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# A Stable Hybrid Containing Haploid Genomes of Two Obligate Diploid *Candida* Species

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***Candida albicans* and *Candida dubliniensis* are diploid, predominantly asexual human-pathogenic yeasts. In this study, we constructed tetraploid (4*n*) strains of *C. albicans* of the same or different lineages by spheroplast fusion. Induction of chromosome loss in the tetraploid *C. albicans* generated diploid or near-diploid progeny strains but did not produce any haploid progeny. We also constructed stable heterotetraploid somatic hybrid strains (2*n* + 2*n*) of *C. albicans* and *C. dubliniensis* by spheroplast fusion. Heterodiploid (*n* + *n*) progeny hybrids were obtained after inducing chromosome loss in a stable heterotetraploid hybrid. To identify a subset of hybrid heterodiploid progeny strains carrying at least one copy of all chromosomes of both species, unique centromere sequences of various chromosomes of each species were used as markers in PCR analysis. The reduction of chromosome content was confirmed by a comparative genome hybridization (CGH) assay. The hybrid strains were found to be stably propagated. Chromatin immunoprecipitation (ChIP) assays with antibodies against centromere-specific histones (*C. albicans* Cse4/*C. dubliniensis* Cse4) revealed that the centromere identity of chromosomes of each species is maintained in the hybrid genomes of the heterotetraploid and heterodiploid strains. Thus, our results suggest that the diploid genome content is not obligatory for the survival of either *C. albicans* or *C. dubliniensis*. In keeping with the recent discovery of the existence of haploid *C. albicans* strains, the heterodiploid strains of our study can be excellent tools for further species-specific genome elimination, yielding true haploid progeny of *C. albicans* or *C. dubliniensis* in future.**

Generally harmless commensals sometimes become virulent in humans with compromised immune systems. *Candida* species belong to such a class of opportunistic yeast pathogens in humans (1). *Candida albicans* is one of the most frequently isolated fungal species from immunocompromised patients (2). *Candida dubliniensis*, another species that belongs to the same CTG clade (1), is most closely related to *C. albicans* (3) but less efficient in colonization and tissue invasion (4, 5). Among the many common features that these two species share, the presence of the same key mating genes (*MTL* loci) is particularly striking, considering their mostly asexual nature of propagation (6–8). Under laboratory conditions, it was shown that *C. albicans* has a parasexual cycle, which provides an alternative pathway to generate strain diversity (9). An elaborate mating system promotes conjugation between mating-competent opaque cells homozygous (**a/a** or α/α) for opposite mating types in this species (10). The resulting tetraploid strains undergo random yet concerted chromosome loss in order to return to the diploid or a near-diploid state. Progeny strains resulting from this parasexual cycle showed altered morphology on laboratory media at different temperatures, demonstrating that this mode of propagation can produce phenotypic variants (11). Such an alternative pathway to meiosis was thus a means to promote a reduction in the ploidy state in this organism. Intriguingly, meiosis was not observed either in *C. albicans* or in *C. dubliniensis* even when certain tetraploid strains of *C. albicans* undergoing a parasexual cycle exhibited Spo11-dependent genetic recombination between homologous chromosomes (11).

While *C. albicans* is the primary cause of a wide spectrum of mucocutaneous diseases in the immunosuppressed host body, *C. dubliniensis* has also been implicated under such conditions (7). Several experiments revealed that the *MTL***a** and *MTL*α strains in each individual species (12) could be engineered to mate either *in vitro* or *in vivo* (13). However, despite establishing mating between *C. albicans* and *C. dubliniensis*, its occurrence in nature is yet unknown. Discovery of the *MTL***a**/*MTL*α locus in *C. dubliniensis* with similarly arranged genes homologous to *C. albicans* genes eventually led to demonstration of interspecies mating between the two, both in suspension and on mouse skin (8). Prior to the discovery of mating in *C. albicans*, somatic hybridization demonstrated that tetraploids could be formed by means of spheroplast fusion (14, 15), and the products of chromosome loss (induced by artificial means) were also cells with a diploid or close to diploid DNA content, indicating that random segregation of chromosomes can occur in tetraploids generated either by mating or by fusion of spheroplasted cells.

Since *C. albicans* and *C. dubliniensis* exhibit an amazing range of karyotypic rearrangements and can tolerate a substantial level of aneuploidy (16–18), an important aspect to study would be the mechanism of chromosome transmission during the parasexual mode of the cell cycle in these organisms. The process of chromosome segregation in mitosis and meiosis is largely powered by a dynamic kinetochore-microtubule interaction. The centromeres of *C. albicans* and *C. dubliniensis* chromosomes were identified to be the binding sites of their respective centromeric histone

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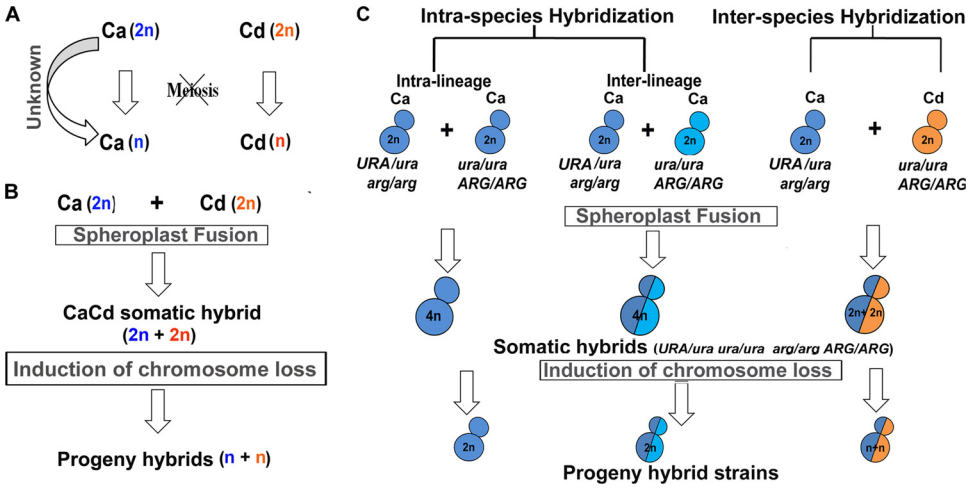


