1 **Differential filamentation of *Candida albicans* and *C. dubliniensis* is**
2 **governed by nutrient regulation of *UME6* expression**

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21 **Abstract**

22 *Candida dubliniensis* is closely related to *C. albicans*, however it is responsible for
23 fewer infections in humans and is less virulent in animal models of infection. *C.*
24 *dubliniensis* forms fewer hyphae *in vivo* and this may contribute to its reduced
25 virulence. In this study we show that unlike *C. albicans*, *C. dubliniensis* fails to form
26 hyphae in YPD supplemented with 10% (v/v) fetal calf serum (YPDS). However, *C.*
27 *dubliniensis* filaments in water plus 10% (v/v) fetal calf serum (WS), and this
28 filamentation is inhibited by the addition of peptone and glucose. Repression of
29 filamentation in YPDS could be partly overcome by preculture in synthetic Lee's
30 medium. Unlike *C. albicans*, inoculation of *C. dubliniensis* in YPDS did not result in
31 increased *UME6* transcription. However, >100-fold induction of *UME6* was observed
32 when *C. dubliniensis* was inoculated in nutrient poor WS medium. Addition of
33 increasing concentrations of peptone to WS had a dose dependent effect on reducing
34 *UME6* expression. Transcript profiling of *C. dubliniensis* hyphae in WS identified a
35 starvation resposne involving expression of genes in the glyoxylate cycle and fatty
36 acid oxidation. In addition a core, shared transcriptional response with *C. albicans*
37 could be identified, including expression of virulence-associated genes including
38 *SAP456*, *SAP7*, *HWP1* and *SOD5*. Preculture in nutrient limiting medium enhanced
39 adherence of *C. dubliniensis,* epithelial invasion and survival following co-culture
40 with murine macrophages. In conclusion, *C. albicans* unlike *C. dubliniensis*, appears
41 to form hyphae in liquid medium regardless of nutrient availability, which may
42 account for its increased capacity to cause disease in humans.

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45 **Introduction.**

46 *Candida dubliniensis* is the closest known relative of *Candida albicans*, the
47 predominant fungal pathogen of humans (26, 27). Epidemiological evidence has
48 shown that *C. albicans* is more prevalent in the human population as a commensal of
49 the oral cavity and is responsible for more infections (both oral and systemic) relative
50 to *C. dubliniensis* (8, 11, 14). *C. albicans* is reponsibe for approximately 60% of cases
51 of candidemia, whereas *C. dubliniensis* accounts for fewer than 2% of cases (11).
52 Evidence from animal infection models also suggest that *C. dubliniensis* is less
53 virulent than *C. albicans* (25, 28). Following oral-intragastric inoculation, *C.*
54 *dubliniensis* strains are more rapidly cleared from the gastrointestinal tract than *C.*
55 *albicans* and are less able to establish disseminated infection (25). Following tail vein
56 inoculation in the systemic mouse model of infection, only a small number of *C.*
57 *dubliniensis* isolates have been shown to establish disseminated infections and most
58 studies conclude that *C. dubliniensis* isolates are generally less virulent compared to
59 *C. albicans* isolates (1, 28).

60 Virulence studies have associated the reduced capacity of *C. dubliniensis* to establish
61 infection with a reduced ability to undergo the yeast to hypha transition (1, 25). In the
62 oral-intragastric infection model, *C. dubliniensis* cells in the stomach and kidney were
63 found to be in the yeast form only, while *C. albicans* cells were found to be in both
64 the yeast and hyphal forms using the same models (25). Asmundsdottir et al. (1) also
65 noted that *C. dubliniensis* produced significantly fewer hyphae than *C. albicans*
66 following dissemination to the liver and kidney in mice. In vitro, *C. dubliniensis*
67 forms true hyphae less efficiently than *C. albicans* in response to serum, pH shifts in
68 Lee's medium, CO_2 and in certain defined media such as RPMI-1640 (15, 25). Poor
69 hypha production has also been observed in *C. dubliniensis* in vitro during co-culture

70 with murine macrophages and during infection of reconstituted oral epithelial tissues
71 (15, 23). This results in an inability of *C. dubliniensis* to evade macrophage killing
72 and limited invasion of epithelial surfaces.

73 Although *C. dubliniensis* produces true hyphae less efficiently than *C. albicans*, *C.*
74 *dubliniensis* can produce abundant pseudohyphae and chlamydospores on certain
75 solid media (26). Recently, Staib et al. (24) demonstrated that the propensity for *C.*
76 *dubliniensis* to form large amounts chlamydospores on these media was due to
77 species-specific down-regulation of the *NRG1* repressor. Further studies have also
78 shown that down-regulation of the *NRG1* transcript is also required for efficient
79 production of true hyphae in *C. albicans* in response to serum (21). We have shown
80 that under conditions where *C. dubliniensis* fails to filament, for example following
81 phagocytosis by murine macrophages, that this species does not down-regulate *NRG1*,
82 whereas *C. albicans* responds to these condition by shutting down *NRG1* transcription
83 (15). Deletion of the *NRG1* gene in *C. dubliniensis* can partly offset the failure of this
84 species to filament in vitro, and leads to more efficient production of hyphae in
85 response to serum, CO_2 and during co-culture with murine macrophages (15).

86 In this study, we have examined in detail the environmental signals required for
87 filamentation in *C. dubliniensis*. We have shown that nutrient rich conditions inhibit
88 efficient hypha formation by suppressing *UME6* expression in *C. dubliniensis*. This
89 study also includes the first description of a *C. dubliniensis*-specific microarray that
90 we used to generate a transcript profile for *C. dubliniensis* true hyphae. The effects of
91 inducing hypha formation in *C. dubliniensis* under these conditions on the ability to
92 infect reconstituted oral epithelial tissues and to evade macrophage killing were also
93 examined.

Materials and methods

Candida *strains and culture conditions.*

All *Candida* strains were routinely cultured on yeast extract-peptone-dextrose (YPD) agar, at 37°C. For liquid culture, cells were grown shaking (200 r.p.m.) in YPD broth at 30°C or 37°C, as indicated (7). Genotypes of strains used in this study are listed in supplementary material, Table S1. Liquid culture was also carried out at 30˚C in the liquid medium of Lee et al. (12) supplemented with 400 mM arginine, 0.001 % (w/v) biotin and trace metals (0.2 mM ZnSO, 0.25 mM CuSO, 1 mM FeCl, 1 mM MgCl, 1 mM CaCl). Where indicated, Lee's medium was buffered to pH 5.0 or pH 7.2 with 0.1 M potassium phosphate buffer. Supplementation of Lee's and other media with peptone was carried out with bacteriological peptone (Oxoid). Peptone supplementation up to 2% (w/v) did not significantly alter the pH of Lee's medium or serum. Hyphal induction was carried out in liquid YPD plus 10% (v/v) fetal calf serum (YPDS) or in sterile Milli-Q H_2O supplemented with 10% (v/v) fetal calf serum (WS) at 37˚C. The proportion of germ-tubes or hyphae in each culture was assessed at intervals by microscopic examination of an aliquot of culture with a Nikon Eclipse 600 microscope (Nikon U.K., Surrey, U.K.).

Genetic manipulation of Candida dubliniensis

Ectopic expression of *CaUME6* in *C. dubliniensis* was achieved using plasmid pCaUme6-3, containing *UME6* under the control of a doxycycline inducible promoter (31). The expression cassette was released from pCaUme6-3 by *Apa*I and *Pml*I digestion and was used to transform Wü284 and CDM10 by electroporation, as described (15). Plasmid pNRG1 was generated from plasmids pNIM1 and pTET42 (17). *NRG1* was removed from pTET42 as a *Sal*I/*Bgl*II fragment and ligated to

118 *Sal*I/*Bgl*II digested pNIM1 to generate pNRG1. The expression cassette was released
119 from pNRG1 by *Sac*II and *Kpn*I digestion and was used to transform Wü284 and
120 CDM10 by electroporation, as described (15). Integration of pNIM1 derivatives at the
121 *ADH1* locus was confirmed by PCR.

122 In order to create strains harboring a P_{ECE1}-*GFP* fusion, we used the integrating vector
123 pCDRI (15). A derivative of this plasmid was created by inserting yEGFP fused to the
124 actin terminator on a *Hin*dIII/*Mlu*I fragment to create pGM175. An *ECE1* promoter
125 fragment from bases -1 to -921 was amplified from *C. albicans* SC5314 with primers
126 ECEAF (GTACGGGCCCAAGAGTCTCATTCAGATAACG) and EXEXR
127 (GCATCTCGAGTTTAACGAATGGAAAATAGTTG) and cloned upstream of
128 yEGFP following digestion of both fragments with *Apa*I and *Xho*I. The plasmid was
129 linearised within the *CDR1* region and used to transform *C. albicans* SC5314, *C.*
130 *dubliniensis* Wü284 and the *nrg1*Δ derivative CDM10 as described (15). Ectopic
131 integration in the *CDR1* gene was confirmed by Southern hybridization.

132

133 *Transcriptional profiling with oligonucleotide microarrays*

134 A set of 5,999 orfs from the CD36 genome was used to design a *C. dubliniensis*
135 expression microarray. Two unique 60mer oligonucleotides were designed specific
136 for each orf using the Agilent eArray probe design tool. Each 60mer was printed in
137 quadruplicate on glass slides by Agilent technologies. To examine the hyphal
138 transcript profile of *C. dubliniensis* strain Wü284, the strain was grown for 18 h in
139 Lee's medium (pH 4.5) at 30°C with shaking, washed in sterile H_2O, and inoculated
140 in 200 ml H_2O plus 10% (v/v) fetal calf serum to a density of 2 x 10^6 cells/ml.
141 Samples (50 ml) were removed for RNA preparation at 1, 3 and 5 h post inoculation.

142 To examine the effects of cell density changes, nutrient depletion, a shift to 37˚C and
143 a shift to alkaline pH, identical 18 h Lee's medium cultures were washed and
144 inoculated at 2 x 10^6 cells/ml in (i) fresh Lee's medium (pH 4.5) at 30˚C, (ii) 10%
145 (v/v) Lee's medium (pH 4.5) at 30˚C, (iii) Lee's medium (pH 4.5) at 37˚C and (iv)
146 Lee's medium (pH 7.2) at 30˚C, respectively. RNA was extracted from these cultures
147 following 3 h incubation under each condition. To identify *NRG1* regulated genes in
148 *C. dubliniensis*, RNA was extracted from Wü284 and its *nrg1Δ* derivative CDM10
149 following growth to $OD600_{nm}$ 1.0 in YPD broth at 30˚C. For RNA preparation, cell
150 pellets were snap frozen in liquid N_2 and disrupted using the Mikro-Dismembrator S
151 system (Sartorius Stedim Biotech, Göttingen, Germany). RNA was prepared using
152 TRI-Reagent (Sigma Chemical Co.) according to the manufacturers instructions.
153 PolyA mRNA was then isolated using the Sigma Genelute mRNA isolation kit. A 200
154 ng aliquot of mRNA was labelled with Cy5 or Cy3 using the Agilent Two-Color Low
155 RNA input Linear Amplification Kit PLUS, according to the manufacturer's
156 instructions. Hybridization and washing of the arrays was carried out using the
157 Agilent Gene Expression Hybridization Kit and Gene Expression Wash Pack
158 according the manufacturer's instructions. For each condition, four biological
159 replicate experiments were performed, including two dye swap experiments. Slides
160 were scanned using the GenePix personal 4100A scanner (Axon) and data were
161 extracted using GenePix Pro 6.1 (Axon). Spots were flagged absent if the signal was
162 less than background +1 standard deviation in both fluorescent channels. Raw data
163 were exported to GeneSpring GX11 and signals for each replicate spot were
164 background corrected and normalized using Loess normalization. Log_2 fluorescence
165 ratios were generated for each replicate spot and averaged. Oligonucleotides were
166 excluded from analysis if >50% of replicates in each condition were flagged absent.

167 Genes differentially expressed across all conditions were identified by ANOVA with
168 the SNK post hoc test in Genespring GX11. A total of 7107 oligonucleotide probes
169 were significantly differentially expressed, with a corrected *p* value (Benjamini-
170 Hochberg FDR) ≤ 0.05. Hierarchical clustering was used to compare gene expression
171 in each condition using the default settings in Genespring GX11. Some individual
172 samples (serum 1h, 3h and 5h) were also analysed using a one-sample *t*-test in order
173 to identify genes exhibiting significant differential expression (2-fold or greater) from
174 preculture cells. All *p* values were adjusted using the Benjamini-Hochberg multiple
175 correction test to limit false differential gene expression and oligonucleotides with *p*
176 values ≤ 0.015 were selected for analysis. Results from all 32 microarrays have been
177 submitted to the GEO archive (Accession: GSE20537).

