The Closely Related Species *Candida albicans* and *Candida dubliniensis* Can Mate

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Because *Candida dubliniensis* is closely related to *Candida albicans*, we tested whether it underwent white-opaque switching and mating and whether white-opaque switching depended on *MTL* homoyzogosity and mating depended on switching, as they do in *C. albicans*. We also tested whether *C. dubliniensis* could mate with *C. albicans*. Sequencing revealed that the *MTL* locus of *C. dubliniensis* was highly similar to that of *C. albicans*. Hybridization with the *MTL* loci of *C. albicans* and *C. dubliniensis* was far less frequent in suspension cultures, due to the absence of mating-dependent clumping. Mating did occur, however, at higher frequencies on agar or on the skin of newborn mice. The increases in *MTL* homoyzogosity, the increase in switching frequencies, the decrease in the quality of switching, and the decrease in mating efficiency all reflected a general deterioration in the regulation of developmental processes, very probably due to the very high frequency of recombination and genomic reorganization characteristic of *C. dubliniensis*. Finally, interspecies mating readily occurred between opaque *C. dubliniensis* and *C. albicans* strains of opposite mating type in suspension, on agar, and on mouse skin. Remarkably, the efficiency of interspecies mating was higher than intraspecies *C. dubliniensis* mating, and interspecies karyogamy occurred readily with apparently the same sequence of nuclear migration, fusion, and division steps observed during intraspecies *C. albicans* and *C. dubliniensis* mating and *Saccharomyces cerevisiae* mating.

Beginning in 1990, a number of studies of the genetic relatedness of *Candida albicans* isolates derived from human immunodeficiency virus (HIV)-positive individuals identified atypical strains (9, 29, 31, 33, 49). These strains were subsequently grouped into the separate species *C. dubliniensis* in 1995 by Coleman, Sullivan, and coworkers (52). This new species shared a number of phenotypic characteristics with *C. albicans*, including two that in the past had been used to distinguish *C. albicans* from other species, namely, chlamydospore and true hypha formation (52). Like *C. albicans*, *C. dubliniensis* was diploid, contained a homolog to the dispersed complex repeat sequence RPS, and underwent the bud-hypha transition and colony-based 3153A-like phenotypic switching (10, 17, 19, 37, 50, 51). The basic phenotypic characteristics that distinguished *C. dubliniensis* from *C. albicans* were different colony color on CHROM-agar, failure to fluoresce under Wood’s light on methyl blue-Sabouraud agar, chlamydospore formation on Staib’s agar, lack of expression of β-glucosidase, and failure to grow at 42°C (1, 10, 37, 51, 52). More importantly, a number of genetic traits distinguished *C. dubliniensis* from *C. albicans*, including diminished hybridization with the related DNA fingerprinting probes 27A (51, 52) and Ca3 (20, 31) and differences in restriction fragment length polymorphism patterns (52), randomly amplified polymorphic DNA patterns (52), multilocus enzyme electrophoresis patterns (9, 24, 33, 51), hybridization with microsatellite DNA sequences (49, 52), and ribosomal DNA (rDNA) sequences (52). It was subsequently demonstrated that *C. dubliniensis* contained a dispersed species-specific midrepeat sequence, which represented a portion of the complex DNA fingerprinting probe Cu25 (20). Furthermore, it was demonstrated that *C. dubliniensis* contained on average twice as many full-length copies of the common repeat sequence RPS as did *C. albicans* and underwent RPS reorganization on average at twice the rate per genome as did *C. albicans* (19). The presence of a potentially recombinogenic species-specific midrepeat sequence and twice as many recombinogenic RPS elements, both dispersed throughout the genome, explained in part the high degree of karyotypic instability of *C. dubliniensis* (19). Together, these results suggested that *C. albicans* and *C. dubliniensis* had diverged into separate species but had done so only recently in evolutionary time.

The unusual level of relatedness of these two species led us to test whether *C. dubliniensis* strains, like *C. albicans* strains, were normally *MTL* heterozygous and underwent *MTL* homozygosis, whether *MTL* zygosity regulated the white-opaque transition, whether mating was dependent on a switch from white to opaque, and whether skin facilitated mating. It also led us to test whether *C. albicans* and *C. dubliniensis* could mate and undergo karyogamy.

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TABLE 1. White-opaque switching and MTL zygosity of C. dubliniensis