**FIG 1** Somatic hybridization followed by parasexual chromosome loss as a means to generate intra- and interspecies progeny hybrid strains. (A) Haploid *C. albicans* (Ca) or *C. dubliniensis* (Cd) strains cannot be generated by means of meiosis; however, haploid *C. albicans* strains can exist in nature, but the mechanism for this is unknown. (B) Schematic to create heterodiploid ($n + n$) strains carrying the haploid genome content of each species. (C) Strategy for constructing an intra- or interlineage tetraploid somatic product of *C. albicans* or a heterotetraploid hybrid between *C. albicans* and *C. dubliniensis*. These hybrid products were further used for generation of progeny strains after induction of chromosome loss using a method described before (9) (see Materials and Methods).

(CenH3) homologs, *C. albicans* Cse4 (CaCse4) and *C. dubliniensis* Cse4 (CdCse4) (2, 19). The properties of the centromeres in these organisms are different from those of the centromeres of most other species studied thus far, with each of the eight chromosomes carrying a unique *CEN* region rich in CenH3 molecules (20). Thus, each *CEN* can be used as a chromosome-specific marker to identify individual chromosomes of each of these two *Candida* species. Recently, it has been shown that even though the *CEN* regions are longer in *C. albicans* than *Saccharomyces cerevisiae*, only one microtubule is associated per kinetochore in both species (21). It is possible that the unique *CEN* properties along with the single kinetochore-microtubule interaction make the chromosome segregation machinery flexible, accommodating a wide range of variations in chromosome number of *C. albicans*.

A recent report suggests that *C. albicans* can rarely exist in the haploid state, which is unstable, and haploid isolates often switch to the diploid state (22). However, how these haploid strains originated remains unknown. In our study, we sought to investigate whether an altered ploidy state other than the diploid or tetraploid state can be created from a hybrid of *C. albicans* and *C. dubliniensis*. A haploid strain of either *C. albicans* or *C. dubliniensis* has not yet been generated by either a sexual or a parasexual process (Fig. 1A). In order to bypass this obstacle, a hybrid strain of these two diploid *Candida* species was created by spheroplast fusion (Fig. 1B). Subsequently, this somatic heterotetraploid hybrid can be induced to lose chromosomes through the process of parasexual reduction, which may generate a possible heterodiploid hybrid progeny strain with the haploid chromosome complement of each species. Here, we report on such a hybrid strain that carries either two sets (heterotetraploid) or one set (heterodiploid) of the chromosome complement of each of *C. albicans* and *C. dubliniensis* (Fig. 1C).

## MATERIALS AND METHODS

A complete description of all materials and methods can be found in the supplemental material.

**Animals.** Female BALB/c mice (*Mus musculus*) approximately 6 to 8 weeks old were maintained and bred under pathogen-free conditions.

**Ethics statement.** Use of mice was approved by the Animal Ethics Committee of Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India. All animal experimentations were performed according to the National Regulatory Guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

**Strains and media.** The strains of *C. albicans* and *C. dubliniensis* used in this study are listed in Table 1. J118 was constructed by deleting one copy of the *DAD2* (orf19.3551) gene (present on CaChr2) by *HIS1*, and the other copy was tagged by the tandem affinity purification (TAP) tag at the C terminus with *URA3* as the marker in strain BWP17. The resulting strain (J118) thus created is heterozygous for the *URA3* gene (23). These strains were grown in 1% yeast extract–2% peptone–2% dextrose (YPD) with 10 mg/ml uridine supplement [YPD(U)] for routine growth or in supplemented synthetic dextrose (SD) minimal medium as described previously (19). Chromosome loss induction was performed with *S. cerevisiae* presporulation (prespo) medium, which contains 1% yeast extract, 0.8% peptone, and 10% dextrose, and L-sorbose-containing medium (0.7% yeast nitrogen bases without amino acids, 2% L-sorbose, 2% agar), as described previously (9). Screening for the diploid hybrid progeny was performed in SD minimal medium supplemented with uridine and 5-fluoroorotic acid (5-FOA). The media used to observe growth and hyphal induction in this study were CHROMagar, Spider medium, and YPD plus serum. All details about the composition of these media have been provided elsewhere (24).

**Construction of hybrid strains.** Spheroplasts were isolated and fused in *S. cerevisiae* according to a standard protoplast fusion protocol (25), along with a few modifications. Approximately 50 ml (containing about $5 \times 10^7$ cells) of exponential-phase cultures grown in YPD(U) was harvested, washed by centrifugation, and suspended in spheroplasting buffer in each case. This suspension was treated with 0.01% β-mercaptoethanol and 20 μg/ml lyticase (Sigma Aldrich) and incubated at 30°C for 1 h. Spheroplasts of the two auxotrophic strains were mixed, harvested, treated with an equal volume of 30% polyethylene glycol (PEG; molecular weight, 3,350), and incubated at room temperature for 10, 20, or 30 min. Following PEG treatment, several washes were given to the mixed cell pellet in MP buffer (1 M sorbitol, 0.1 M NaCl, 0.01 M acetic acid). The