178 The *C. albicans* hypha-induced gene set used in this study included the hypha-
179 regulated genes identified by Nantel et al. (16) and by Kadosh & Johnson (10).
180 Additional *C. albicans* hypha-regulated genes were identified in the data set of
181 Kadosh and Johnson (10) following analysis of the dataset with GeneSpring GX11.
182 These additional genes were included if they exhibited significant >2-fold regulation
183 (*t* test $p \leq 0.01$) in the 2 h and 3 h data sets (10).

184 *Real-time PCR analysis of gene expression*

185 Cultures for RNA preparation for QRT-PCR were set up in identical fashion to those
186 used for microarray analysis. RNA for QRT-PCR was isolated using the RNeasy
187 Mini-kit (Qiagen). Cells were disrupted using a FastPrep bead beater (Bio101).
188 RNA samples were rendered DNA free by incubation with Turbo-DNA free reagent
189 (Ambion, Austin, TX). cDNA synthesis was carried out as described by Moran et al.
190 (15). Primers used in this study are listed in Table S2 and were designed using Primer

191 Express software v1.5 (Applied Biosystems, Foster City, CA). These primers yielded
192 single, specific amplimers from genomic DNA and cDNA templates. Primer pairs for
193 *UME6* and *NRG1* were selected that yielded similar amplification efficiencies as the
194 *TEF1* primer pair against a serial dilution of template DNA. Real-time detection of
195 amplimers was carried out using the *Power* SYBR® Green PCR Master Mix (Applied
196 Biosystems, Foster City, CA) and the ABI 7500 sequence detector, performing
197 separate reactions for each gene. Gene expression levels were normalized against the
198 expression levels of the constitutively expressed *TEF1* gene in the same cDNA
199 sample.

200

201 *Epithelial adhesion and invasion studies*

202 Adherence of *Candida* strains to the oral epithelial cell line TR146 was determined
203 using the assay of Rotrosen et al. (19). Monolayers of TR146 cells were cultured in 6-
204 well tissue culture dishes in complete medium (CM), which consisted of Dulbecco's
205 modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100
206 units/ml) and streptomycin (100 µg/ml). A suspension of 2 x 10^2 yeast cells per ml
207 was prepared in CM and 1 ml was added to triplicate wells and incubated at 37˚C, 5%
208 (v/v) CO_2 for the indicated time periods. The same suspension was also plated on
209 YPD agar to enumerate CFU in the starting inoculum. Following incubation, non-
210 adherent cells were removed from the monolayer by washing with 10 ml PBS. The
211 monolayer was then overlaid with 2 ml YPD agar and incubated at 37˚C overnight.
212 The number of colonies present on the monolayers relative to the starting inoculum
213 was determined and expressed as percentage adherence. Statistical analysis of the data
214 was performed using ANOVA in Prism 4.0 (GraphPad Software).

215 Invasion of reconstituted human oral epithelial (RHE) tissue of TR146 cells was

216 determined using RHE tissues purchased from Skinethic Laboratories (Nice, France)
217 and used as described previously (22, 25). The release of lactate dehydrogenase
218 (LDH) from epithelial cells into the cell-culture medium was measured to quantify the
219 extent of epithelial cell damage using the CytoTox 96® non-radioactive cytotoxicity
220 assay (Promega Corp., Madison, WI) as described by Moran et al. (15)
221

222 *Macrophage cell culture and infection with* Candida
223 Infection of the murine macrophage-like cell line RAW264.7 with *Candida* isolates
224 was carried out as described by Moran et al. (15). Evaluation of yeast cell
225 proliferation in co-culture with macrophages was assessed after 18 h incubation using
226 an XTT dye reduction assay (Sigma-Aldrich), also described by Moran et al. (15).
227

228 **Results**

229 *Effect of nutrient concentration on hypha formation in* C. dubliniensis.

230 Previous studies have examined the transcript profile of *C. albicans* hyphae when
231 induced in YPD supplemented with 10% (v/v) fetal calf serum (YPDS) at 37˚C (10,
232 16). In this study, we wished to compare the transcript profile of *C. dubliniensis*
233 Wü284 hyphae induced in YPDS. However, preliminary experiments demonstrated
234 that *C. dubliniensis* did not produce sufficient numbers of true hyphae under these
235 conditions over a period of 5 hours (Fig. 1a). This differential filamentation
236 phenotype was confirmed with an additional 11 *C. dubliniensis* isolates and 5 *C.*
237 *albicans* isolates (Fig. 1d). On average, 21% (range 9 to 43%) of yeast cells in *C.*
238 *dubliniensis* YPDS cultures produced germ-tubes or filaments following 2 h
239 incubation (Fig. 1d). In contrast, 80% (range 61 to 95%) of cells in *C. albicans*

240 cultures produced germ-tubes or filaments under the same conditions (Fig. 1d).
241 Previous studies by Stokes et al. (25) demonstrated that water supplemented with 10%
242 fetal calf serum (WS) was a more potent inducer of *C. dubliniensis* hyphae. Under
243 these conditions, *C. dubliniensis* Wü284 was approximately 90% hyphal after 3 h
244 incubation (Fig. 1b). Eleven additional *C. dubliniensis* isolates exhibited significantly
245 increased rates of filamentation in WS compared to YPDS, whereas the rate of
246 filamentation in 6 *C. albicans* isolates was similar in both media (Fig. 1d). Induction
247 of hypha-specific gene expression was examined by observing induction of yEGFP
248 expression from the *CaECE1* promoter in both species. *C. albicans* produced
249 fluorescent hyphae in WS and YPDS, whereas cells of *C. dubliniensis* only produced
250 fluorescence in WS (Fig. 1c).

251 These data suggest that efficient filamentation in *C. dubliniensis* requires nutrient
252 depletion. We investigated whether the addition of nutrients present in YPD medium
253 such as glucose or peptone to *C. dubliniensis* incubated in WS could inhibit
254 filamentation. The addition of 2% (w/v) glucose to WS cultures had no significant
255 effect on the rate of filamentation of *C. dubliniensis* Wü284 (Fig. 1e). However, a
256 reduction in filamentation was observed upon the addition of 2% (w/v) peptone and a
257 greater effect was observed when WS was supplemented with both glucose and
258 peptone (Fig. 1e).

259

260 *Preculture in Lee's medium pH 4.5 enhances filamentation in* C. dubliniensis.

261 We investigated whether preculture in Lee's medium, a peptone free synthetic
262 medium, could affect subsequent filamentation of *C. dubliniensis* in YPDS. Cells
263 precultured in Lee's medium (pH 4.5) at 30˚C showed a greater capacity to form true

264 hyphae compared to cells precultured in YPD (pH 5.6), also at 30˚C (Fig. 2a).
265 Following preculture in Lee's medium approximately 56% of cells were observed to
266 produce germ-tubes (Fig. 2a). However, budding growth resumed after several hours
267 incubation, indicating that Lee's medium preculture alone could not maintain hyphal
268 elongation under these conditions (Fig. 2a). We examined whether the pH shift, the
269 temperature shift or the nutrient composition of Lee's medium was responsible for
270 this phenotype. Preculture in Lee's medium at 37˚C or in Lee's medium buffered to
271 pH 7.2 could inhibit filamentation in strain Wü284, indicating that a pH and
272 temperature shift was required (Fig. 2b). However, we also showed that addition of
273 1% peptone to Lee's medium could also inhibit subsequent filamentation by Wü284
274 in YPDS, indicating that the medium composition also played a role (Fig. 2b). In *C.*
275 *albicans* SC5314, the addition of peptone (1%) to the Lee's preculture medium could
276 not inhibit filamention in YPDS (Fig. 2b), whereas preculture of *C. albicans* at 37˚C
277 in Lee's medium increased the numbers of pseudohyphae relative to true hyphae (Fig
278 2b).

279 Lee's medium preculture enhanced filamentation in 10 of 12 additional *C.*
280 *dubliniensis* isolates examined, exhibiting an average rate of filamentation of 48%
281 following 2 h incubation in YPDS (Fig. 1d). Analysis of six independent *C. albicans*
282 isolates showed that Lee's medium preculture also enhanced filamentation in YPDS
283 by approximatley 10% in these isolates relative to cells precultured in YPD (Fig. 1d).

284 *Regulation of* UME6 *and* NRG1 *transcription*

285 Previous studies have shown that in *C. albicans*, filamentation in YPDS is associated
286 with down regulation of *NRG1* transcript levels and increased expression of *UME6*
287 (15). Examination of *NRG1* transcript levels in *C. dubliniensis* in YPDS demonstrated

288 that *NRG1* transcript levels increased following 1 h incubation in YPDS at 37˚C (Fig.
289 3a). However, inoculation of cells precultured in Lee's medium resulted in a transient
290 drop in *NRG1* transcript levels by approximately 50% following 1 h (Fig 3a).
291 Inoculation of *C. dubliniensis* in WS yielded a 70% decrease in *NRG1* transcript
292 levels by 3 h (Fig. 3a), similar to those observed during filamentration of *C. albicans*
293 in YPDS (data not shown). Analysis of *UME6* transcript levels in *C. dubliniensis* in
294 YPDS revealed no significant change (Fig 3b). However, when cells were precultured
295 in Lee's medium (pH 4.5), we observed a ~30-fold increase in *UME6* expression in
296 YPDS (Fig 3b). In addition, we observed >100-fold induction of *UME6* in *C.*
297 *dubliniensis* following inuculation in WS medium (Fig 3b).

298 Addition of peptone to WS cultures showed that peptone could decrease *UME6*
299 expression in *C. dubliniensis* in a concentration dependent manner, with 2% (w/v)
300 peptone reducing *UME6* expression by approximately 80%. Glucose (2% w/v) alone
301 did not significantly decrease *UME6* expression, although the combination of glucose
302 plus peptone had an additive effect on *UME6* expression.

303 *Overexpression of* UME6 *enhances filamentation in* C. dubliniensis

304 We further investigated the roles of *NRG1* and *UME6* in hypha formation in the *C.*
305 *dubliniensis nrg1*Δ mutant CDM10. Previously, we have shown that the *nrg1*Δ strain,
306 unlike wild-type, forms hyphae in response to CO_2 and filaments more rapidly in
307 response to serum in water (15). In this study, a derivative of CDM10 harboring a
308 P_{ECE1}-*GFP* promoter fusion (M10EGFP) formed elongated filaments in YPDS,
309 however these filaments posessed the characteristic constrictions of pseudohyphae
310 (Fig. 4a). Strain M10EGFP was weakly fluorescent in YPD and YPDS (Fig. 4a),
311 whereas in WS the same strain emitted strong fluorescence and formed masses of true

312 hyphae (Fig. 4a). We tested whether overexpresion of *UME6* from a doxycycline
313 inducible promoter could enhance true hypha production by CDM10 in YPDS.
314 Addition of 20 µg/ml doxycycline promoted conversion of pseudohyphae to true
315 hyphae in this strain (Fig 4b). Similary, introduction of the same construct in the
316 parent isolate Wü284 could promote the formation of true hyphae in YPDS medium
317 (Fig. 4b).

318 We also tested whether constitutive *NRG1* expression from the doxycycline inducible
319 promoter could prevent filamentation. Constitutive expression of *NRG1* in Wü284
320 and CDM10 could block pseudohypha formation in YPDS. However, expression of
321 *NRG1* from this promoter was not sufficient to block true hypha formation in WS
322 (data not shown).