| Isolate | Source| Geographical origin | MTL genotype | Genetic group | Wh/Op transition | Reference |
|---------|-------|---------------------|--------------|---------------|-----------------|-----------|
| d900006 | HIV+  | UK                  | a/a          | I             | +               | 20        |
| d81217  | NA    | TX (USA)            | a/a          | I             | +               | 20        |
| PC35    | Healthy | South Africa       | a/a          | I             | +               | 7         |
| UP29    | Healthy | South Africa       | a/a          | I             | +               | 7         |
| d90013  | HIV+  | UK                  | a/a          | I             | +               | 20        |
| d88014  | HIV+  | UK                  | a/a          | I             | +               | 20        |
| UP26    | Healthy | South Africa       | a/a          | I             | +               | 7         |
| d1046   | Drug addict | Spain          | a/a          | I             | +               | 20        |
| d1425638 | Cancer | NY (USA)          | a/a          | I             | +               | 20        |
| ANSA26  | HIV+  | Belgium             | a/a          | I             | –               | 20        |
| ANSA27  | HIV+  | Belgium             | a/a          | I             | –               | 20        |
| d932634 | Gynecology | Belgium         | a/a          | I             | –               | 20        |
| d14149-2| HIV+  | France              | a/a          | I             | –               | 20        |
| P62     | HIV+  | South Africa        | a/a          | I             | –               | 7         |
| d163    | HIV+  | IA (USA)            | a/a          | I             | –               | 20        |
| d930713 | HIV+  | Belgium             | a/a          | I             | –               | 20        |
| d920710 | HIV+  | Belgium             | a/a          | I             | –               | 20        |
| d961    | Drug addict | Spain          | α/α          | I             | +               | 20        |
| P86     | HIV+  | South Africa        | α/α          | I             | +               | 7         |
| d126423 | Cancer | IA (USA)            | α/α          | I             | +               | 20        |
| ANSA5   | HIV+  | Belgium             | α/α          | I             | +               | 20        |
| ANSA9   | HIV+  | Belgium             | α/α          | I             | +               | 20        |
| UP24a   | HIV+  | South Africa        | α/α          | I             | +               | 7         |
| ANSA28  | HIV+  | Belgium             | α/α          | I             | +               | 20        |
| d940613 | Gynecology | Belgium         | α/α          | I             | +               | 7         |
| d1003   | Drug addict | Spain          | α/α          | I             | +               | 20        |
| d75004  | Diabetes | UK                  | α/α          | I             | +               | 20        |
| UP6a    | Healthy | South Africa       | α/α          | I             | +               | 7         |
| UP12    | Healthy | South Africa       | α/α          | I             | +               | 7         |
| UP16    | Healthy | South Africa       | α/α          | I             | +               | 7         |
| UP24a   | Healthy | South Africa       | α/α          | I             | +               | 7         |
| UP36    | Healthy | South Africa       | α/α          | I             | +               | 7         |

a HIV+, HIV-positive. All strains were isolated from the oral cavity. NA, not available.
b Geographical locale in which isolate was collected.
c These three MTL-heterozygous isolates became homozygous at high frequency. The colonies that underwent the white-opaque transition were MTL homozygous (see Fig. 5).
d White-opaque switching was analyzed by plating cells from each 5-day colony at low density (~50 CFU per plate) onto two plates. This was repeated three times.

MATERIALS AND METHODS

Strain origin and maintenance. The collection of 82 C. dubliniensis strains used in this study were DNA fingerprinted with the complex probe C25 (7, 11, 20). It included 61 from the group I clade and 21 from group II, a less closely related group of isolates than those of group I (see Fig. 1). The 32 isolates analyzed for switching are listed in Table 1, with host disease state, geographical origin, and relevant reference. The origin of C. albicans strains WO-1 (α/α) and P37005 (α/a) and their mating characteristics have been previously described (25, 26, 40). All strains were maintained in 20% glycerol at 20°C.

Isolation and sequence analysis of the MTLα locus of C. dubliniensis. To obtain the full-length MTLα locus and flanking sequences, 5 × 10⁶ plaques of a C. dubliniensis genomic library of strain d1558 (20) were screened with the 555-bp DNA fragment spanning the MTLα2 open reading frame (ORF) of C. albicans (15). Six positive clones were subjected to a secondary screen, and clones λCDPL1.1 and λCDPL2.1 were chosen for further characterization. To generate the first consensus sequence for locus walking, a 7.5-kb EcoRI fragment containing the MTLα2 regions was subcloned into pGEM-7Z (Promega Corp., Madison, Wis.). Using the initial consensus DNA sequence generated from the plasmid, the full-length MTLα locus sequence was obtained by clone walking with custom primers and an ABI sequencing apparatus (PE-ABI Inc., Foster City, Calif.). Comparisons of protein and nucleotide sequences were performed using the multiple-alignment editor of Clustal W software (http://clustalw.genome.jp).

Analysis of mating-type zygosity. The mating type of each of the 82 C. dubliniensis strains was analyzed by Southern blot hybridization with the C. albicans MTLα1, MTLα1, and MTLα2 ORFs (13). DNA probes were generated by PCR. A subset of strains was also analyzed with the C. albicans MTLα2 ORF (53) and with C. dubliniensis MTLα1 and MTLα2 ORFs, generated by PCR. Southern analysis was performed as previously described with EcoRI-digested DNA (26).

Mating on skin. The methods for analyzing mating on skin were similar to those previously described (21, 22). In brief, a cotton patch saturated with equal numbers of two test strains was taped to the skin on the back of a newborn White Swiss/Webster ND-4 mouse (Sprague-Dawley, Madison, Wis.). After 24 h, the animal was sacrificed, the patch was removed, and the skin under the patch was excised and processed for scanning electron microscopy.