**TABLE 1** Strains used in this study[a]

| Strain | Genotype | Reference |
|---|---|---|
| SC5314 | Wild type | 37 |
| J118 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*his1*::*hisG*/Δ*his1*::*hisG* Δ*arg4*::*hisG*/Δ*arg4*::*hisGDAD2*-TAP *URA3*/*ORF19.3551*::*HIS1* | 23 |
| Wü284 | Clinical isolate | 38 |
| CdUM1A | *URA3-1*/*ura3*::FRT/*ura3* Δ*ura3*::FRT | 39 |
| CdUM4b | *ura3*Δ*1*::FRT/*ura3* Δ*ura3*::FRT | 39 |
| BWP17 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*his1*::*hisG*/Δ*his1*::*hisG* Δ*arg4*::*hisG*/Δ*arg4*::*hisG* | 40 |
| UBWP17 | BWP17 *RPS10*/*rps10*::*URA3* | This study |
| WUM1B | *URA3-1*/*ura3-2* Δ::MPA$^r$-FLIP | 41 |
| WUM5A | *ura3-1*::FRT/*ura3-2* Δ::FRT | 41 |
| RM1000 | CAI4 *ura3*::λ *imm434*/*ura3*::λ *imm434 his1*::*hisG*/*his1*::*hisG* | 42 |
| URM1000 | RM1000 *RPS10*/*rps10*::*URA3* | This study |
| HBT1 | Prototroph, tetraploid somatic hybrid of J118 and CdUM4b | This study |
| HBT2 | Prototroph, tetraploid somatic hybrid of J118 and CdUM4b | This study |
| CAT1 | Prototroph, tetraploid somatic hybrid of J118 and RM1000 | This study |
| CAT2 | Prototroph, tetraploid somatic hybrid of J118 and RM1000 | This study |
| CAT3 | Prototroph, tetraploid somatic hybrid of BWP17 and WUM1b | This study |
| CAP101 to CAP204 | *ura3*, progeny of CAT1 and CAT2 | This study |
| UCAP101 | CAP101 *RPS10*/*rps10*::*URA3* | This study |
| UCAP102 | CAP102 *RPS10*/*rps10*::*URA3* | This study |
| UCAP103 | CAP103 *RPS10*/*rps10*::*URA3* | This study |
| UCAP104 | CAP104 *RPS10*/*rps10*::*URA3* | This study |
| UCAP201 | CAP201 *RPS10*/*rps10*::*URA3* | This study |
| UCAP202 | CAP202 *RPS10*/*rps10*::*URA3* | This study |
| UCAP203 | CAP203 *RPS10*/*rps10*::*URA3* | This study |
| UCAP204 | CAP204 *RPS10*/*rps10*::*URA3* | This study |
| CAP301 to CAP321 | *ura3* progeny of CAT3 | This study |
| UCAP301 | CAP301 *RPS10*/*rps10*::*URA3* | This study |
| UCAP302 | CAP302 *RPS10*/*rps10*::*URA3* | This study |
| UCAP303 | CAP303 *RPS10*/*rps10*::*URA3* | This study |
| UCAP304 | CAP304 *RPS10*/*rps10*::*URA3* | This study |
| UCAP305 | CAP305 *RPS10*/*rps10*::*URA3* | This study |
| UCAP306 | CAP306 *RPS10*/*rps10*::*URA3* | This study |
| UCAP307 | CAP307 *RPS10*/*rps10*::*URA3* | This study |
| UCAP308 | CAP308 *RPS10*/*rps10*::*URA3* | This study |
| UCAP309 | CAP309 *RPS10*/*rps10*::*URA3* | This study |
| UCAP310 | CAP310 *RPS10*/*rps10*::*URA3* | This study |
| HBP1 to HBP30 | *ura3* progeny HBT1, derived from prespo and L-sorbose media | This study |
| UHBP1 | HBP1 *RPS10*/*rps10*::*URA3* | This study |
| UHBP2 | HBP2 *RPS10*/*rps10*::*URA3* | This study |
| UHBP3 | HBP3 *RPS10*/*rps10*::*URA3* | This study |
| UHBP4 | HBP4 *RPS10*/*rps10*::*URA3* | This study |
| UHBP5 | HBP5 *RPS10*/*rps10*::*URA3* | This study |
| UHBP6 | HBP6 *RPS10*/*rps10*::*URA3* | This study |
| UHBP7 | HBP7 *RPS10*/*rps10*::*URA3* | This study |
| UHBP8 | HBP8 *RPS10*/*rps10*::*URA3* | This study |
| UHBP9 | HBP9 *RPS10*/*rps10*::*URA3* | This study |
| UHBP10 | HBP10 *RPS10*/*rps10*::*URA3* | This study |
| UHBP11 | HBP11 *RPS10*/*rps10*::*URA3* | This study |

[a] FRT, flippase recombination target; MPA, mycophenolic acid; MPAr-FLIP, MPA resistant flipper. Ura$^+$ derivatives have U as a prefix to their names.

fused spheroplast suspension was subsequently mixed in the molten regeneration medium (0.67% yeast nitrogen base, 2% dextrose, 18.2% sorbitol, 1.2% agar) and finally overlaid on selective synthetic medium (complete medium [CM], uridine, arginine).

**Induced chromosome loss experiment.** To test for chromosome loss, the tetraploid hybrid strains obtained by somatic fusion were grown on different media, such as *S. cerevisiae* prespo medium and L-sorbose medium, and incubated at 30°C and 37°C for 7 to 15 days (9). Following incubation, cells were streaked on synthetic medium containing uridine and 5-FOA. Chromosome loss was observed in three independent experiments with cells grown on prespo or L-sorbose medium.