323 *Transcript profiling of* C. dubliniensis *in serum*

324 This study has shown that under nutrient depleted conditions, *C. dubliniensis* can
325 form hyphae as effectively as *C. albicans*. In order to determine whether *C.*
326 *dubliniensis* hyphae can express the same range of virulence-associated factors as *C.*
327 *albicans* hyphae, we carried out whole genome transcript profiling of *C. dubliniensis*
328 during growth in WS medium. Samples were analysed at 1h, 3h and 5h post
329 inoculation in WS. Within 1 h, we observed a 2.5 fold or greater change in
330 transcription in 1095 genes relative to preculture cells (*t* test $p < 0.015$; Table S3). This
331 corresponds to 18% of the genome. Analysis of the up-regulated genes (n=526) for
332 significant shared GO terms identified large groups of genes associated with transport
333 (102 genes), organelle organization (73), the cell cycle (44) and translation (43) (Fig.
334 5a). Many of these genes were associated with processes known to be involved in
335 hyphal development, such as the assembly of actin cables (*TPM2, ARF3, MEA1,*

336 *ARP9* and *YEL1*), Spitzenkörper assembly (*MLC1*), and GTPases with roles in actin
337 organisation (*RSR1, RAC1*, *RDI1* and *RHO3;* Fig. S1). These data also highlighted
338 some processes not previously associated with hypha formation, such as down
339 regulation of vacuolar metabolism, including vacuolar protein catabolysis (8/10
340 annotated genes, Fig. S1), suggesting a shut down in autophagic processes. However,
341 increased expression of genes with roles in vacuolar biogenesis and inheritance was
342 also observed (*VAM3, YPT7, YPT72* and *YKT6;* Fig. S1). Reorganisation of
343 membrane lipid structure was indicated by a significant decrease in sphingolipid
344 metabolism (9/25 annotated genes, Fig. S1). Reorganisation of the cell surface was
345 indicated by an increase in expression of genes associated with GPI anchor
346 biosynthesis (*DPM1, MCD4, orf19.538*) and glycosylation (*PMI1, PMT2, PMT5,*
347 *ALG5, ALG6, ALG7, GFA1, DPM1, orf19.2298, orf19.7426*).

348 Within 1 h, significant up-regulation of *RAS1*, an upstream regulatory element of the
349 cAMP-PKA pathway was detected (Fig 5b). Regulation of several transcriptional
350 regulators of filamentous growth was also observed, including *EFH1, TEC1* and
351 *UME6* (Fig. 5b). Induction of the pH regulator *RIM101* was also observed. Down
352 regulation of *EFG1* and the transcriptional repressor *NRG1* was also observed by 1 h
353 (Fig 5b). We also observed increased expression of Cd36_54430, the putative
354 orthologue of *CaSFL2*, a novel regulator of hypha production that we have previously
355 shown to be uniquely expressed by *C. albicans* during infection of oral epithelial
356 tissues in vitro (Fig. 5b) (23).

357 By 3 hours, approximately 90% of cells in WS produced true hyphae. At this time
358 point, 345 genes exhibited a >2.5 fold induction and 348 exhibited a >2.5 fold
359 decrease in expression, relative to the preculture cells (*t* test $p \leq 0.015$; Table S4). A
360 significant proportion of those genes upregulated at three hours were orthologous to

361 *C. albicans* genes annotated with the GO term 'pathogenesis' (n=20, $p \leq 0.044$)
362 including the secreted proteinase *SAP7* and *CdSAP456,* the single *C. dubliniensis*
363 orthologue of *C. albicans SAP4, 5* and *6* genes (Fig. 5c). We also observed induction
364 of the predicted GPI-anchored proteins *SOD5, HWP1* and *ALS1* and down regulation
365 of the orthologues of *ALS4*, *ALS9* and *RBT5* (Fig 5c).

366

367 *Environmental regulation of gene expression in* C. dubliniensis

368 In order to understand how different environmental stimuli shaped the transcriptional
369 response to growth in 10% serum, we also analysed the transcript profile of *C.*
370 *dubliniensis* following a change in cell density, a shift to 37°C, nutrient depletion
371 (10% v/v Lee's medium) or a shift to alkaline pH (pH 7.2). None of these conditions
372 alone could induce morphogenesis in *C. dubliniensis*. Although both *UME6* and
373 *NRG1* exhibited regulation under the conditions examined, the changes did not reach
374 the levels seen in WS cultures, indicating perhaps that multiple environmental signals
375 are required to alter their expression sufficiently to allow filamentation to proceed
376 (Fig. S2). We carried out ANOVA to identify differentially regulated transcripts ($p \leq$
377 0.05) and visualised the results using hierarchical clustering. From this analysis we
378 could identify two large clusters of genes regulated by changes in cell density (Fig
379 6a). Cluster I genes (n=163) were induced in all experiments involving a change in
380 cell density and were significantly enriched for genes encoding ribosomal subunits or
381 proteins involved in ribosome biogenesis (Fig. 6b). Cluster IV (n=167) included genes
382 down regulated by cell density changes and was significantly enriched for genes
383 involved in glycolysis and trehalose biosynthesis (Fig. 6b). Three clusters of serum-
384 specific genes could also be identified (clusters II, III and V; Fig. 6a) and these were

385 largely involved in metabolism of alternative carbon sources (*ECI1, ICL1, PXP2;* Fig.
386 6c) and nutrient transport (e.g. *HGT1, JEN1*; Fig 6c). These data show that growth in
387 WS resulted in a switch from carbohydrate catabolism to fatty acid oxidation and the
388 glyoxylate cycle for energy production (Fig. S3). Smaller clusters of genes were
389 identified that were induced by alkaline pH or relief of *NRG1* repression (Fig. 6d and
390 6e). The regulation of genes in response to nutrient depleted Lee's medium and the
391 temperature shift was more complex (Fig. 6f and 6g). Some transcripts exhibited clear
392 temperature induction (*MET14, CEK2*; Fig. 6f) or nutrient depletion induction (*GAP2,*
393 *MNN4*; Fig. 6g). Other transcripts responded to several conditions (e.g. *HGT12* was
394 *NRG1* repressed and nutrient regulated whereas *CFL11* was induced by both
395 temperature and pH).

396

397 *Comparison of the C. albicans and C. dubliniensis hypha-regulated gene set*s

398 We compared the list of *C. dubliniensis* hypha-expressed genes (Table S3) with a list
399 of genes regulated during hypha formation in serum by *C. albicans* (see material and
400 methods). We identified a core set of 65 hypha-induced genes in both species (Table
401 1). Sixty-seven genes were found to be downregulated by both species (Table 2). This
402 analysis could identify common sets of cell surface, stress response and regulatory
403 genes induced or repressed in hyphae of both species. The specific transcriptional
404 response of *C. dubliniensis* to WS was largely associated with the nutrient poor
405 conditions used and included genes genes of the glyoxylate cycle and fatty acid beta-
406 oxidation (Fig S3, Fig 6c). Increased expression of several species-specific
407 hypothetical genes in *C. dubliniensis* could also be detected (Cd36_41370,

408 Cd36_63200, Cd36_65070) as well as down regulation of a putative glutamate
409 decarboxylase (Cd36_10760) and a predicted orf (Cd36_34790).

410 The specific response of *C. albicans* included several predicted GPI-anchored
411 proteins, including *RBT4*, *PGA54* and *PGA55* (Table S5). Nine of the *C. albicans*-
412 specific genes had no direct orthologue in *C. dubliniensis* (i.e. genes without Blast
413 matches in *C. dubliniensis*, or where the top Blast hit in *C. dubliniensis* was not
414 reciprocal). These included *EED1, SAP4, SAP5, ALS3* and *HYR1* and several
415 members of the *C. albicans* telomeric *TLO* gene family (Table 3). The transcriptional
416 regulator *BCR1* was also induced in *C. albicans*, which may contribute to the
417 concomitant upregulation of the *BCR1* regulated genes *HYR1, ALS3*, *GCN1* and
418 orf19.6079.

419

420 *Can stimulation of hypha formation in* C. dubliniensis *result in tissue damage?*

421 We wished to determine whether induction of hyphae can increase the invasive
422 potential of *C. dubliniensis* using simple infection models. Previous studies have
423 demonstrated that *C. dubliniensis*, in contrast to *C. albicans*, does not invade a
424 reconstituted oral epithelium tissue model when precultured in YPD medium at 37˚C
425 (15, 23, 25). These findings were confirmed here when *C. dubliniensis* Wü284 was
426 inoculated on the surface of RHE cultures following preculture in YPD at 37˚C. Cells
427 grown under these conditions remained exclusively in the yeast phase and attached
428 poorly to the surface of the tissue. Penetration of the tissue by filaments did not occur
429 (Fig. 7a). In contrast, when *C. dubliniensis* cells precultured in Lee's medium pH 4.5
430 at 30˚C were inoculated on the tissue, we observed a mixture of morphologies (yeasts,
431 pseudohyphae and some true hyphae) and the cells adhered more closely to the

432 surface of the epithelial tissue (Fig. 7b). In addition, localised invasion was observed
433 by hyphae and pseudohyphae at 24 h post infection (Fig 7b, 7c). When a quantitative
434 assessment of epithelial damage was used by measuring the release of lactate
435 dehydrogenase from epithelial cells, we observed a significant increase in damage
436 caused by cultures incubated at 30˚C in Lee's pH 4.5 compared to YPD grown
437 cultures (Fig. 7d). Increased cell damage was also recorded in RHE infections with *C.*
438 *dubliniensis* strain CD36 following preculture in Lee's medium (7.0 +/- 0.6 LDH U/l)
439 relative to YPD (5.2 +/- 0.2 LDH U/l).

440 These data suggest that the difference in tissue damage and invasion elicited by YPD
441 medium and Lee's medium grown *C. dubliniensis* cells may be due to differences in
442 adherence. We carried out a more detailed investigation of the adhesion of *C.*
443 *dubliniensis* to TR146 cell monolayers over 90 min. Within 30 min of inoculation, 10-
444 20% of yeast cells had adhered to the monolayer (Fig. 7e). Adherence of *C. albicans*
445 SC5314 increased by 60 min, and this was independent of preculture conditions and
446 corresponded with germ tube formation by *C. albicans* (Fig. 7e) In contrast, only *C.*
447 *dubliniensis* cells precultured in Lee's medium at 30˚C exhibited an increase
448 adherence over time (Fig 7e). The difference in adherence at 90 min was highly
449 significant ($p < 0.01$, 2-way ANOVA). An additional *C. dubliniensis* strain CD36 was
450 also shown to exhibit increased adhesion to TR146 monolayers following preculture
451 in Lee's medium (Fig. S4). In additional experiments, we altered the preculture
452 conditions in order to determine the role of the temperature shift, the pH shift and the
453 nutrient composition of Lee's medium in this phenotype (Fig 7f). Preculture at 37˚C
454 or at pH 7.2 reduced adhesion by 48% and 35%, respectively (Fig. 7f). The addition
455 of 1% (w/v) peptone to the preculture medium also significantly reduced adhesion by

456 43% ($p < 0.05$, ANOVA). Preculture at 37˚C with peptone did not have any
457 significant additive effect on adhesion ($p > 0.05$).

458 In addition, we have previously observed that *C. dubliniensis* is engulfed and killed
459 more efficiently than *C. albicans* by RAW264.7 murine macrophages (15). This
460 phenotype was associated with the inability of *C. dubliniensis* to filament and destroy
461 the macrophage. However, preculture of *C. dubliniensis* in Lee's medium at pH 4.5 at
462 30˚C lead to an increase in the rate of filamentation following phagocytosis by murine
463 macrophages compared to YPD 37˚C grown cells (Fig 8). Assessment of candidal
464 growth in co-culture with the macrophage cells demonstrated that Lee's pH 4.5 grown
465 cells could proliferate to a significantly greater level compared to YPD grown cells
466 (Fig. 8a). No difference in proliferation was noted with *C. albicans* cultures pregrown
467 in YPD at 37˚C or Lee's medium grown at 30˚C (data not shown).

