural strains tested, these species may undergo other forms
461 of switching. For example, *C. lusitaniae* switches spontaneously among three
462 colored phenotypes on $CuSO_4$ containing medium: white, light brown, and dark
463 brown (27).

464 In our opinion, our discovery of white-to-opaque switching regulating mating in
465 *C. tropicalis* has revived an important question: why do white *C. tropicalis* and
466 *C. albicans* cells have to switch to opaque to achieve mating (39)? Based on
467 the observation of pheromone induced white cell response in *C. albicans*,
468 Daniels et al. have proposed that pheromone stimulation may lead to a chain
469 event where a minority of opaque cells drive white cells to form a biofilm in

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470 which opaque cells are able to mate (10). The underlying mechanism of
471 white-opaque switching and mating regulation could be much more complex
472 than the hypothesis proposed by Daniels et al. (10). The unique biological
473 features in *C. tropicalis* and *C. albicans* not only provide mechanistic plasticity
474 and diversity of sexual reproduction, but could also improve the fitness and
475 adaptation in their natural environments. For example, white cells can better
476 survive in systemic infections and are more resistant to antifungals than
477 opaque cells (39). Under natural conditions, sexual reproduction may not be
478 necessary for *Candida* species, since the costs of sexual reproduction are
479 considerable. Maintaining a white state could be an efficient way to shut down
480 all the mating related gene machinery. A similar strategy for sexual mating
481 adopted by the yeast *Clavispora opuntiae* is to enter stationary phase to
482 become maximally mating competent (19).

483 The present study not only validates the recent report published by Porman et
484 al. (2011) (33), but also provides new insights into the evolution of
485 white-opaque switching and mating in pathogenic *Candida* species. Both of the
486 two studies have found that *C. tropicalis* can undergo white-opaque switching
487 and sexual mating. Only opaque cells of *C. tropicalis* mate efficiently and the
488 switching and mating are not sensitive to high temperatures. The switching
489 frequency of white-to-opaque in this organism is extremely low in glucose
490 containing medium. However, several findings make our project of special
491 novelty. Firstly, natural *MTL* homozygous strains were used in our study, while

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492 *MTL***a**/**a** and α/α strains engineered from an *MTL***a**/α strain were used in the
493 Porman et al. report (33). Secondly, we identified a possible intermediate
494 phase between white and opaque. Thirdly, we have shown GlcNAc induces
495 white-to-opaque switching, thus facilitating mating. Aged opaque colonies or
496 sectors could be stained red on phloxine B containing media. Fourthly, we
497 have found that not only *MTL* homozygous but also *MTL* heterozygous strains
498 undergo switching in the presence of GlcNAc. Therefore, it is very possible
499 that most natural strains have the ability to undergo switching in ecological
500 niches where inducing factors such as GlcNAc are present (e.g. physiological
501 conditions). However, this was not the case with *C. albicans* where only a
502 minority of natural *C. albicans* strains (8%), which are homozygous at the *MTL*
503 locus, switched from white-to-opaque (22). This finding indicates that
504 white-opaque switching very possibly evolved before the association with
505 mating in *Candida* species. Finally, inhibition of filamentous growth by GlcNAc
506 in *C. tropicalis* provides new insights for the evolution of the opaque phenotype.
507 Taken together, these two studies on the topic of white-opaque switching and
508 mating in *C. tropicalis* are significant and provide new implications to
509 understand the evolutionary trajectory of sexual reproduction in pathogenic
510 *Candida* species.
511

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518

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659 **FIGURE LEGENDS**

660 FIG. 1. Overexpression of *C. tropicalis WOR1* in a *C. albicans MTL*a/a strain
661 promotes opaque cell formation.
662 WT, GH1012. White cells of the strains WT+ vector and WT+TETp-ctWOR1
663 were plated onto Lee's + glucose plates and cultured at 25°C for 5 days under
664 non-inducing (0 μg/ml doxycycline) or inducing (50 μg/ml doxycycline)
665 conditions.
666
667 FIG. 2. A comparison of white-to-opaque switching in *C. albicans* and *C.*
668 *tropicalis*.
669 A. Morphology of white-opaque switching in *C. albicans*. White cells of *C.*
670 *albicans* were patched or plated onto Lee's + glucose or GlcNAc plates and
671 cultured at 25°C for 7 days.
672 B. Morphology of white-opaque switching in *C. tropicalis*. White cells of *C.*
673 *tropicalis* were patched or plated onto Lee's + glucose or GlcNAc plates
674 and cultured at 25°C for 7 days.
675 C. Examples of SEM images of *C. albicans* white and opaque cells.
676 D. Examples of SEM images of *C. tropicalis* white and opaque cells.
677
678 FIG. 3. GlcNAc induces opaque cell formation and inhibits filamentous growth
679 of natural *C. tropicalis* strains.
680 A. Experiments at 25 °C. White cells of 5 natural strains were patched or