**Flow cytometry, cytological analysis, and indirect immunofluorescence.** Asynchronous cultures of the strains were grown in YPD(U) to an $A_{600}$ of 0.6 and processed for flow cytometry and other cytological analysis as described before (2). Intracellular Cse4 was visualized by indirect immunofluorescence as described previously (2) (details of the procedure are given in the supplemental material).

**ChIP assay.** Chromatin immunoprecipitation (ChIP) followed by PCR analysis was performed as described previously (19, 20).

**Virulence assay in mouse model for systemic candidiasis.** Cultures of strains used in the virulence assay were grown in YPD(U) to an $A_{600}$ of 1.000.

**Preparation of inoculum for injection.** BALB/c mice (females; age, 6 to 8 weeks; weight, approximately 20 to 22 g) were injected through the lateral tail vein (26). Approximately 10-ml cultures were centrifuged at 4,000 rpm for 5 min to pellet down the cells at room temperature. The cell pellets were resuspended in 10 ml 0.85% saline, and the actual concentration was then verified by counting the cells in a hemocytometer and by plating to determine the viable cell count. Different concentrations of cells ($5 \times 10^5$ cells/50 μl, $5 \times 10^6$ cells/50 μl) were intravenously injected into the tail vein of the mice. Each *Candida* strain was injected into four or five mice at each of the concentrations of cells indicated above. Survival was monitored after every 6 to 8 h. Readings were taken on the basis of at least four separate experiments with each strain tested. The survivability graphs for each strain in mice were constructed in GraphPad Prism software, where the number of days postinfection was plotted on the *x* axis and the percent survival of the individual mice was plotted on the *y* axis. Mean survival times were compared among the different strains by using the log-rank (Mantel-Cox) test (Prism software, version 5.0). *P* values calculated by this test along with the survival curves are indicated in Fig. 7.

**Microarray data accession number.** A custom comparative genome hybridization (CGH) microarray, the *C. albicans* and *C. dubliniensis* Cross-Species CGH microarray (4×180K), was designed using an Agilent platform (see the supplemental material). Microarray data have been submitted to the GEO database (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE24269.

## RESULTS

**An induced parasexual cycle in *C. albicans* and *C. dubliniensis* resulted in the generation of interspecific somatic hybrid progeny.** We performed PEG-mediated somatic hybridization between diploid *C. albicans* strains belonging to the same or different lineages (Fig. 2A and B; Table 2). Flow cytometry indicated that two strains, CAT1 and CAT2 (where CAT represents ***C. a**lbicans* **t**etraploid), obtained from intralineage fusion and CAT3, obtained from interlineage fusion, were tetraploids (Fig. 2, right; Table 1 and Table 2). Two culture conditions (9) were used to induce chromosome loss in them: *Saccharomyces cerevisiae* prespo and L-sorbose-containing synthetic media (at 30°C and 37°C). All the tetraploid strains had only one functional copy of the *URA3* gene on *C. albicans* chromosome 2 (CaChr2). To select for progeny that might have undergone parasexual chromosome loss, colonies from prespo and L-sorbose media were incubated in 5-FOA-containing medium supplemented with uridine. Loss of at least this chromosome should allow growth on the 5-FOA-containing medium. Four progeny strains were obtained from each of CAT1 grown on prespo medium (CAP101 to CAP104, where CAP represent ***C. a**lbicans* **p**rogeny) and CAT2 grown on L-sorbose medium (CAP201 to CAP204) (Table 2). Twenty-one progeny strains (CAP301 to CAP321) were derived from CAT3 (Table 2) after induced chromosome loss; of these, 18 were from prespo medium and the others were from L-sorbose medium. However, progeny strains isolated from either medium were phenotypically indistinguishable. Flow cytometric measurement of the DNA content of these *C. albicans* progeny strains (Table 2) indicated that the ploidy of these strains belonged to two classes: (i) 2*n* to 4*n* and (ii) ~2*n* (Fig. 3A; see Fig. S1A and B in the supplemental material).

Using a similar strategy, we generated interspecies hybrid strains of *C. albicans* and *C. dubliniensis*. Spheroplasts obtained from *C. albicans* strain J118 (*URA/ura arg/arg*), a derivative of BWP17, and *C. dubliniensis* strain CdUM4b (*ura/ura ARG/ARG*), a derivative of Wü284, were fused (Fig. 2C). The fusant cells first appeared to form syncytia, a large undivided, multinucleated mass