468

469 **Discussion**

470 In our attempts to generate a hyphal transcript profile for *C. dubliniensis*, we initially
471 encountered problems in inducing ~100% hyphal growth in liquid medium with this
472 species. This led us to carry out a thorough investigation of the environmental
473 conditions that favour the yeast to hypha transition in *C. dubliniensis* in liquid media.
474 Nutrient depletion was found to be the most important requirement for filamentation
475 of *C. dubliniensis* in liquid media. Highly efficient filamentation was observed in *C.*
476 *dubliniensis* when a nutrient poor inducing medium (water plus 10% v/v FCS) was
477 used and this could be suppressed by the addition of peptone and glucose. Although
478 nutrient limitation has been shown to induce hypha formation in *C. albicans* in liquid
479 and solid medium, this species still filaments efficiently in nutrient rich YPD in the

480 presence of a shift to alkaline pH at 37˚C (4). In *C. dubliniensis*, a shift from YPD
481 medium to nutrient rich YPDS (pH ~7.5) could not induce significant morphological
482 changes. However, filamentation of *C. dubliniensis* was partly induced in YPDS when
483 this species was precultured in synthetic Lee's medium. This Lee's medium induction
484 could also be suppressed by the addition of 1% peptone to the preculture medium.
485 These data indicate that nutrient sensing mechanisms, specifically those that sense
486 complex mixtures of peptides, may somehow suppress pH and temperature-induced
487 filamentation in *C. dubliniensis.* We have shown that the mechanism of inhibition
488 involves suppression of *UME6* induction. Addition of peptone to WS medium could
489 inhibit filamentation in *C. dubliniensis* and suppressed *UME6* induction in a
490 concentration-dependent manner. We also observed induction of *NRG1* transcription
491 in *C. dubliniensis* following inoculation in YPDS and this may play also a significant
492 role in preventing filamentation in this medium as Saville et al. (21) have shown that
493 induced *NRG1* transcription can prevent filamentation in YPDS by *C. albicans*. In
494 previous studies we have hypothesized that the lack of filamentation observed in *C.*
495 *dubliniensis* in certain media may be due to lack of *NRG1* down regulation (15).
496 However in the present study, examination of the *nrg1*Δ mutant in YPDS showed that
497 although removal of Nrg1 repression could enhance filamentation in this medium, the
498 mutant still exhibited pseudohyphal characteristics and only exhibited moderate
499 fluorescence from a P_{ECE1}-*GFP* fusion, suggesting an additional mechanism of
500 nutrient repression (Fig 3a). Induction of *UME6* expression from a doxycycline
501 inducible promoter promoted true hypha formation in the *nrg1*Δ mutant in YPDS
502 (Fig. 3b). In addition, overexpression of *UME6* in the wild type strain could also
503 induce filamentation in YPDS, indicating that differential expression of *UME6* may
504 be the key reason for reduced filamentation of *C. dubliniensis* in these media. In *C.*

505 *albicans*, it has been shown that *UME6* may also play a role in suppressing *NRG1*
506 transcription during filamentation, and the differential expression of *NRG1* observed
507 in *C. dubliniensis* may also be *UME6* dependent (2, 6). Unexpectedly, constitutive
508 expression of *NRG1* from the doxycycline-inducibe promoter could not prevent hypha
509 formation in WS medium, suggesting that high level *UME6* expression may also
510 affect *NRG1* function post-transcriptionally.

511 To further examine the response of *C. dubliniensis* a nutrient poor medium, we
512 examined the transcript profile of Wü248 grown in 10% (v/v) Lee's medium. Nutrient
513 depletion induced expression of genes involved in amino acid, carbohydrate and iron
514 uptake (*GAP2, HGT12* and *FET3*). In *C. albicans*, expression of the hexose
515 transporter *HGT12* is induced by glucose limitation, and the glucose sensor Hgt4
516 mediates this induction (5). In addition, *HGT4* is required for filamentation under
517 some conditions (spider medium) in *C. albicans* (5). However, low glucose
518 stimulation is not essential for filamentation in *C. albicans*, as *HGT4* mutants form
519 filaments normally in glucose rich YPDS (5). It is also unlikely that *HGT4* signalling
520 is required for filamentation of *C. dubliniensis* in WS, as addition of 2% glucose to
521 WS medium did not significantly inhibit filamentation in *C. dubliniensis*. Nutrient
522 depletion did not induce any other obvious transcriptional changes associated with
523 filamentous growth in *C. dubliniensis*, indicating that any additional effects of
524 nutrient depletion on filamentation may be post transcriptional. Repression of
525 filamentation was most apparent when cells were exposed to a complex mixture of
526 carbohydrate and peptides, indicating that a general nutrient sensing mechanism may
527 be involved. Interestingly it has recently been shown in *C. albicans* that an orthologue
528 of the general nutrient sensor Tor1 can modulate *NRG1* expression in spider medium
529 (3). In addition, it has also been shown that a *C. albicans MDS3* mutant can only form

530 hyphae in the presence of the Tor1 inhibitor rapamycin (29). We are currently
531 assessing whether the *C. dubliniensis* Tor1 could play novel role in nutrient sensing
532 and filamentation.

533 The transcript profiling data presented here also indicate important roles for pH,
534 temperature and cell density changes in activating the transcription of hypha-specific
535 genes in *C. dubliniensis*. The transcript profiling data presented here show a key role
536 for the pH response in activating the filamentous growth regulators *SFL2, UME6,*
537 *TEC1* and *RIM101 (4, 6)*. *UME6* was also found to be *NRG1* repressed whereas *TEC1*
538 also exhibited induction due to cell density changes. Temperature changes also
539 induced *EFH1* and *CPH1*. These data show that induction of filamentation under the
540 conditions examined in *C. dubliniensis* involves multiple environmental signals.

541 The microarray data presented here highlighted some novel processes regulated
542 during filamentation in *C. dubliniensis* as well as identifying a strong core
543 transcriptional response shared with *C. albicans*. The data show rapid induction of
544 genes involved in regulating polarised growth, including genes involved in actin
545 polymerisation, vesicle transport and septin formation. The data also provide evidence
546 for processes not previously described during the morphological switch. This includes
547 evidence for changes in lipid composition, with a shutdown in transcription of genes
548 involved in sphingolipid synthesis and an increase in fatty acid biosynthesis gene
549 expression. Changes in vacuole function are also indicated with an increase in
550 expression of genes involved in vacuolar biogenesis and inheritance, and decreases in
551 expression of vacuolar proteases, suggesting that the vacuole plays a structural rather
552 than metabolic role in hyphae. Comparison of this transcript profile with previously
553 published studies of gene expression in *C. albicans* allowed us to identify a core
554 transcriptional response to filamentation in both species, consisting of 132 genes

555 regulated 2-fold or greater (10, 16). This strongly conserved core response supports
556 the hypothesis that a specific programme of transcriptional changes may be essential
557 for filamentation to proceed in both species, in addition to post-transcriptional events.
558 Induction of several secreted and cell wall-associated proteins was specific to *C.*
559 *albicans* under the conditions examined, including *RBT4*, *PGA54* and *PGA55*. Several
560 species-specific genes were also induced in *C. albicans* including *HYR1*, *ALS3* and
561 *EED1*. *C. albicans* expresses three *SAP* genes, *SAP4,5* and *6* during filamentation,
562 whereas *C. dubliniensis* possesses only one orthologue of these genes, termed
563 *CdSAP456*, which is also induced during hyphal growth (9, 20). However, SAP
564 activity in *C. dubliniensis* may be supplemented by *SAP7* expression, which exhibited
565 an 8-fold increase in expression. *C. albicans* also expresses the putative invasin *ALS3*
566 (18). However, we did not identify any compensatory expression of *ALS* genes in *C.*
567 *dubliniensis*, although orthologues of *C. albicans ALS2, 4* and *9* all exhibited
568 decreased expression during hyphal growth.

569 Overall, transcript profiling revealed that *C. dubliniensis* hyphae express a number of
570 genes associated with virulence, suggesting that induction of filamentation in *C.*
571 *dubliniensis* could promote tissue invasion. Recently, Spiering *et al.* concluded that
572 the reduced virulence of *C. dubliniensis* in the RHE model was a result of a failure to
573 initiate filamentation and the specific transcriptional programme associated with this
574 (23). In the present study we have shown that induction of *UME6* expression in *C.*
575 *dubliniensis* by preculturing in Lee's medium at 30˚C could enhance filamentation in
576 the RHE model. This resulted in greater attachment of *C. dubliniensis* cells to the
577 tissue surface and localised invasion of the epithelium. We have never previously
578 identified RHE invasion in a wild-type strain of *C. dubliniensis* (15, 23, 25).
579 Examination of adhesion of *C. dubliniensis* to TR146 monolayers demonstrated that

580 this adherent phenotype could be partly inhibited by the addition of peptone to the
581 preculture medium, as well as by removing the pH or temperature shift. However, the
582 level of damage to the RHE tissues was still significantly lower than that routinely
583 observed when tissues are infected with *C. albicans*. There may be several reasons for
584 this; firstly the transition following Lee's preculture is largely short-lived and by 24 h
585 most cells have reverted to budding growth. Secondly, although *C. dubliniensis* can
586 be induced to form hyphae, the absence of several *C. albicans*-specific hypha-
587 associated genes (*ALS3, SAP5*, *HYR1*, *EED1*) may also attenuate the virulence of this
588 species (18, 20, 30). Studies are currently underway to determine if these genetic
589 differences are crucial to the greater pathogenicity of *C. albicans*.

590 Finally, this study suggests that the ability of *C. albicans* to form filaments at alkaline
591 pH, irrespective of nutrient availability, may enable it to colonise and infect a wider
592 range of niches relative to *C. dubliniensis*. *C. dubliniensis* may have lost or perhaps
593 failed to acquire this morphological flexibility since the divergence of the two species.
594 The genome sequence of *C. dubliniensis* suggests that due to gene loss and
595 pseudogenization, *C. dubliniensis* may be undergoing niche specialization. It may be
596 possible that reduced filamentation is part of this specialization process and may even
597 be of benefit to *C. dubliniensis* in certain niches, particularly where tissue damage,
598 inflammation and attraction of the of host's defences is unfavourable.

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759 **Table 1.** Selected genes[a] commonly up-regulated during hypha formation in response
760 to serum in *C. albicans* and *C. dubliniensis.*

Category	GeneDB ID	CGD ID	Fold change[b]		Name	Description
			Ca	Cd		
Cell surface/secreted	Cd36_43360	orf19.1321	71	48	*HWP1*	Hyphal wall protein
	Cd36_52240	orf19.4255	5.9	4.8	*ECM331*	GPI-anchored protein
	Cd36_64370	orf19.5760	4.3	5.0	*IHD1*	GPI-anchored protein
	Cd36_63420	orf19.5542	75	28*	*SAP456*	Secreted aspartyl proteinase
	Cd36_43260	orf19.3374	87	52	*ECE1*	Secreted cell elongation protein
	Cd36_44230	orf19.3829	7.7	8.8	*PHR1*	GPI-anchored protein
Stress response	Cd36_60850	orf19.85	9.8	9.1	*GPX1*	Glutathione peroxidase
	Cd36_15620	orf19.2060	11.2	15.9	*SOD5*	Copper-zinc superoxide dismutase
	Cd36_33470	orf19.3710	8.6	3.4*	*YHB5*	Protein related to flavohemoglobins
DNA replication	Cd36_23200	orf19.201	3.5	8.0	*CDC47*	DNA helicase
	Cd36_20640	orf19.5487	5.7	10.8	*CDC46*	Part of ARS replication complex
	Cd36_21620	orf19.1901	3.3	4.4	*MCM3*	Part of ARS replication complex
	Cd36_63950	orf19.5597	2.5	4.7	*POL5*	DNA polymerase V, 5-prime end
	Cd36_41670	orf19.4616	5.2	9.5	*POL30*	Accessory for DNA polymerase delta
Cytoskeleton	Cd36_03010	orf19.3013	5	4.7	*CDC12*	Septin
	Cd36_29930	orf19.548	2.5	2.9	*CDC10*	Septin
	Cd36_11250	orf19.5265	9.5	9.7	*KIP4*	Kinesin heavy chain homolog
GTPases	Cd36_81390	orf19.1702	14.8	2.8	*ARF3*	GTP-binding ADP-ribosylation
	Cd36_18700	orf19.815	2.7	2.8*	*DCK1*	DOCK180 protein
	Cd36_71380	orf19.6573	5.3	3.8	*BEM2*	Bud-emergence protein
	Cd36_24270	orf19.1760	2.6	2.2	*RAS1*	Small monomeric GTPase
	Cd36_84970	orf19.5968	3.4	2.8*	*RDI1*	Rho GDP dissociation inhibitor
	Cd36_73140	orf19.6705	7.2	11	*YEL1*	Conserved hypothetical protein
Secretion	Cd36_86230	orf19.7409	3.3	2.1	*ERV25*	Component of ER- derived vesicles
	Cd36_40670	orf19.4181	4.2	3.3	*SPC2*	Subunit of signal peptidase complex
	Cd36_72140	orf19.6476	3.0	3.8	*AVL9*	Conserved Golgi protein
	Cd36_51450	orf19.586	3.9	2.1	*ERV46*	Component of ER- derived vesicles
Glycosylation	Cd36_07530	orf19.5073	4.3	2.3	*DPM1*	Dolichol-P-mannose synthesis
	Cd36_60365	orf19.1203.	9.0	3.3	*DPM2*	Regulator of dolichol-P-mannose
	Cd36_32420	orf19.1843	2.3	2.3	*ALG6*	Glucosyltransferase
	Cd36_02340	orf19.2937	8.4	5.5	*PMM1*	Phosphomannomutase
	Cd36_23720	orf19.1390	3.9	3.7	*PMI1*	Mannose-6-phosphate isomerase
Transcription factors	Cd36_81290	orf19.1715	5.5	4.9	*IRO1*	Transcription factor
	Cd36_01290	orf19.3328	3.9	2.0	*HOT1*	Osmostress transcription factor
	Cd36_05880	orf19.1822	21	4.3	*UME6*	Regulator filamentation
Kinases/phosphatases	Cd36_08920	orf19.4809	4.3	4.2	*ERG12*	Mevalonate kinase
	Cd36_40980	orf19.4698	2.6	4.7	*PTC8*	Serine/threonine phosphatase
	Cd36_42970	orf19.2678	3.3	2.8	*BUB1*	Protein kinase in mitosis checkpoint

761 [a] Excluding ribosomal proteins

[b] Refers to expression relative to yeast cells. Ca refers to *C. albicans* and Cd refers to *C. dubliniensis*. *C. albicans* values are taken from the data of Kadosh and Johnson (10, 16) except those marked * taken from Nantel et al. (16).