Fluorescent staining of live cells. The methods for staining live cells were previously reported in detail (26). In brief, cells from one strain were vitally stained with rhodamine-conjugated concanavalin A (ConA) and cells from the other strain were stained with fluorescein isothiocyanate (FITC)-conjugated ConA (Vector Laboratories, Burlingame, Calif.). After mating, cells were stained with calcofluor white MR (Molecular Probes, Eugene, Ore.). The cells were visualized with a Radiance 2100 MP multiphoton laser-scanning confocal microscope (Bio-Rad, Hemel Hempstead, United Kingdom). For nuclear staining of fusants, cells stained with rhodamine- and FITC-conjugated ConA were fixed with 4% paraformaldehyde and stained with a solution of 1 μg/ml of Hoechst 33342 (Molecular Probes, Eugene, Ore.) per ml.

Analysis of released chemotaxant. The methods for testing yeast cells for the release of chemotaxant for polymorphonuclear leukocytes (PMNs) were presented in detail in previous publications (12, 13). In brief, cells were suspended in buffer for 3 h and pelleted, and the supernatant (conditioned buffer)
was placed in one well of a quartz single-cell chemotaxis chamber (38, 39) fashioned as described by Zigmond (54). Buffer alone was added to the other well. Human PMNs were placed over the bridge between wells. After 5 min at 37°C, cell behavior was continuously videorecorded and analyzed with DIAS software (42, 47). Instantaneous velocity, chemotactic index, and percent positive chemotaxis were computed as previously described (12, 42).

**RESULTS**

**Mating type.** To assess the *MTL* zygozygosity (a/a, α/α, or α/α) of natural *C. dubliniensis* strains, Southern blots of EcoRI-digested DNA from natural isolates were probed with the *C. albicans* *MTLα1*, *MTLα1*, and *MTLα2* ORFs (15). Of the 82 tested isolates, 61 were members of the group I clade and 21 were members of group II (Fig. 1) (7, 11, 20). EcoRI-digested DNA of all 82 isolates hybridized to either *MTLα1* alone (a/a), the two *MTLα* probes alone (α/α), or all three probes (α/α) (Fig. 2). EcoRI-digested DNAs from a subset of 10 a/a, 10 α/α, and 10 α/α strains were subsequently probed with the recently identified *MTLα2* ORF (53). While all 20 a/a and α/α strains hybridized to the probe, the 10 α/α strains did not, demonstrating that as in *C. albicans* (53), a1 and a2 are linked (data not shown).

Of the 82 natural *C. dubliniensis* strains tested, 55 (67%) were *MTL* heterozygous (a/α), 17 (21%) were *MTLα* homozygous (a/a), and 10 (12%) were *MTLα* homogyzous (α/α) (Fig. 1). The proportion of natural *MTL-homozygous* *C. dubliniensis* strains was therefore 33%, which is approximately 10-fold higher than the proportion of natural *C. albicans* strains that are *MTL* homozygous (25). As is evident in the dendrogram in Fig. 1, all but one of the *MTL-homozygous* strains of the *C. dubliniensis* collection were members of the group I clade. While 43% of group I isolates were *MTL* homozygous, only 5% of group II isolates were *MTL* homozygous. This difference was significant (P = 8 × 10⁻⁴, Fisher’s exact test).

The hybridization results suggested a high level of identity between the *MTLα* and *MTLα* genes of *C. albicans* and the *C. dubliniensis* homologs. To test this directly, the *MTLα* locus was sequenced and compared to that of *C. albicans* strain SC5314 (a/α) (15) (Fig. 3). The organization of the *C. dubliniensis* *MTLα* locus was identical to that of *C. albicans* (Fig. 3). The nucleotide sequences of the five genes in the locus were between 87.5 and 90.0% identical to that of *C. albicans* homologs (Fig. 3). The deduced amino acid sequences exhibited between 96.8 and 99.5% similarity (Fig. 3). The identity of the bands hybridizing to the *C. albicans* *MTLα1* and *MTLα2* probes was confirmed by using a select group of 10 strains with PCR-generated *C. dubliniensis* *MTLα1* and *MTLα2* probes (data not shown). Recently, data from the *C. dubliniensis* genome sequencing project of the Pathogen Sequencing Unit at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/C_dubliniensis/) revealed that the *MTLα* locus of *C. dubliniensis* also has high homology and similar structure to the *C. albicans* *MTLα* locus.