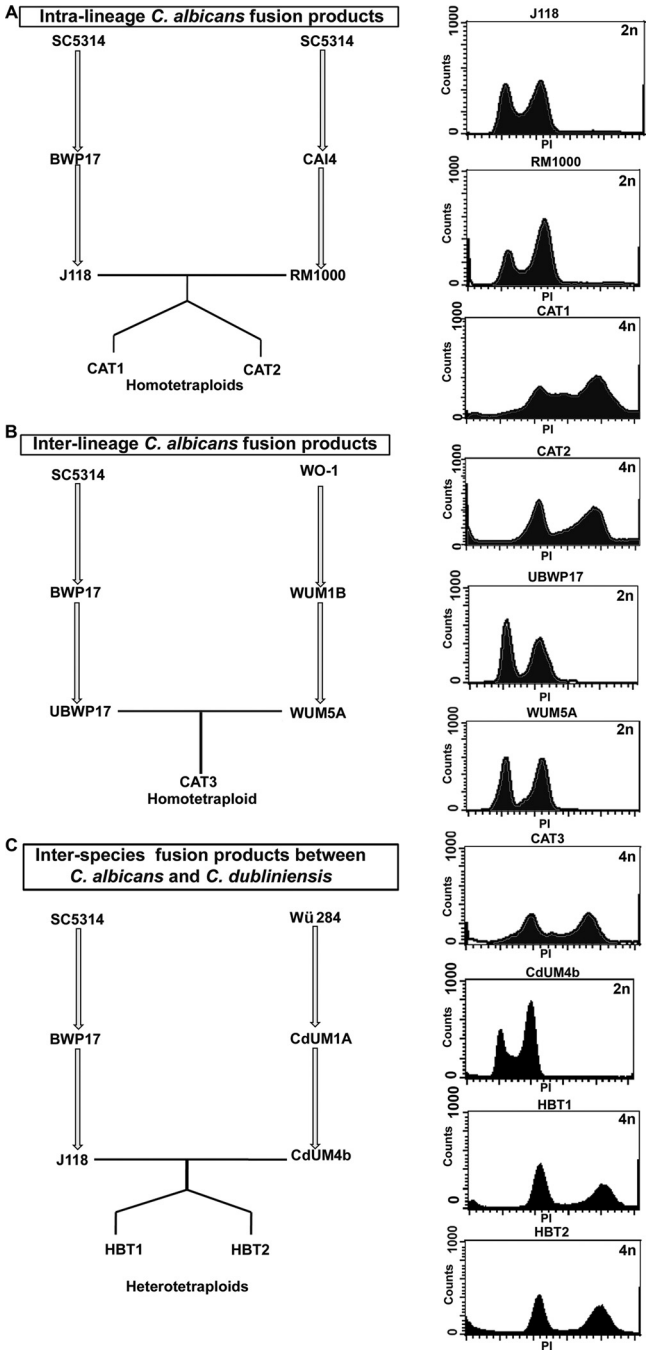


**FIG 2** Flow cytometry (fluorescence-activated cell sorting) analyses determined the ploidy of the various somatic hybrid strains. (A) Schematic representing intralineage spheroplast fusion between J118 and RM1000 generating homotetraploid fusion products CAT1 and CAT2. (B) Schematic representing interlineage spheroplast fusion between UBWP17 and WUM5A generating homotetraploid fusion product CAT3. (C) Schematic representing interspecies spheroplast fusion between J118 and CdUM4b generating heterotetraploid fusion products HBT1 and HBT2. The ploidy of all the parents and their respective hybrid strains was determined by fluorescence-activated cell sorter analyses (right). For ploidy analysis, cells were stained with PI to examine the cellular DNA content. The *x* axis of each graph (PI) represents a linear scale of fluorescence, and the *y* axis (counts) represents a linear scale of cell number.

**TABLE 2** Description of strains constructed in this study[a]

| Nature of fusion | Parent 1 | Parent 2 | Product | Progeny | Ura$^+$ progeny[b] |
|---|---|---|---|---|---|
| Intralineage | J118 (*C. albicans*) | RM1000 (*C. albicans*) | CAT1 (4*n*) | CAP101 to CAP104 | UCAP101 to UCAP104 |
| Intralineage | J118 (*C. albicans*) | RM1000 (*C. albicans*) | CAT2 (4*n*) | CAP201 to CAP204 | UCAP201 to UCAP204 |
| Interlineage | UBWP17 (*C. albicans*) | WUM5A (*C. albicans*) | CAT3 (4*n*) | CAP301 to CAP321 | UCAP301 to UCAP310 |
| Interspecies | J118 (*C. albicans*) | CdUM4b (*C. dubliniensis*) | HBT1 (2*n* + 2*n*) HBT2 (2*n* + 2*n*) | HBP1 to HBP30[c] | UHBP1 to UHBP11[c] |

[a] CAT, *C. albicans* tetraploid; CAP, *C. albicans* progeny from CAT; HBT, hybrid heterotetraploid of diploid *C. albicans* and *C. dubliniensis*; HBP, hybrid progeny from HBT.
[b] Ura$^+$ derivatives have U as a prefix to their names.
[c] Not determined.

of cells (see Fig. S2A in the supplemental material), completed nuclear fusion, and were subsequently found to be mononuclear (see Fig. S2A to D in the supplemental material). Analysis of propidium iodide (PI)-stained cells by flow cytometry indicated that two hybrid strains, HBT1 and HBT2 (where HBT represents **h**y**b**rid **t**etraploid; Table 2), were heterotetraploid (2*n* + 2*n*) (Fig. 2, right; see Fig. S3A in the supplemental material). The stability of these homo-and heterotetraploid hybrid strains was measured by passaging them in nonselective media for at least 30 generations, and the ploidy of the cells was examined before and after passaging by flow cytometry (see Fig. S4 in the supplemental material). No observable change in the ploidy states of these strains was seen even after passaging. By inducing chromosome loss, we further constructed hybrid progeny strains from HBT1 with reduced DNA content. Thirty progeny strains were obtained from HBT1 (HBP1 to HBP30, where HBP represents **hyb**rid **p**rogeny), with 24 strains being derived from prespo medium and 6 strains being derived from L-sorbose medium (Table 2). They belonged to two categories of ploidy content: (i) (*n* + *n*) to (2*n* + 2*n*) and (ii) close to (*n* + *n*) (Fig. 3B; see Fig. S1C in the supplemental material). We studied nuclear division in DAPI (4′,6-diamidino-2-phenylindole)-stained cells of these strains (Table 3) and found no visible nuclear segregation defects in any case.