792 **Table 2.** Genes commonly down-regulated during hypha formation in response to
793 serum in *C. albicans* and *C. dubliniensis*

Category	**GeneDB ID**	**CGD ID**	**Fold Change**[a]		**Common**	**Description**
			Cd	**Ca**		
Cell surface	Cd36_64800	orf19.1097	7.1	14.3	*CdALS21*	Agglutinin-like sequence protein
	Cd36_65010	orf19.1097	10.0	14.3	*CdALS22*	Agglutinin-like sequence protein
	Cd36_64610	orf19.4555	6.7	7.1	*ALS4*	Agglutinin-like sequence protein
	Cd36_26450	orf19.2531	2.5	5.0	*CSP37*	Cell surface protein
	Cd36_51670	orf19.575	3.7	3.8	*HYR5*	Similar to *HYR1*
	Cd36_29770	orf19.532	3.8	3.7	*RBR2*	Hypothetical protein
	Cd36_43810	orf19.5305	5.0	12.5	*RHD3*	Conserved protein reressed in hyphal
	Cd36_22720	orf19.3618	10.0	80.0	*YWP1*	Putative cell wall protein
	Cd36_23050	orf19.220	7.1	25.0	*PIR1*	Cell wall structural constituent with
Transport	Cd36_20820	orf19.23	10.0	4.0	*RTA3*	Putative transporter or flippase
	Cd36_28130	orf19.2425	2.4	5.3	*HGT18*	Putative glucose transporter
	Cd36_29200	orf19.473	2.6	1.7*	*TPO4*	Sperimidine transporter
	Cd36_27990	orf19.2849	2.0	33.3	*AQY1*	Aquaporin
	Cd36_27190	orf19.3749	4.5	2.0	*IFC3*	Peptide transporter
	Cd36_41090	orf19.4679	4.5	5.0	*AGP2*	Amino-acid permease
	Cd36_71860	orf19.6514	2.5	3.0	*CUP9*	Copper homeostasis
	Cd36_35530	orf19.7666	16.7	2.2	*SEO3*	Permease
	Cd36_83640	orf19.6956	5.3	5.9	*DAL9*	Allantoate permease
Mitochondrial	Cd36_17750	orf19.5805	12.5	5.9	*DLD3*	Mitochondrial D-lactate
	Cd36_41790	orf19.4602	4.2	7.1	*MDH1*	Mitochondrial malate dehydrogenase
	Cd36_01500	orf19.3353	6.3	9.1	*CIA30*	Possible complex I intermediate
	Cd36_60630	orf19.3656	2.0	2.0	*COX15*	Cytochrome oxidase assembly factor
Transcription	Cd36_84590	orf19.5924	2.5	5.6	*ZCF31*	Conserved hypothetical protein
	Cd36_12210	orf19.4941	2.4	1.7*	*TYE7*	Basic helix-loop-helix transcription
	Cd36_73890	orf19.7150	5.3	2.0	*NRG1*	Transcriptional repressor
	Cd36_06830	orf19.4438	14.3	33.3	*RME1*	Zinc-finger transcription factor
	Cd36_52720	orf19.4318	3.0	1.7	*MIG1*	Transcriptional regulator
Stress response	Cd36_80290	orf19.5437	2.3	1.7*	*RHR2*	DL-glycerol-3-phosphatase
	Cd36_01850	orf19.4526	33.3	100.0	*HSP30*	Plasma membrane heat shock protein
	Cd36_01930	orf19.3664	2.6	9.1	*HSP31*	Membrane heat shock protein
	Cd36_10070	orf19.2344	4.0	33.3	*ASR1*	Similar to heat shock proteins
Glutamate metabolism	Cd36_01650	orf19.4543	3.0	10.0	*UGA22*	Succinate-semialdehyde dehydrogenase
	Cd36_10950	orf19.1153	2.9	5.3	*GAD1*	Glutamate decarboxylase
	Cd36_45660	orf19.4716	7.1	2.0*	*GDH3*	NADP-glutamate dehydrogenase

794 [a] Refers to expression relative to yeast cells. Ca refers to *C. albicans* and Cd refers to
795 *C. dubliniensis*. *C. albicans* values are taken from the data of (10)except those marked
796 * taken from Nantel et al. (16).

797

798 **Table 3.** *C. albicans*-specific genes expressed during hyphal development.

orf19 number	Common Name	Description
orf19.5716	*SAP4*	Secreted aspartyl proteinase
orf19.5585	*SAP5*	Secreted aspartyl proteinase
orf19.4975	*HYR1*	Predicted GPI anchored cell wall protein
orf19.1816	*ALS3*	ALS family; role in epithelial adhesion, endothelial invasiveness
orf19.7561	*EED1*	Protein required for filamentous growth and for escape from epithelial cells
orf19.7544	*TLO1*	Member of a family of telomere-proximal genes
orf19.4054	*TLO12*	Member of a family of telomere-proximal genes
orf19.7127	*TLO16*	Member of a family of telomere-proximal genes
orf19.3074	*TLO10*	Member of a family of telomere-proximal genes

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811 **Figure Legends**

812 **Figure 1.** (a) Hypha formation in YPD plus 10% fetal calf serum (YPDS) by *C.*
813 *dubliniensis* Wü284 (grey lines) and *C. albicans* SC5314 (black lines) following
814 preculture in YPD at 30˚C (solid lines) or 37˚C (dashed lines). (b) Enhanced
815 filamentation of *C. dubliniensis* Wü284 in water plus 10% fetal calf serum (WS)
816 following preculture in YPD at 30˚C (solid black line), YPD at 37˚C (dashed black
817 line) or in Lee's medium pH 4.5 at 30˚C (grey line). Error bars correspond to standard
818 deviation in at least three replicate experiments. A sigmoidal curve was fitted to the
819 data for visualization using Prism 4.0 (GraphPad Software Inc.). (c) Examination of
820 induction of GFP expression from the hypha-specific *ECE1* promoter in *C. albicans*
821 and *C. dubliniensis* in YPDS and WS medium. (d) Average percent hypha formation
822 in *C. dubliniensis* (12 isolates) and *C. albicans* (6 isolates) at 37˚C in YPD plus 10%
823 serum (YPDS), in YPDS following preculture in Lee's pH 4.5 and in Water plus 10%
824 serum (WS). Error bars correspond to the standard error of the mean (SEM). (e)
825 Filamentation of *C. dubliniensis* in WS supplemented with 2% peptone, 2% glucose
826 or both peptone and glucose.

827

828 **Figure 2.** (a) Filamentation rate of strain Wü284 in YPDS at 37˚C following
829 preculture in YPD broth at 30˚C (grey line) or following preculture in Lee's pH 4.5 at
830 30˚C (black line). Error bars correspond to standard deviation in three replicate
831 experiments. (b) Photomicrographs showing typical morphology of *C. dubliniensis*
832 Wü284 and *C. albicans* SC5314 following 2 h incubation in YPD plus 10% (v/v) FCS
833 following preculture in Lee's medium. Cells were precultured for 24 h in modified

834 Lee's medium, buffered to pH 5.0 or 7.2 with 0.1 M potassium phosphate buffer, or
835 supplemented with 1% (w/v) peptone.

836 **Figure 3.** Real-time PCR analysis showing (a) relative levels of *NRG1* transcript and
837 (b) relative levels of *UME6* transcript in *C. dubliniensis* incubated in serum
838 containing medium. Expression levels were normalised to *TEF1* expression levels in
839 each sample. The solid grey line indicates expression in *C. dubliniensis* in WS
840 following preculture in Lee's medium, 30°C. The black line indicates expression
841 levels in *C. dubliniensis* in YPDS following preculture in Lee's medium 30°C and the
842 dashed grey line indicates expression levels in *C. dubliniensis* in YPDS following
843 preculture in YPD 30°C. Error bars represent standard deviation of results from three
844 replicate RNA preparations. In the case of *UME6* expression in WS, representative
845 data from one replicate is shown; additional experiments all showed >100-fold
846 induction at 1 h (c) Relative expression of *UME6* in *C. dubliniensis* in WS
847 supplemented with additional nutrients. Cells were precultured in YPD at 30°C and
848 inoculated in WS alone or supplemented with the indicated concentrations of peptone
849 or glucose. *UME6* expression levels were normalised to *TEF1* expression levels in the
850 same sample.

851 **Figure 4.** (a) Photomicrographs showing morphology of a derivative of the *C.*
852 *dubliniensis nrg1*Δ mutant harbouring a P_{ECE1}-*GFP* construct (CDM10). Top panel
853 shows morphology in YPD medium, YPDS and WS. Lower panel shows levels of
854 fluorescence expressed from the P_{ECE1}-*GFP* fusion under each condition. (b)
855 Morphology of CDM10 and Wü284 derivative strains harbouring plasmid pCaUME6,
856 containing the *UME6* gene under the control of a doxycycline inducibe promoter.

857 Morphology is shown following 3 h incubation in YPDS with or without 20 μg/ml
858 doxycycline.

859 **Figure 5**. (a) Graphical representation of the changes in expression in selected Gene
860 Ontology (GO) groups during filamentation in *C. dubliniensis*. The total number of
861 genes up or down regulated 2.5-fold in each group are shown at each time-point. (b)
862 Microarray expression of selected regulators of filamentous growth during hypha
863 formation in *C. dubliniensis* Wü284 in WS. Columns (left to right) for each gene
864 show expression levels relative to preculture cells at 1, 3 and 5 h post inoculation. (c)
865 Microarray expression of selected virulence-associated genes during hypha formation
866 in *C. dubliniensis* Wü284 in WS. Columns (left to right) for each gene show
867 expression levels relative to preculture cells at 1, 3 and 5 h post inoculation. Error
868 bars in (b) and (c) represent standard deviations from the mean generated in
869 Genespring GX from two distinct oligonucleotide probes per gene in four biological
870 replicate experiments.

871 **Figure 6.** (a) Hierarchical cluster analysis showing expression patterns of
872 differentially regulated genes in *C. dublinienis* (ANOVA, $p \leq 0.05$) induced 2-fold or
873 greater in WS at 3 h. Clustering was carried out in Genespring GX11 using default
874 hierarchical clustering parameters. Colours refer to Log_2 ratio values as depicted in
875 bar legend. Conditions include a change in cell density (density shift), a switch to
876 nutrient 10% v/v Lee's medium (nutrient shift), a switch to growth at 37°C
877 (temperature shift), a shift to pH 7.5 (pH shift), or expression in an *nrg1Δ* background
878 (*nrg1Δ)*. Solid bars to the right (labelled I, II, III, IV and V) indicate major clusters of
879 co-regulated genes (see text). The star shows the location of the major group of *NRG1*
880 regulated genes and the circle the position of the main pH regulated group. (b-g).

881 Graphs showing expression plots of representative genes indentified from clusters in

882 (a)

883

884 **Figure 7.** Interaction of *C. dubliniensis* Wü284 with reconstituted human oral

885 epithelium (RHE) following 24 h incubation. (a) Photomicrograph of *C. dubliniensis*

886 yeast cells at the surface of RHE following preculture in YPD at 37˚C. Bar equals 25

887 μm (b) Localized invasion of the surface of the RHE by *C. dubliniensis* following

888 preculture in Lee's medium pH 4.5 at 30˚C. Bar equals 25 μm (c) High magnification

889 photomicrograph of a hyphal *C. dubliniensis* cell penetrating the surface of the RHE,

890 following preculture in Lee's medium pH 4.5 at 30˚C. Bar equals 10 μm (d) Damage

891 to the RHE tissues estimated by measurement of lactate dehydrogenase (LDH) release

892 in control (uninfected) tissues, tissues infected with YPD pregrown cells and tissues

893 infected with Lee's pH 4.5 pregrown cells after 24 h incubation. (e) Adherence of *C.*

894 *dublinienis* Wü284 and *C. albicans* SC5314 to TR146 monolayers over time.