**White-opaque switching.** To test whether natural strains of *C. dubliniensis* underwent the white-opaque transition and whether it was regulated by *MTL* zygozy, as it is in *C. albicans* (25, 30), cells from 17 a/a strains, 10 α/α strains, and 5 α/α strains were plated on phloxine B-containing agar. In *C. albicans*, phloxine B distinguishes opaque sectors and colonies by differentially staining them red, as demonstrated for *C. albi-
C. dubliniensis strain d1558 reveals the same organization of genes as in the C. albicans strain WO-1 in Fig. 4A (2). Over half of the natural C. dubliniensis a/a and a/α strains produced red sectors and/or red colonies that contained cells with the signature opaque-cell phenotype, as described in the next section (Table 1; Fig. 4B through F). However, two general characteristics of white-opaque switching in C. dubliniensis made the process harder to study than in C. albicans. First, in contrast to switching in wild-type C. albicans (Fig. 4A), a second switching system appeared to be operational in at least half of the strains, resulting in colony phenotypes that appeared to express the opaque phenotype as well as variant characteristics of the second switching system (e.g., Fig. 4B and C). Second, high frequencies of reversibility from opaque to white were the norm, such as at the edge of the opaque sectors of strain d90006 in Fig. 4D and E. Among C. dubliniensis strains that showed a white-opaque phase transition, estimated frequencies of the transition from the white to the opaque phase were similar to those of C. albicans (25). White-phase populations accumulated opaque phase cells at frequencies of 10^{-3} to 10^{-4}. In contrast, opaque-phase cell populations of most C. dubliniensis strains accumu-

lated white phase cells at a much higher frequency (>10^{-1}). We were able to identify only five strains with opaque-phase cells stable enough (i.e., with low reversion rates to white) to perform many of the subsequent experiments reported here. These strains, d90006 (a/a), d81217 (a/α), d80014 (α/α), ANSA5 (α/a), and d126423 (α/α), exhibited switching frequencies from opaque to white of approximately 10^{-1}, 10^{-3}, 10^{-3}, 10^{-3}, and 10^{-2}, respectively. However, opaque-phase cells of strains d88014 and ANSA5 also switched to additional phenotypes, presumably part of a second switching system, at frequencies of ca. 10^{-1} and 10^{-2}, respectively.

Three of the five tested MTL-heterozygous (a/α) strains (UP6a, UP16, and UP12) also formed red colonies and/or sectors on agar containing phloxine B (Table 1). For C. albicans, it was demonstrated that a/α strains that underwent the white-opaque transition did so because they underwent high-frequency MTL homozygosis and that it was in fact the MTL-homozygous offspring in every case that were undergoing white-opaque switching (25). We therefore picked white and opaque-phase cells of strains d88014 and ANSA5 also selected clones that were white (W1 through W5) proved to be a/α while both clones (O1 and O2) that were opaque proved to be a/α (Fig. 5A). For UP16, two of the four clones that were white (W1 and W4)
proved to be a/α while the remaining two (W2 and W3) were a/a (Fig. 5B). The three clones (O1 through O3) that were opaque were a/a (Fig. 5B). Finally, for UP12, the five clones that were white (W1 through W5) proved to be a/α while the two that formed rough-edged (RE) red colonies and sectors that contained both large round (LR) cells and a minority of opaque cells proved to be a/α (Fig. 5C). These results are consistent with the conclusion that *C. dubliniensis* cells undergoing white-opaque switching are *MTL* homozygous, as with *C. albicans* (25, 30).

**Opaque-phase cell phenotype.** In *C. albicans*, the surface of white cells is smooth and the cell shape round to slightly ellipsoidal while the surface of opaque cells is pimpled and the shape is elongate (2, 40). The surface of *MTL*-homozygous *C. dubliniensis* cells in the white phase was smooth, and the shape was round to ellipsoidal (Fig. 6A), like white cells of *C. albicans*. The surface of the majority of cells in the opaque phase was pimpled, and the shape was elongate (Figure 6B to D), like opaque cells of *C. albicans* (2). This opaque-phase signature phenotype was expressed by both *C. dubliniensis* a/α and /H9251/H9251 cells. Opaque-phase cells of *C. dubliniensis* sometimes grew into very large, elongate cells that tapered apically and were pimpled, suggesting that such *C. dubliniensis* cells may have entered the apical mode of growth characteristic of hyphae while still expressing the opaque phenotype. This unusual phenotype has not been observed in *C. albicans*. In fact, it has been demonstrated that hyphae formed by *C. albicans* opaque cells are devoid of pimples (3). In addition, very large, round cells were sometimes found mixed with opaque cells in rough-edged red sectors or colonies (data not shown). Since these cells were usually found in sectors or colonies also exhibiting characteristics of a second switching system (e.g., Fig. 4C), we tentatively conclude that these aberrant cellular phenotypes may have resulted from overlapping switching systems.

**C. dubliniensis** opaque a/α and α/α cells can mate. To test whether *C. dubliniensis* a/α and α/α cells could mate and whether mating depended on a switch, from white to opaque, mixtures of a/α and a/α cells were incubated in shaker cultures overnight and then examined for fusants by using differential interference contrast (DIC) optics. This method was used to obtain high frequencies of fusants in mating mixtures of *C. albicans* (26). When white a/α cells (strain d88014) and white α/α cells (strain ANSA5) were incubated together, cells neither "shmooed" (i.e., extended conjugation tubes constricted at the
mother-tube junction) nor fused (Fig. 7A and B). When opaque a/a cells (strain d88014) and opaque α/α cells (strain ANSA5) were incubated together, over 80% formed shmoos after 4 h (Fig. 7C), and the shmoo extensions grew into conjugation tubes (Fig. 7D to F). Fusants formed after 24 h (Fig. 7F to H) but were rare (~0.1%). Similar results were obtained with mixtures of opaque d81217 (a/a) and d126423 (α/α) cells (data not shown).