When grown at 30°C in rich medium, colonies from different progeny strains displayed a wide range of phenotypes, from smooth (more of the yeast-phase cells) to wrinkled (more of the filamentous cells) colonies (Fig. 4A). Microscopic studies revealed the presence of both yeast and pseudohyphal cells in wrinkled colonies, while the smooth colonies had a majority of the yeast cells (see Fig. S5 and S6 in the supplemental material). In order to clinically demarcate the heterodiploid progeny strains from their wild-type and hybrid parents, we used CHROMagar, a commercially available chromogenic agar-based medium, and grew the strains at 37°C. *C. albicans* colonies were Persian green, and *C. dubliniensis* colonies appeared dark blue. Colonies of *C. dubliniensis* were significantly more slowly growing at 37°C than those of *C. albicans* (data not shown). HBT1 developed an intermediate coloration and formed a hyphal mat in medium (Fig. 4B). The heterodiploid progeny could easily be differentiated from their homodiploid counterparts.

PCR analysis with chromosome-specific *CEN* primer pairs (see Table S1 in the supplemental material) confirmed that HBT1 and HBT2 had each of the 8 chromosomes of *C. albicans* and *C. dubliniensis* (Fig. 3C; see Fig. S3 in the supplemental material). Of all the progeny hybrid strains derived from HBT1, 22 heterodiploid progeny strains with close to diploid DNA content retained all 16 *CEN* regions from both parents, indicating that at least one copy of each of the *C. albicans* and *C. dubliniensis* chromosomes was present. Similar analysis revealed that at least 6 progeny strains had lost both homologs of certain chromosomes of either *C. albicans* or *C. dubliniensis* (see Fig. S7 in the supplemental material). Since these strains were viable, cross-species functional complementation of lost chromosomes must have occurred. We tested the doubling time of each of these strains of various classes obtained through this process (see Table S2 in the supplemental material). An increase in the generation time of the tetraploid and heterotetraploid strains and their progeny strains (105 to 125 min) compared to that of *C. albicans* (95 min) or *C. dubliniensis* (90 min) was observed. It should be noted that this increase in doubling time observed in the hybrid progeny compared to that observed in their diploid parent strains was not due to the absence of the *URA3* gene.

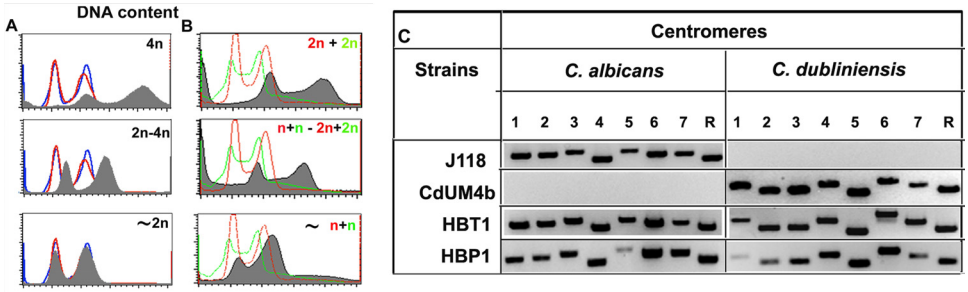


**FIG 3** Ploidy and species-specific chromosome identity of the progeny hybrid strains were determined by flow cytometry (fluorescence-activated cell sorting) and chromosome-specific PCR. (A) The fluorescence-activated cell sorter analysis profiles of parent *C. albicans* tetraploid strain CAT3 and its progeny strains represent two major types of ploidy states (gray histograms) among the homotetraploid progeny. Red and blue dotted lines, fluorescence-activated cell sorting profiles of diploid parents UBWP17 and WUM5A, respectively. The *x* axis of each graph represents the fluorescence intensity, and the *y* axis represents cell number. (B) The fluorescence-activated cell sorting profiles of HBT1 and its progeny strains represent two major types of ploidy states (gray histograms). Red and green dotted lines, fluorescence-activated cell sorting profiles of diploid parents J118 and CdUM4b, respectively. (C) Genomic DNAs isolated from the indicated strains were analyzed by PCR using centromere-specific primers of *C. albicans* (Ca*CEN*) and *C. dubliniensis* (Cd*CEN*) (see Table S1 in the supplemental material).

**TABLE 3** Percentage of cells with the indicated nuclear morphologies based on DAPI staining

| Strain | % cells | | | | |
|---|---|---|---|---|---|
| | | | Large budded | | |
| | Unbudded | Small budded | Properly segregated nuclei | Improperly segregated nuclei | Total no. of cells |
| J118 | 68 | 14 | 17.6 | 0.4 | 260 |
| CdUM4b | 21 | 45 | 34 | | 139 |
| RM1000 | 70 | 16 | 13 | 1 | 100 |
| UBWP17 | 75 | 10 | 15 | | 120 |
| WUM5A | 22.5 | 52.5 | 25 | | 200 |
| HBT1 | 55 | 24 | 18 | 3 | 293 |
| UHBP1 | 13 | 33 | 50 | 4 | 278 |
| CAT1 | 45 | 14 | 36 | 5 | 188 |
| UCAP102 | 53 | 28 | 13.4 | 5.6 | 142 |
| CAT3 | 34 | 48 | 13 | 5 | 258 |
| UCAP301 | 75 | 15 | 8 | 2 | 300 |