895 Adherence was determined in cells precultured in YPD at 37˚C and Lee's medium at

896 30˚C and expressed as the percentage of adherent CFU relative to the inoculum. Error

897 bars represent standard deviation from the mean of three replicate experiments (f)

898 Examination of adherence of *C. dublinienis* Wü284 precultured in various

899 modifications of Lee's medium, including media buffered to pH 5.0, pH 7.2,

900 incubated at 37˚C or supplemented with peptone. Error bars represent standard

901 deviation from the mean of three replicate experiments.

902 **Figure 8.** Survival of *C. dubliniensis* Wü284 following co-culture with murine

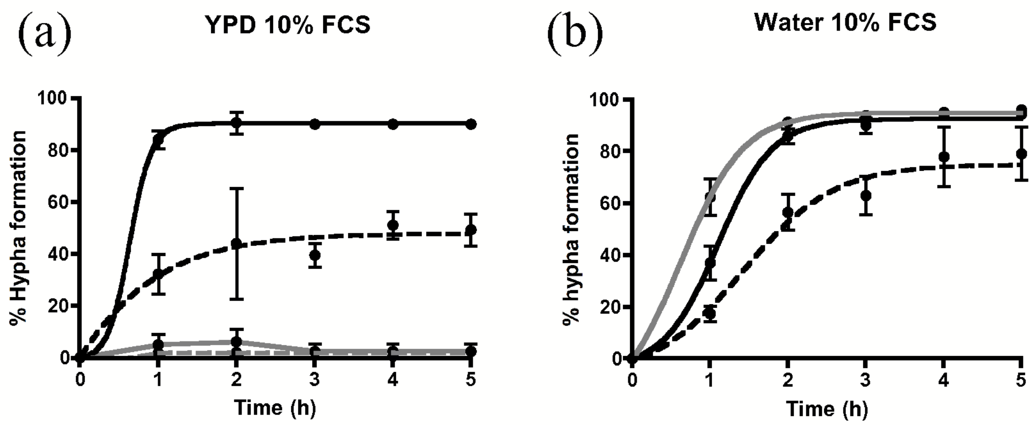
903 RAW264.7 macrophages. (a) Proliferation of viable *Candida* cells was assayed using

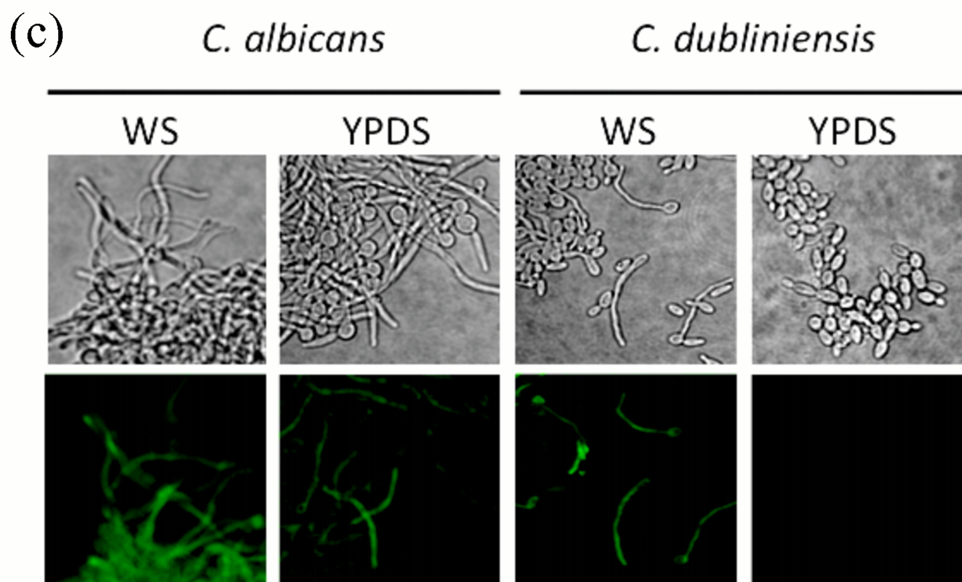
904 an XTT dye reduction assay following 18 hour co-culture at several multiplicities of

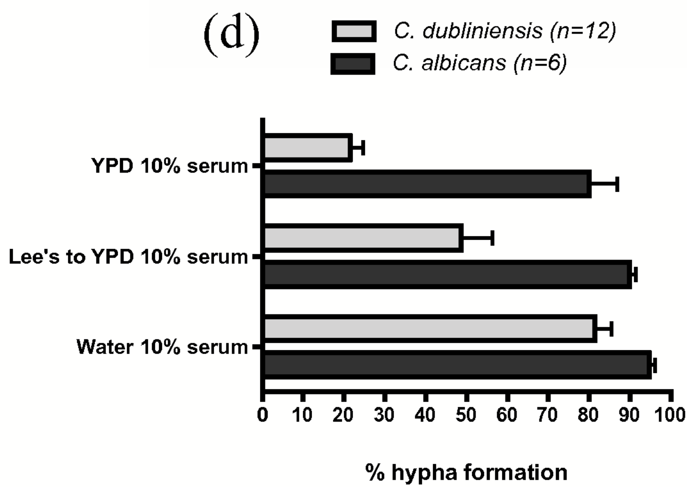
905 infection (MOI; *Candida*:macrophages). Wü284 cells precultured in Lee's medium
906 pH 4.5 exhibited significantly greater proliferation at MOIs of 1:8 and 1:32. (b)
907 Morphology of YPD grown and (c) Lee's pH 4.5 grown *C. dubliniensis* Wü284 cells
908 following 5 h incubation with murine RAW264.7 macrophages. Error bars represent
909 standard deviation from the mean of three replicate experiments.

910

Fig. 1







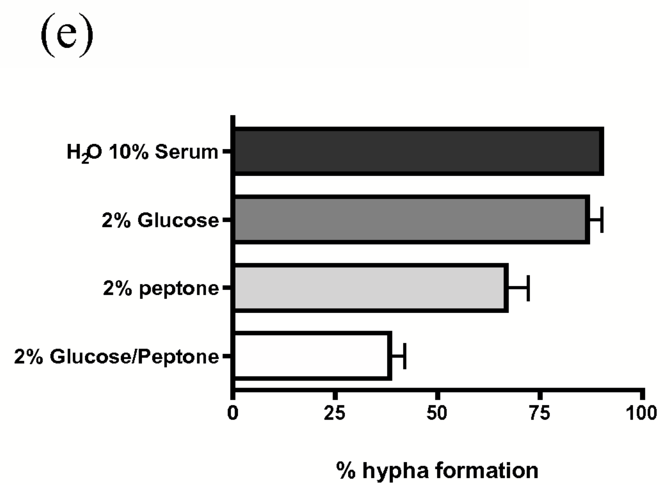
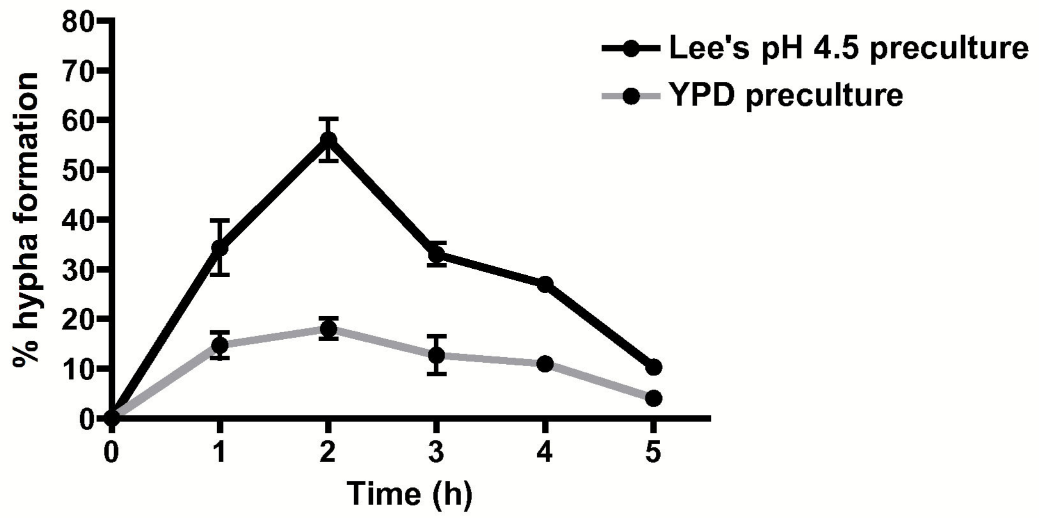


Fig. 2

(a)



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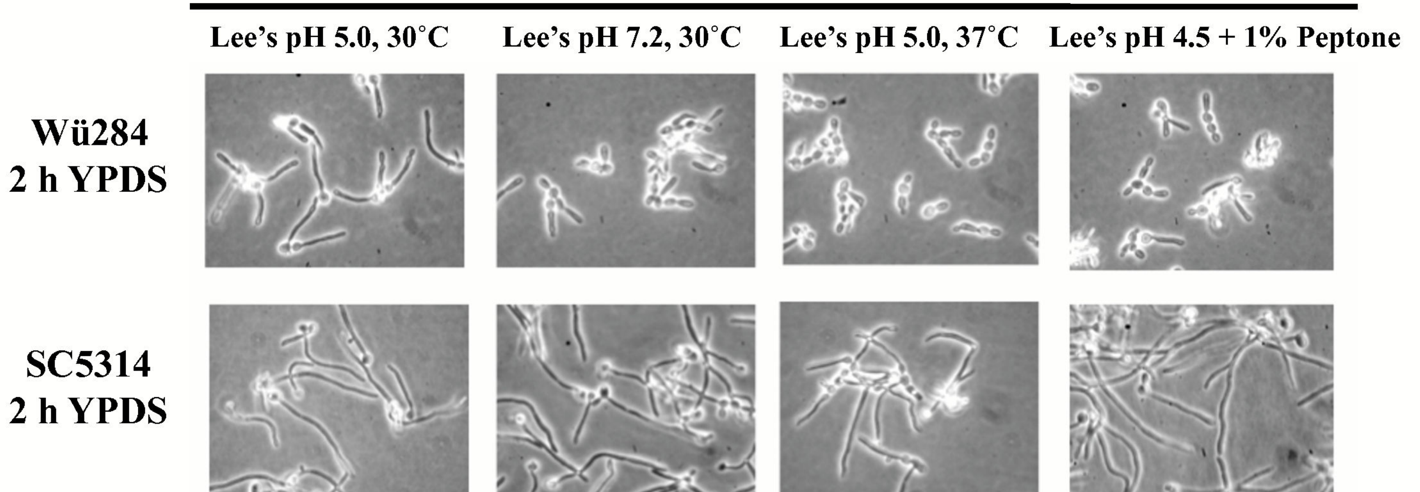
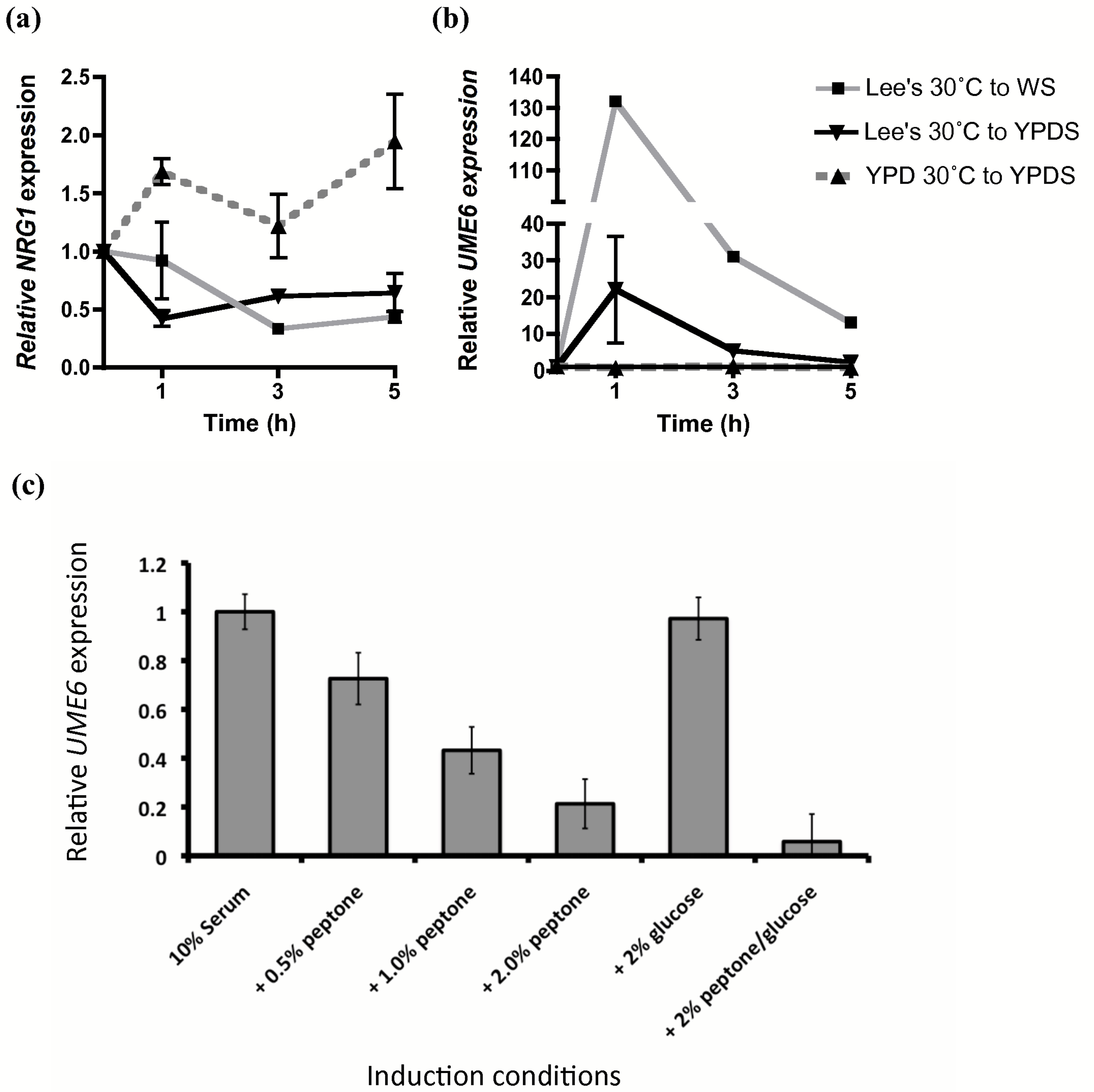