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681 plated onto Lee's +glucose or Lee's +GlcNAc plates and cultured for 7 days.
682 Opaque (op) sectors were indicated. Strains used (up to down; left to right):
683 JX1002, JX1004; JX1001, JX1003 and JX1005.
684 B. Experiments at 37 °C. White cells of 3 natural strains were patched plated
685 onto Lee's +glucose or Lee's +GlcNAc plates and cultured for 4 days. Opaque
686 (op) sectors were indicated. Strains used (left to right): JX1009; JX1008 and
687 JX1010.
688
689 FIG. 4. Mating in *C. tropicalis*.
690 A. Strategy for quantitative mating in *C. tropicalis*. JX1374u, *MTL***a/a** *ura3*- ,
691 ClonNAT resistant. JX1003, WT, *MTL*$\alpha/\alpha$ *URA3/URA3*, ClonNAT sensitive.
692 $10^6$ of **a**/**a** and $\alpha/\alpha$ cells were mixed and dropped onto Lee's +glucose or
693 Lee's +GlcNAc plates and cultured for 4 days at 25°C. Then, $10^7$ of the
694 mating mixture cells were plated onto selectable plates containing 150
695 μg/ml ClonNAT and depleted of uridine. The colonies growing out from
696 selectable plates were used for further experiments.
697 B. FACS analysis indicated the mating fusant from selectable plates were
698 tetraploid.
699 C. PCR of *MTL***a** and *MTL*$\alpha$ genes. Parental strains (**a**/**a**, $\alpha/\alpha$) and two random
700 colonies of the mating fusant (Mat1, Mat2) were tested.
701 D. Cell microscopy of mating. S: shmoos; C: conjugation tubes.
702

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703 FIG. 5. Examples of SEM images of mating in *C. tropicalis.*

704 Strains used for mating: JX1374u (*MTL***a/a**) and JX1003 (*MTL*α/α).

705 FIG. 6. GlcNAc increases *C. tropicalis* mating efficiency.

706 White cells of *MTL***a/a** and α/α were first grown on glucose or GlcNAc medium
707 at 25°C for 5 days, then mixed and cultured on corresponding plates for 4 days
708 at 25 or 37°C. ~$10^7$ of the mating mixture cells were plated onto selectable
709 plates. The images represent three independent experiments.

710

711 FIG. 7. GlcNAc inhibits invasive growth in *C. tropicalis*.

712 2000 cells of 6 clinically independent *C. tropicalis* strains were dropped onto
713 Lee's +glucose or Lee's +GlcNAc plates and cultured for 7 (at 25°C) or 4 days
714 (37°C). Images of plates were taken before and after washing with dd$H_2O$.
715 Strains used (up to down): JX1004 (**a/a**), JX1374 (**a/a**); JX1369 (α/α), JX1003
716 (α/α); JX1016 (**a**/α), JX1009(**a**/α).

717

718 **TABLE 1.** White-to-opaque switching in the CTG clade species

| | White-to-opaque switching | | | |
|---|---|---|---|---|
| **Species name** | Glucose 25°C | Glucose 37°C | GlcNAc 25°C | GlcNAc 37°C |
| *C. albicans* | **Y** | N | **Y** | **Y** |
| *C. dubliniensis* | **Y** | N | **Y** | **Y** |
| *C. tropicalis* | **Y** | **Y** | **Y** | **Y** |
| *C. parapsilosis* | N | N | N | N |
| *L. elongisporus,* | N | N | N | N |
| *C. guilliermondii* | N | N | N | N |
| *C. lusitaniae* | N | N | N | N |
| *D. hansenii* | N | N | N | N |

719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741

742 Lee's glucose and Lee's GlcNAc media were used for this experiment. For the
743 species which grew very slow on Lee's glucose and Lee's GlcNAc media,
744 SD-glucose and SD-GlcNAc were used to verify their phenotypes. Y: yes,
745 switching observed. N: no, switching not observed.