**Parasexual chromosome loss resulted in a euploid or aneuploid copy number of chromosomes.** To determine the copy number of each chromosome of *C. albicans* and *C. dubliniensis* in HBT1 (prototroph) and HBP1 (a Ura$^-$ heterodiploid progeny), we performed array comparative genome hybridization (aCGH) using custom-designed whole-genome microarrays representing the *C. albicans* and *C. dubliniensis* genomes (see Materials and Methods). It was evident that the copy number of all chromosomes in HBT1, based on *C. albicans*-specific probes (see Table S3 in the supplemental material), was close to 2 (range, 1.6 to 2.2) (Fig. 5A). We adapted *C. dubliniensis* probes based on the chromosome annotation that was submitted to EMBL (http://www.ebi.ac.uk/embl/) (3), and most of the chromosomes showed copy numbers close to 2 (range, 1.5 to 2.0), except for *C. dubliniensis* chromosome 4 (CdChr4) and CdChr6 (Fig. 5B; see Table S3 in the supplemental material). The *C. dubliniensis* annotated chromosome assembly (based on the sequenced strain, Cd36) was used here for the *C. dubliniensis* probes while designing the tiling arrays relative to each other. The relative copy number of most of the *C. albicans* and *C. dubliniensis* chromosomes (12 out of 16 chromosomes) in HBP1 was found to be close to 1 (Fig. 5C and D); the exceptions were for CaChr2, CdChr2, CdChr3, and CdChr4, where the values were found to be less than 1 (see Table S3 in the supplemental material). It is possible that these numbers do not actually reflect the absolute number of copies of specific chromosomes per cell; rather, they may indicate an overall greater variation in chromosomal composition in the population of cells of HBP1. This heterodiploid progeny was found to have half the genome content of the heterotetraploid (for *C. albicans* chromosomes, 0.56 ± 0.10; for *C. dubliniensis* chromosomes, 0.45 ± 0.13) (see Table S3 in the supplemental material). It is to be noted here that the *C. dubliniensis* parent strain used in this study is a derivative of Wü284, and as two different *C. dubliniensis* clinical isolates differ significantly in karyotype (27), the copy number differences observed in certain *C. dubliniensis* chromosomes of the hybrid genome can well be expected.

**The chromosomes of the hybrid strains segregate faithfully during mitosis.** Since high-fidelity chromosome segregation depends on proper centromere formation on a chromosome, we examined binding of the centromeric histones (CenH3) CaCse4 and CdCse4 at the centromeres of all the chromosomes from *C. albicans* and *C. dubliniensis* in the hybrid and its progeny strains. Indirect immunofluorescence microscopy using affinity-purified polyclonal anti-CaCse4 or anti-CdCse4 antibodies (against amino acid residues 1 to 18 of CaCse4) (2) revealed bright dot-like signals in all the cells (Fig. 6) of the interspecies hybrid as well as its representative progeny. The localization patterns appeared to be identical to those of CaCse4 in *C. albicans* (2) and CdCse4 in *C. dubliniensis* (19) at corresponding stages of the cell cycle (Fig. 6A and B). Coimmunostaining of fixed cells with antitubulin and anti-Ca/CdCse4 antibodies showed proper spindle morphology in the dividing cells, analogous to the typical localization patterns of kinetochore proteins in *C. albicans* and *C. dubliniensis*. Standard ChIP assays with anti-Ca/CdCse4 antibodies revealed enrichment of Cse4 on the *CEN* regions of *C. albicans* and *C. dubliniensis* in HBT1 and one of its progeny strains, HBP1 (see Materials and Methods). The immunoprecipitated DNA was assayed with a set of primer pairs designed to amplify *CEN* regions, where Cse4 binding was reported to be maximum (see Table S1 in the supplemental material). None of these *CEN* primers showed any cross-species PCR amplification (Fig. 3C). ChIP-PCR analysis revealed significant enrichment of CaCse4 and CdCse4 to their native *CEN* loci on all the respective chromosomes of the hybrid except in chromosome 4 of *C. dubliniensis* (Fig. 6C to F). Binding of the evolutionarily conserved kinetochore protein CaCse4 or CdCse4 to each of the species-specific *CEN* regions strongly suggests that the centromere identity of the chromosomes of both the species is maintained in these hybrid strains.

**Homotetraploid *C. albicans* strains show better fitness as pathogens than their heterotetraploid counterparts.** Virulence is a measurable trait of the biological fitness of pathogenic organisms. In this study, we sought to compare the biological fitness of these yeasts in their natural ploidy states with that in an altered one. Of all the biological processes thought to be related to virulence, the ability to show filamentous growth from yeast cells both *in vivo* and *in vitro* is considered to be an important trait in *C. albicans* and *C. dubliniensis* (5). Both grow as budding yeasts in standard growth medium, YPD, at 30°C. In response to various stimuli, for instance, elevated temperatures, the presence of serum, or nutrient starvation conditions, these yeasts switch to filamentous forms.

In this study, we compared the growth patterns of the hybrid strains with those of their diploid parent strains under 3 different temperature conditions: 30°C, 37°C, and 42°C (Fig. 4A). *C. albicans* tetraploids and their progeny strains showed proper growth at all the temperatures, with CAT1 and its UCAP102 progeny strain (Ura$^+$ derivatives have U as a prefix to their names) being significantly hyphal at 42°C (see Fig. S5 and S6 in the supplemental material). On the other hand, CdUM4b did not grow at this temperature at all, as expected from previous reports of *C. dubliniensis* (7). In contrast to their parents, cells of the heterotetraploid hybrid and its UHBP1 progeny were constitutively filamentous when grown at all these temperatures (see Fig. S6 in the supplemental material). Extensive filamentation in these two strains caused flocculation in broth at both 37°C and 42°C (Fig. 4A; see Fig. S6 in the supplemental material). These two strains essentially grew as budding yeast cells at a lower temperature, such as 28°C.