Fig. 3



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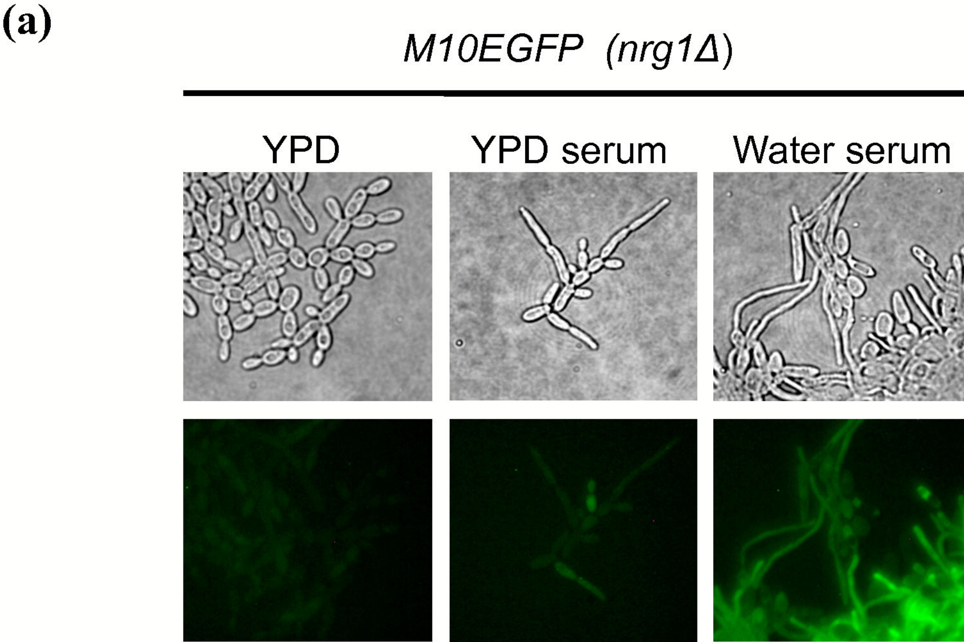

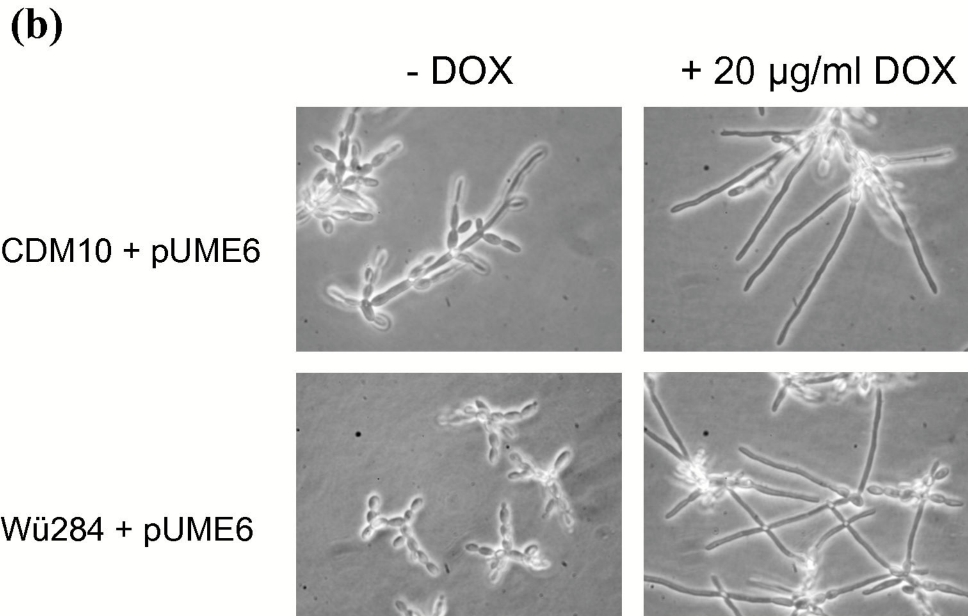

Fig. 5

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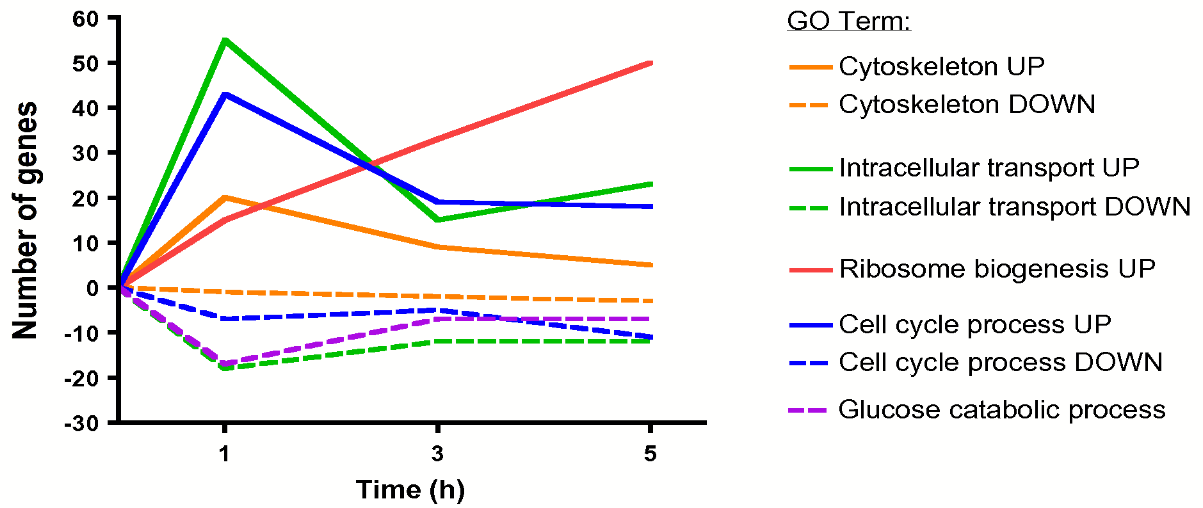

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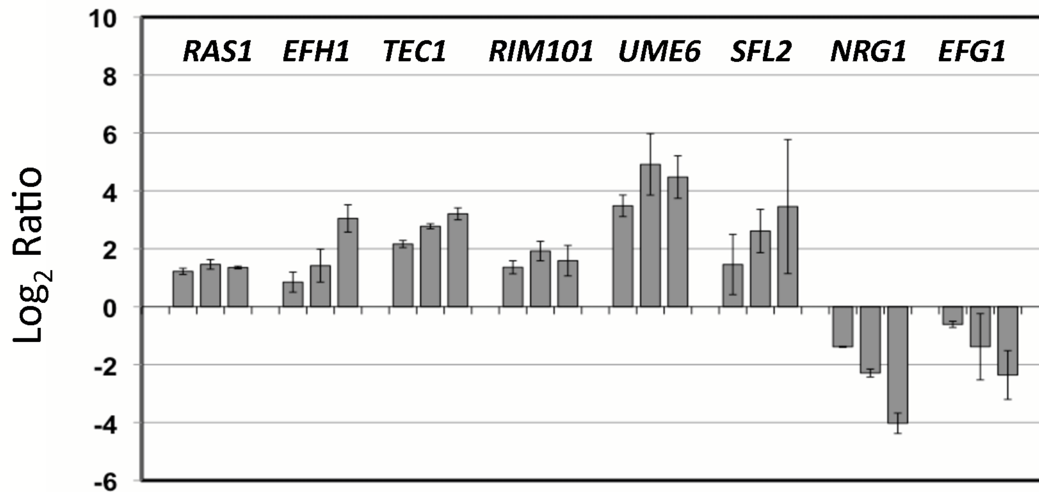