Therefore, although mixtures of C. dubliniensis opaque a/a and α/α cells in suspension cultures readily formed shmoos that grew into long conjugation tubes, like C. albicans, the frequency of fusion was less than 1/20 that of C. albicans (26). The reason for this appeared to be in the degree of cell clumping. Suspended mixtures of C. albicans opaque a/a and α/α cells formed large, stable clumps that facilitated mating (26). However, C. dubliniensis opaque a/a and α/α cells did not clump. When samples were taken from suspended mating mixtures, the cells adhered loosely to one another, usually side by side as doublets, as in Fig. 7E. We therefore tested whether the frequency of fusion increased in dense undisturbed cultures by simply incubating mixtures of a/a and α/α cells on nutrient agar. As expected, the frequency increased approximately five-fold over that in suspension cultures. It was still, however, far below that observed in comparable suspension cultures of mating C. albicans cells (26).

To test unambiguously that fusions occurred only between C. dubliniensis a/a and α/α cells, a/a opaque cells were vitally stained green with FITC-conjugated ConA and α/α opaque cells were vitally stained red with rhodamine-conjugated ConA prior to mixing (26). If fusion occurred and if it was mating-type dependent, then 100% of fusants would include one green (a/a) and one red (α/α) parent cell. The experiment was performed with both suspension and standing dish cultures. All the fusants (20 fusants analyzed) included a green and a red parent cell (Fig. 8). None exhibited a homogeneous color com-

FIG. 6. C. dubliniensis opaque-phase cells have pimples like C. albicans but can become abnormally large and long. (A) white cells; (B through D) opaque cells; (E and F) large, elongate opaque cells. Note the pimples on opaque cells. Bars, 2 μm.

FIG. 7. Cell biology of C. dubliniensis mating. Equal numbers of opaque a/a and α/α cells were mixed and cultured in suspension for 5 h. (A and B) A cross between white a/a and α/α cells. (C) Shmooing in a cross between opaque a/a and α/α cells. (D and E) Conjugation tube formation in a cross between opaque a/a and α/α cells after 7 h. (G and H) Examples of fusants in a cross between opaque a/a and α/α cells after 7 h. dc, daughter cell. Bar, 5 μm.
bination. These results demonstrate that, as in *C. albicans* (26), fusion occurs only between *C. dubliniensis* opaque a/a cells and opaque a/a cells.

**Mating between species.** Because of the high degree of genetic relatedness of *C. albicans* and *C. dubliniensis*, the possibility of interspecies mating was entertained. We first tested whether *C. dubliniensis* opaque a/a cells responded to the *C. albicans* α-pheromone peptide by shmooing and subsequent conjugation tube growth, like *C. albicans* a/a cells (6, 27, 32). When white a/a cells of *C. dubliniensis* strains d81217 and d88014 were treated with 10^{-5} M chemically synthesized *C. albicans* α-pheromone (27), they did not shmoo (Figure 9A to C). However, when opaque a/a cells of the two strains were treated with 10^{-5} M *C. albicans* α-pheromone, they formed unconstricted evaginations after 2 h in the process of shmooing (Fig. 9D to F). These evaginations elongated, forming conjugation tubes, which reverted apically to the budding-growth form after 5 h of incubation (Fig. 9G to I), as do the conjugation tubes of α-pheromone-treated *C. albicans* a/a cells (27).

We then tested whether *C. dubliniensis* mated with *C. albicans* in a mating-type-dependent fashion. Mixtures of equal numbers of *C. albicans* opaque a/a cells (P37005) stained with FITC-conjugated ConA (green) and *C. dubliniensis* opaque α/α cells (d126423) stained with rhodamine-conjugated ConA (red) were incubated for 10 h in shaker cultures and then analyzed for fusants by laser-scanning confocal microscopy. Fusants were found throughout the population at frequencies approximately 10 times higher than in homospecies suspension mixtures of *C. dubliniensis* a/a and α/α opaque cells. All the fusants (50 analyzed) exhibited the green and red color combination (Fig. 10), demonstrating that *C. albicans* and *C. dub-

*FIG. 8. Demonstration by laser-scanning confocal microscopy that only a/a and α/α cells fuse in mating cultures of *C. dubliniensis*. FITC-conjugated ConA (green)-stained a/a and rhodamine (Rho)-conjugated ConA (red)-stained α/α cells were allowed to mate in suspension cultures for 5 h. Crosses were between opaque d88014 a/a and ANSA5 α/α cells and between opaque d88014 a/a and d126423 α/α cells. (A and E) Fusant stained with calcifluor for cell wall to visualize the entire zygoite. (B and F) Selective imaging of FITC-conjugated ConA. (C and G) Selective imaging of rhodamine-conjugated ConA. (D and H) Overlays of calcifluor, FITC-conjugated ConA, and rhodamine-conjugated ConA images. Bar, 5 μm.*
C. dubliniensis, strain d81217 (a/a) Wh, α-pheromone, 2 hr