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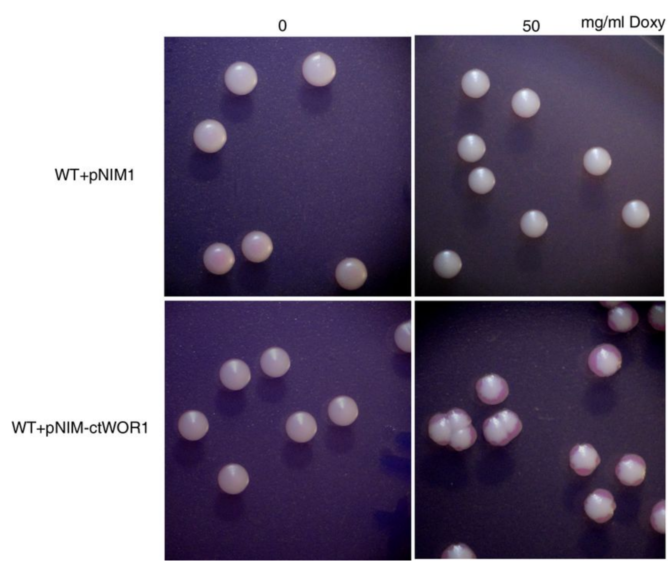

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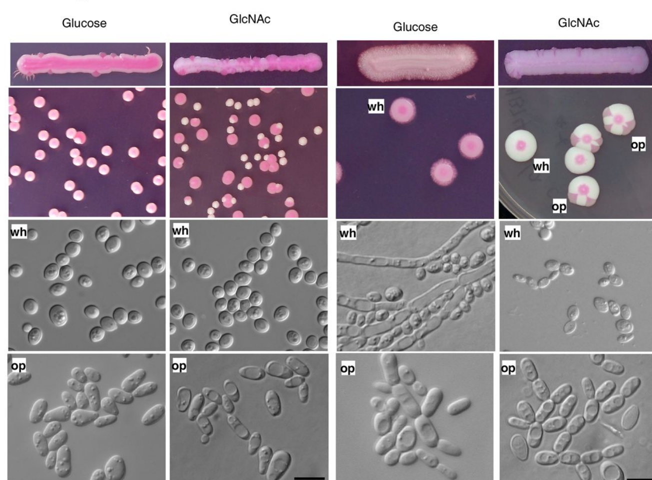


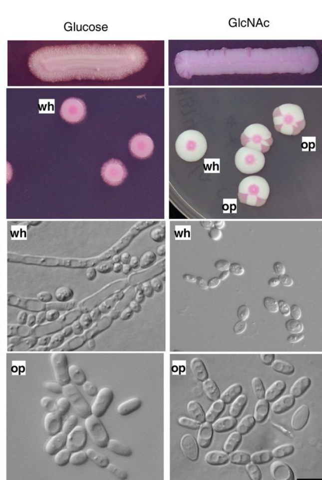


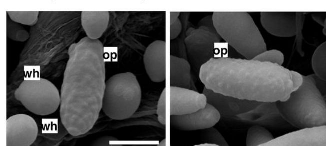


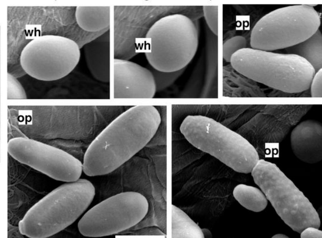

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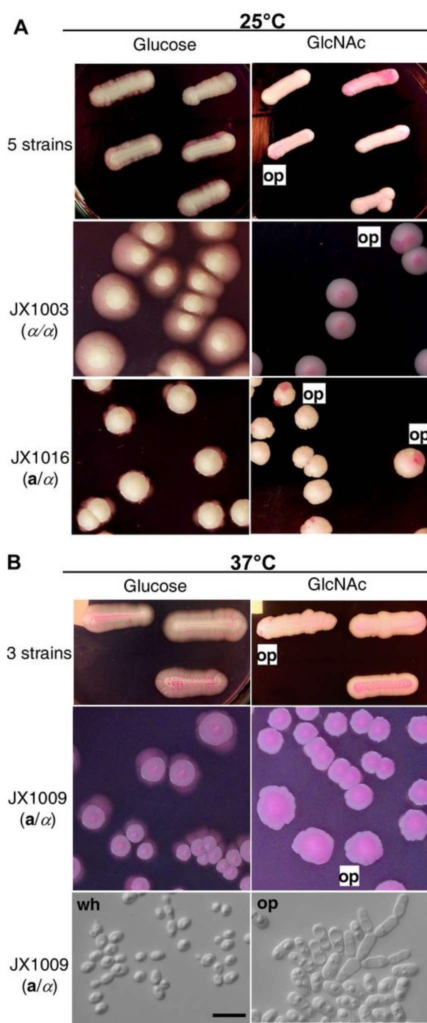