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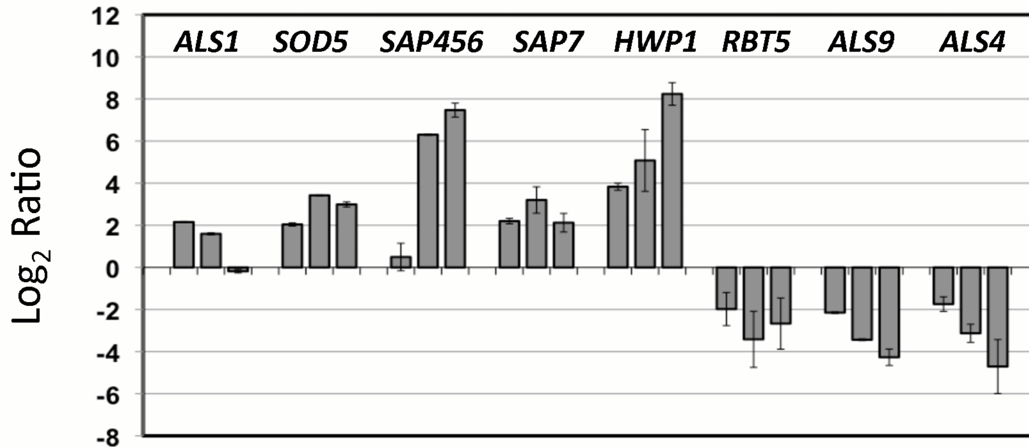
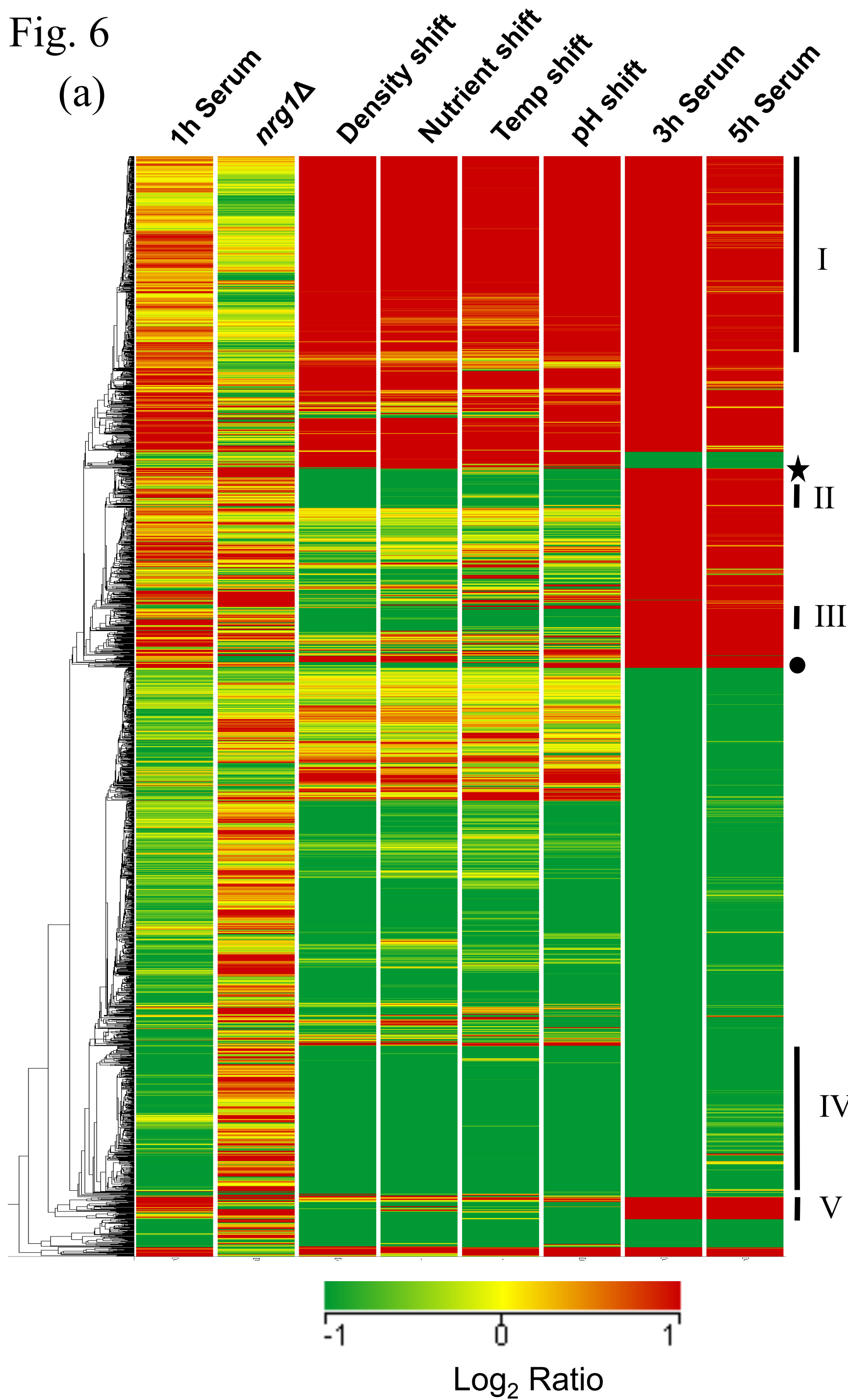
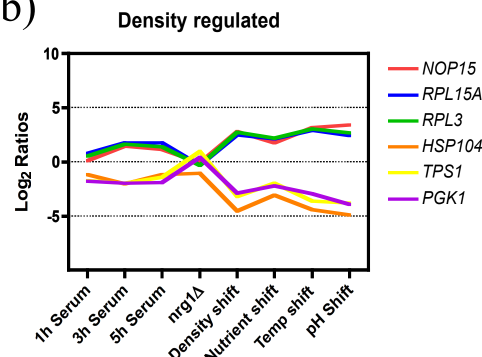
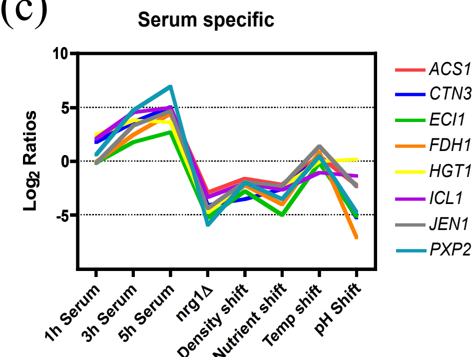
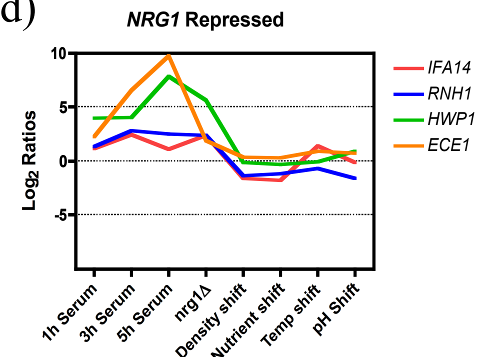


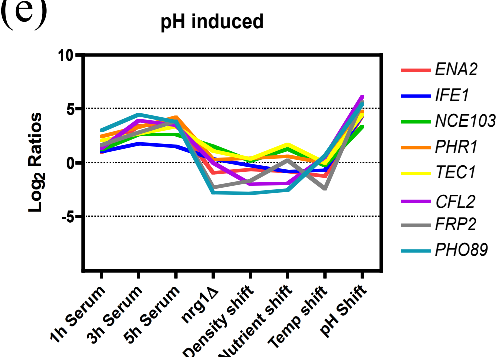
Fig. 6

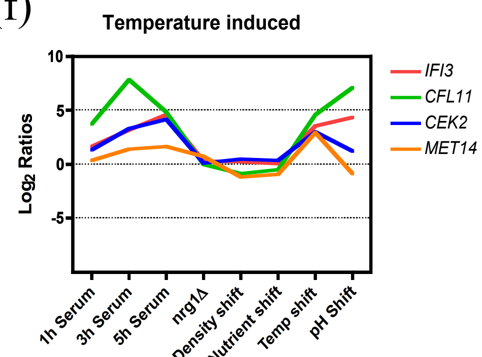












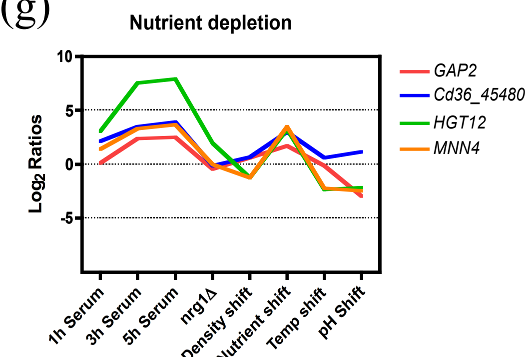
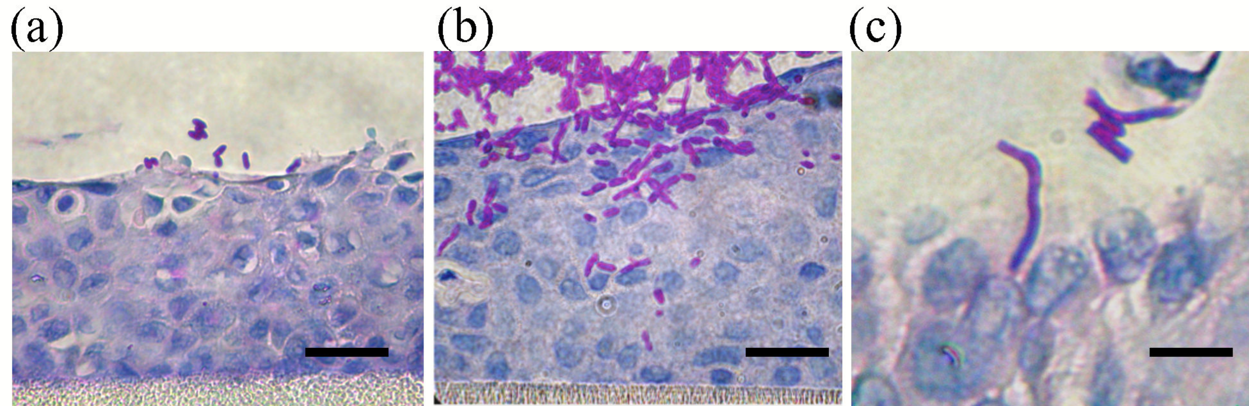


Fig. 7



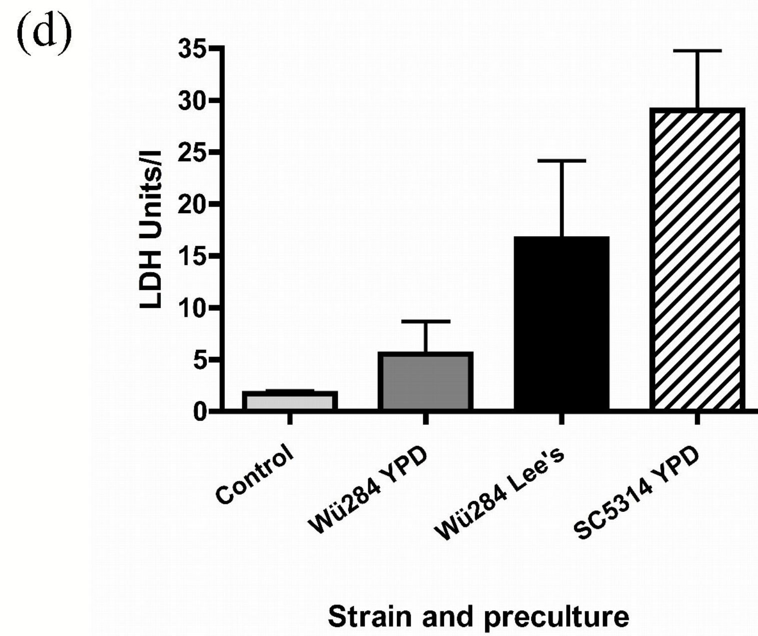
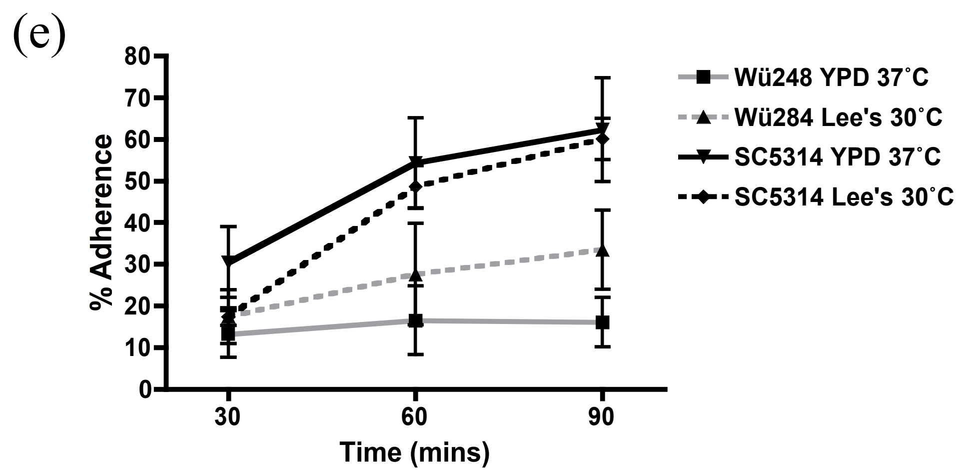


Fig. 7

(e)



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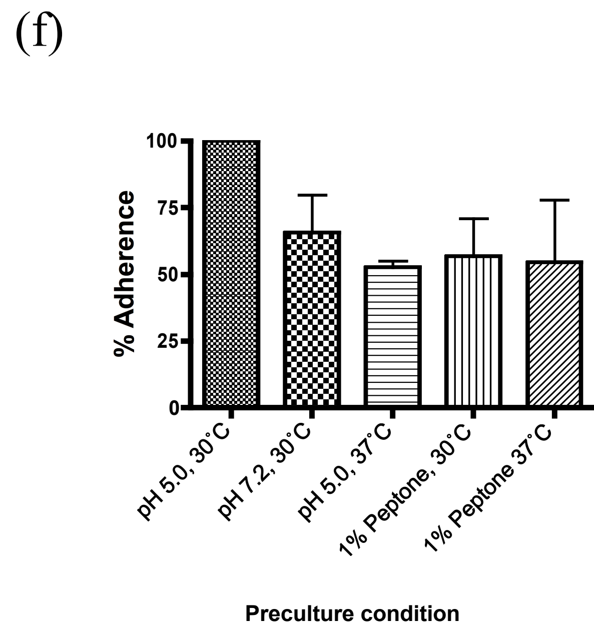

Fig. 8