A. B. C.

C. dubliniensis, strain d81217 (a/a) Op, α-pheromone, 2 hr

D. E. F.

C. dubliniensis, strain d88014 (a/a) Op, α-pheromone, 5 hr

G. H. I.

FIG. 9. C. dubliniensis opaque a/a cells shmoo in response to C. albicans α-pheromone (26). (A to C) White a/a cells do not shmoo after treatment with C. albicans α-pheromone for 5 h. (D to F) Opaque a/a cells shmoo after treatment with C. albicans α-pheromone for 2 h (arrows indicate unconstricted evaginations). (G to I) Opaque a/a conjugation tubes revert apically to the budding-growth form after a 5-h treatment (arrows indicate apical buds). Bar, 5 μm.

A qualitative analysis of intraspecific C. dubliniensis fusants and C. albicans fusants revealed similar nuclear patterns (data not shown).

Mating on skin. Since newborn mouse skin has been demonstrated to facilitate mating between C. albicans opaque a/a cells and opaque α/α cells (22), we tested whether skin supported C. dubliniensis mating and then tested whether it supported interspecies mating. When C. dubliniensis opaque a/a cells (strain d88104) were incubated alone for 24 h on skin, no shmoo, cells with conjugation tubes, or fusants were observed (Fig. 12A and B). However, when C. dubliniensis opaque a/a cells were incubated with opaque α/α cells on skin for 24 h in two different mating crosses (strains d88104 [a/a] and d126423 [α/α] or strains d81217 [a/a] and ANSA5 [α/α]), cells with conjugation tubes and fusants were observed (Fig. 12C and D). The frequency was 10 to 20 times higher than that for the same mixture in suspension cultures. When interspecies crosses of C. dubliniensis strain d81217 a/a opaque cells and C. albicans strain WO-1 α/α opaque cells were incubated on skin for 24 h, fusants were also observed at comparable frequencies (Fig. 12E to H). When C. dubliniensis strain d126423 α/α opaque cells were incubated with C. albicans strain L26 a/a opaque cells on skin for 24 h, fusants were also observed (data not shown).

White-opaque switching in C. dubliniensis regulates the release of PMN chemoattractant. Recently, it was demonstrated by a single-cell chemotaxis assay (54) that white-opaque switching in C. albicans regulates the release of a potent PMN chemoattractant (13). While white cells secrete the attractant, opaque cells do not. It was suggested that the absence of attractant in opaque cultures may play a role in the overall mating strategy (13). We therefore tested whether white-opaque switching in C. dubliniensis regulated the release of attractant by using a single-cell chemotaxis assay in which cells were placed on a gradient chamber bridge bordered by one trough containing buffer alone and one trough containing cell-conditioned buffer (12, 13). Gradients of low-molecular-weight molecules form between 5 and 20 min of incubation (12, 13, 38, 39). The “chemotactic index” (C.I.) was calculated as the distance moved by a cell in the direction of the trough with conditioned buffer divided by the total distance moved. A C.I. of +1.00 represents persistent movement in a straight line toward the source with conditioned buffer (positive chemotaxis), a C.I. close to 0.00 represents random movement (no chemotaxis), and C.I.s increasing from 0.00 to +1.00 reflect increasing efficiencies of positive chemotaxis. The parameter “percent positive chemotaxis” represents the proportion of cells exhibiting a positive chemotactic index. A measure of 100% means that all test cells exhibited net movement toward the source of chemooattractant. A measure of 50% positive chemotaxis reflects random movement (i.e., no chemotaxis). Conditioned buffer from white and opaque cells of three different strains of C. dubliniensis (ANS5, d81217, and d88014) was tested. In every case, conditioned medium from white cells induced chemotaxis (average C.I. between +0.32 and +0.62; percent positive chemotaxis between 91 and 93%) (Table 2). In contrast, conditioned medium from opaque cells did not induce chemotaxis (average C.I. between −0.01 and +0.10; percent positive chemotaxis between 42 and 48%) (Table 2). Therefore, just as for C. albicans (13), switching in C. dubliniensis regulates the release of a PMN chemoattractant.

DISCUSSION

The C. dubliniensis MTL locus and MTL zygosity. Using the C. albicans mating-type genes MTLα1, MTLα2, and MTLα2 as probes (15), we have demonstrated by Southern analysis that a/a, a/a, and α/α strains of C. dubliniensis exist in nature, like C. albicans (25). Conservation of the MTL locus was confirmed by comparing the sequence of the MTLα locus of C. dubliniensis with that of C. albicans (15). The arrangement of the five genes harbored by the MTLα locus was the same as in C. albicans. The nucleotide sequences and deduced amino acid sequences were highly similar. Recently, sequencing data provided by the Wellcome Trust Sanger Institute demonstrated that this was also the case for MTLα and confirmed our results for MTLα.