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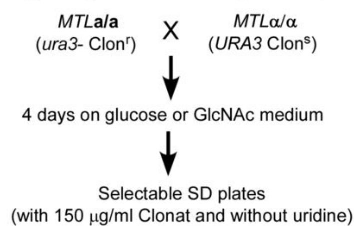
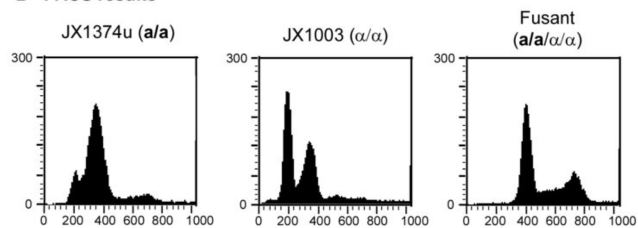
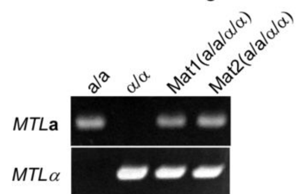
**A Strategy for quantitative mating in *C. tropicalis***



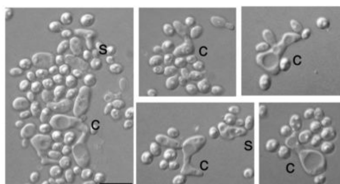

**B FACS results**




**C PCR of *MTL*a or *a* genes**




**D Cell fusion**



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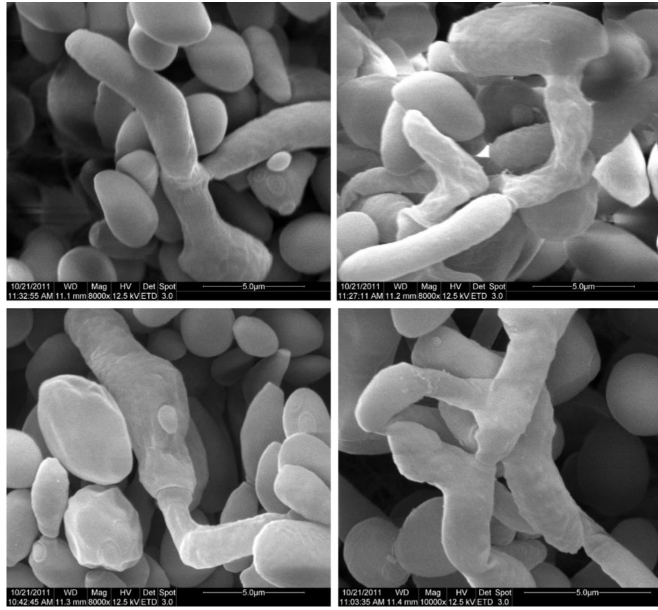

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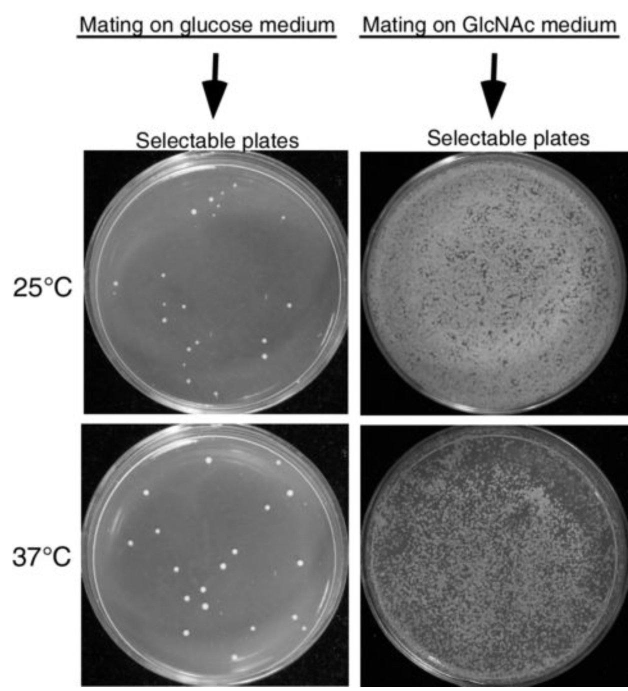

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