In the collection of 82 C. dubliniensis strains analyzed here, roughly one-third were MTL homologous. This represents 10 times the frequency of MTL homozygosity found in natural C. albicans strains (25), suggesting that MTL homozygosity occurs more frequently in C. dubliniensis. Recently, mitotic re-
combination has been demonstrated to be one mechanism for spontaneous MTL homozygosis in natural strains of *C. albicans* (W. Wu, S. Lockhart, and D. R. Soll, unpublished data). The high frequency of MTL homozygotes in the *C. dubliniensis* collection is therefore consistent with previous observations that *C. dubliniensis* undergoes genomic reorganization at high frequency, leading to karyotype instability (19). Presumably, the high rate of genomic reorganization results, at least in part, from the high rate of mitotic recombination, which in turn results from the twofold-larger number of recombinogenic RPS sequences (19) and the additional *C. dubliniensis*-specific repeat sequence dispersed throughout the genome (20).

We also found twice as many a/a as α/α strains in the *C. dubliniensis* collection, suggesting an adaptive hierarchy in nature of α/α > a/a > α/α. Finally, we found that MTL homozygotes were almost exclusively members of the group I clade. Members of this clade have been shown by DNA fingerprinting to be genetically more closely related than members of the group II clade (20) and to be more frequently recovered from HIV-positive individuals than group II strains (11). Clade-specific characteristics have begun to emerge in *C. albicans* (46). Natural flucytosine resistance was found to be restricted to one of the five clades of *C. albicans* (34). Flucytosine resistance represents the first identified clade-specific phenotypic characteristic of *C. albicans* (34, 46). The high frequency of MTL homozygosis in group I represents a new clade-specific characteristic in *C. dubliniensis*.

**White-opaque transition in C. dubliniensis.** We have demonstrated for the first time here that MTL-homozygous strains of *C. dubliniensis* undergo white-opaque switching, like MTL-homozygous strains of *C. albicans* (25, 30). However, white-opaque switching in *C. dubliniensis* was less uniform and sometimes harder to assess than in *C. albicans* for two reasons. First, it appeared to occur sometimes concurrently with other high-frequency switching systems. These other systems affected both colony and cellular phenotype and in many cases appeared to distort or camouflage the opaque phenotype. The second reason that switching is less uniform in *C. dubliniensis* was the high frequency of reversion from opaque to white, which was obvious at the edges of opaque sectors. In fact, we found that only a minority of the *MTL*-homozygous *C. dubliniensis* strains that undergo the white-opaque transition do so at frequencies low enough to perform many of the experiments reported here.

The cellular phenotype of *C. dubliniensis* opaque cells was similar to that of *C. albicans* opaque cells, including unique opaque-specific pimples (2), selective adhesion to skin (21, 22), the absence of secreted PMN chemoattractant (13), and mating competency (25, 30). However, opaque-phase cells of *C. dubliniensis* differed from those of *C. albicans* in their propensity to grow to enormous sizes by elongation while continuing to form pimples on their surface. The tapered, tubular shape of these cells suggested that they may have begun to grow in an apical mode, as do hyphae, even though they continued to form pimples in the wall. These aberrant opaque cell morphologies have not been reported for *C. albicans*, and hyphae formed by opaque cells are free of pimples (3).

**Intraspecies and interspecies mating.** We have presented evidence that *C. dubliniensis* mating follows the same general rules as those for *C. albicans* mating (18, 45, 48), namely, that mating-type zygosity regulates white-opaque switching, that
FIG. 11. Nuclear migration, division, and localization during mating between opaque a/a \textit{C. albicans} (P57072) and opaque a/a \textit{C. dubliniensis} (d90006) cells. The former were vitally stained with FITC-conjugated ConA and the latter were stained with rhodamine-conjugated ConA prior to mixing. After 6 h, mating mixtures in suspension cultures were fixed and stained with the nuclear dye Hoechst 33342. Using a two-photon laser-scanning confocal microscope, FITC-conjugated ConA-stained \textit{C. albicans} cells were visualized as green, rhodamine-conjugated ConA-stained \textit{C. dubliniensis} cells were visualized as red, and nuclei were visualized as blue. For each fusant, three images are presented: the DIC image, the FITC, rhodamine, and Hoechst 33342 images overlaid, and the Hoechst 33342 image alone. Twenty fusants that had not formed daughter buds and thirty-nine that had formed daughter buds were scored for number and location of nuclei. The images shown in panels A through G represent the major patterns. Cells that were not part of the fusion are marked with an X in the DIC images and have been digitally removed from the fluorescent images. The sequence of stages was deduced from the major patterns, the quantitation of which is presented in the Results section. dc, daughter cell.
cells must switch from the white to the opaque phase in order to mate, that fusion occurs only between opposite mating types, that α-pheromone induces shmooing and conjugation tube formation in a/a cells, and that the cellular stages of mating are similar to those in C. albicans. We have also demonstrated that skin facilitates mating, as it does in C. albicans (22). However, we have also found that in suspension cultures, although the majority of opaque a/a and α/α cells shmoo, fusion is infrequent, presumably because C. dubliniensis opaque a/a and α/α cells do not clump as do C. albicans opaque a/a and α/α cells in suspension. Clumping appears to facilitate mating by stabilizing cell-cell associations (26). Low frequencies of fusion and the absence of clumping were observed in crosses between all tested sets of strains. We therefore tested whether the absence of clumping was the reason for the low frequency of fusion in suspension cultures. We therefore tested whether the absence of clumping was the reason for the low frequency of fusion by assessing fusion in undisturbed a/a and α/α mixes on agar. The frequency was approximately fivefold higher than in suspension, supporting this conclusion. Furthermore, skin facilitated higher frequencies of fusion, again supporting the idea that the low frequencies of fusion in suspension are due to the lack of clumping.

Finally, we have demonstrated that C. albicans and C. dubliniensis can mate in suspension cultures. Surprisingly, the frequencies of interspecies mating were higher than those of intraspecies C. dubliniensis mating in suspension cultures, whether C. dubliniensis was the a/a or the α/α partner. The higher efficiency of interspecies mating correlated with increased clumping. The cellular characteristics of interspecies mating in suspension or on skin were at least superficially similar to those of intraspecies mating.

**Karyogamy.** We have found that in fusants that have not yet formed a daughter cell, the parent cell nuclei move into the bridge, ending up next to each other at the site of daughter cell formation. Migrating nuclei were highly elongate, suggesting tension, presumably the result of associated cytoskeleton involved in migration (8, 14, 35). The number of nuclei and their spatial localization were quantitatively analyzed in interspecies fusants and qualitatively analyzed in intraspecies mating.

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**TABLE 2.** White, but not opaque, cells of C. dubliniensis release a PMN chemoattractant

| Strain          | Cell phenotype-conditioned buffer | Instantaneous velocity (µm/min) | Chemokinetic stimulation (%) | C.I. | % Positive chemotaxis |
|-----------------|----------------------------------|---------------------------------|------------------------------|------|-----------------------|
| None            | fMLP*                            | 14.3 ± 4.6                      | 42                           | +0.46 | 87                    |
| None            | Bufferb                          | 10.2 ± 4.6                      | +0.03                        | 47   |
| ANSAS5 White    |                                  | 17.0 ± 1.4                      | 68                           | +0.43 | 91                    |
| ANSAS5 Opaque   |                                  | 10.6 ± 2.3                      | 5                            | 0.00  | 45                    |
| d81217 White    |                                  | 18.5 ± 2.3                      | 82                           | +0.62 | 93                    |
| d81217 Opaque   |                                  | 12.8 ± 2.2                      | 27                           | −0.01 | 48                    |
| d88014 White    |                                  | 20.9 ± 3.5                      | 106                          | +0.37 | 90                    |
| d88014 Opaque   |                                  | 12.2 ± 3.3                      | 21                           | +0.10 | 42                    |

* 10⁻⁷ M fMLP in source well (positive control).
  
  b Buffer alone in source well (negative control).
sis and C. albicans fusants. From the proportions of cells exhibiting different patterns, we have deduced the tentative temporal and spatial program of nuclear events accompanying interspecies mating. The nuclei that migrate into the bridge fuse and divide. One daughter nucleus then enters the daughter cell, while the other remains in the bridge. The nucleus in the bridge divides, and then the nuclei in the daughter cell divides. This scheme is similar to that deduced from a qualitative analysis of interspecies C. albicans and C. dubliniensis fusants (data not shown). The scheme is also remarkably similar to that of Saccharomyces cerevisiae (36).

Because interspecies mating and karyogamy occur so readily in vitro, it seems likely that the event may occur in nature, since mixed mycotic infections readily occur (44). However, although the two species can readily mate and undergo karyogamy, it is likely that recombination normally occurs in nature, given the divergence of the two species documented at the genetic level (10, 11, 19, 20, 49, 50, 51, 52). Experiments are now in progress to demonstrate at the genetic level whether the fused cells of interspecies mating generate viable allotetraploids similar to the autotetraploids generated by C. albicans fusion (5, 16, 28) and whether these cells revert to diploids by chromosome loss, as do C. albicans tetraploids generated by mating (5).

Conclusion. We have found that although C. dubliniensis possesses the same developmental programs as C. albicans, these programs appear to be less uniform, more variable between strains, and less efficient. It seems likely that this is a result of the high level of genetic instability characteristic of C. dubliniensis, due in part to increased frequencies of mitotic recombination (19). One may therefore consider C. dubliniensis to have lost the balance between mitotic recombination and genomic stability exhibited by C. albicans. Although the higher levels of recombination exhibited by C. dubliniensis could provide an advantage for rapid adaptation, it is obviously detrimental to the maintenance of its developmental programs and MTL heterozygosity in nature. This may explain why C. albicans, not C. dubliniensis, remains the dominant Candida species in human commensalisms and disease.

We have also found that C. dubliniensis and C. albicans, which can cohabit in the same body niches in nature, readily mate in vitro and on skin. The fact that these related species maintain genetic integrity in the same geographical locales suggests that even if they readily mate, they may not undergo recombination in nature. Alternatively, they may mate, undergo karyogamy, and recombine in nature, but the recombinants may be less competitive than either parent. These alternatives are now being tested.

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