When they were compared for their colony morphologies in different morphology media (24), YPD plus serum at 37°C in-

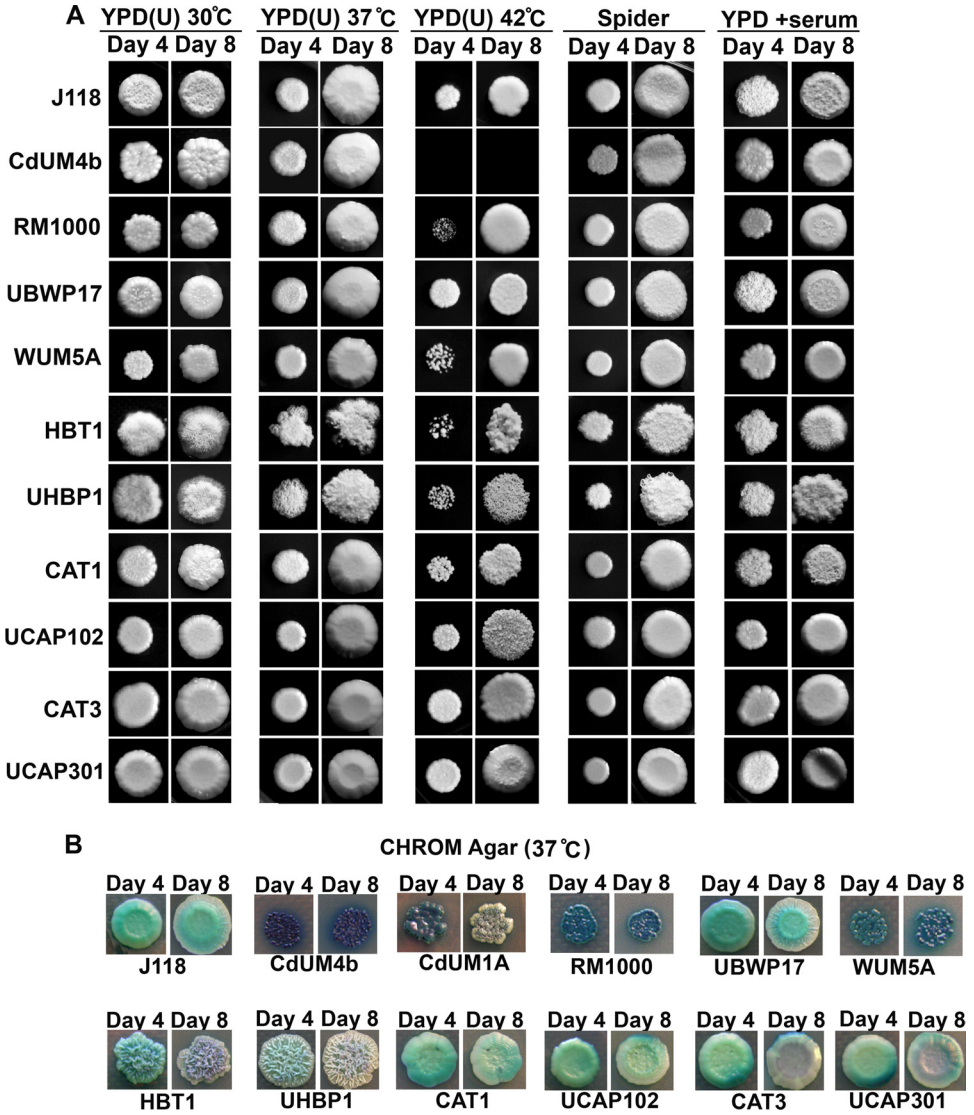


**FIG 4** Colony morphologies observed under different temperature conditions and in different growth media. (A) The heterotetraploid and its progeny strain show a wrinkled phenotype at all three temperatures in hypha-inducing morphology medium. CdUM4b did not show any growth at 42°C. (B) Colony morphologies observed in CHROMagar. The colony phenotype of each hybrid strain was a mix of the diploid parent morphologies. However, both the heterotetraploid and its heterodiploid progeny formed thick hyphal mats in this medium at 37°C, a morphology which was distinctly different from that of the intra- and interlineage strains.

duced wrinkled colony formation in the diploid *C. albicans* strains heterozygous for *URA3*, suggesting a mix of yeast and hyphal cells (Fig. 4A). All the *ura3* mutants grew as smooth colonies under similar conditions of growth. However, the tetraploid derivatives and their corresponding Ura$^+$ *C. albicans* progeny also grew as smooth colonies, suggesting the presence of a majority of yeast cells. In contrast to these strains, the heterotetraploid and its progeny strains always showed a wrinkled hyphal morphology in all these media. Here again we observed that they were mostly filamentous under the above-described conditions (Fig. 4A; see Fig. S5 and S6 in the supplemental material).

In keeping with these observations, we were interested to see if this filamentous growth phenotype affected the virulence of the strains in any way or not. As the uridine auxotrophs of *C. albicans* strains showed compromised pathogenicity, *C. albicans URA3* was integrated into the CaChr1 *RPS10* locus (28) of all the parent Ura$^-$ strains (see the Materials and Methods in the supplemental material). All animal experiments were performed with Ura$^+$ strains of both *C. albicans* and *C. dubliniensis*. A tetraploid *C. albicans* strain has previously been shown to be either as virulent as or less virulent than the diploid parent strains in a mouse model of systemic infection (29). We compared the virulence potential of the strains having altered ploidy with that of their diploid *Candida* parent strains. An intravenous dosage of $5 \times 10^5$ cells of SC5314 (*C. albicans*) was sufficient to kill mice within 12 days postinfection, whereas the same dosage of Wü284 (*C. dubliniensis*) cells could not (Fig. 7A). However, at a higher dosage of 5 million cells, both strains killed the animals within 3 to 4 days postinfection (Fig. 7B). Thus, a dosage of 5 million cells of each strain was administered intravenously in a murine systemic model of infection. The viru-

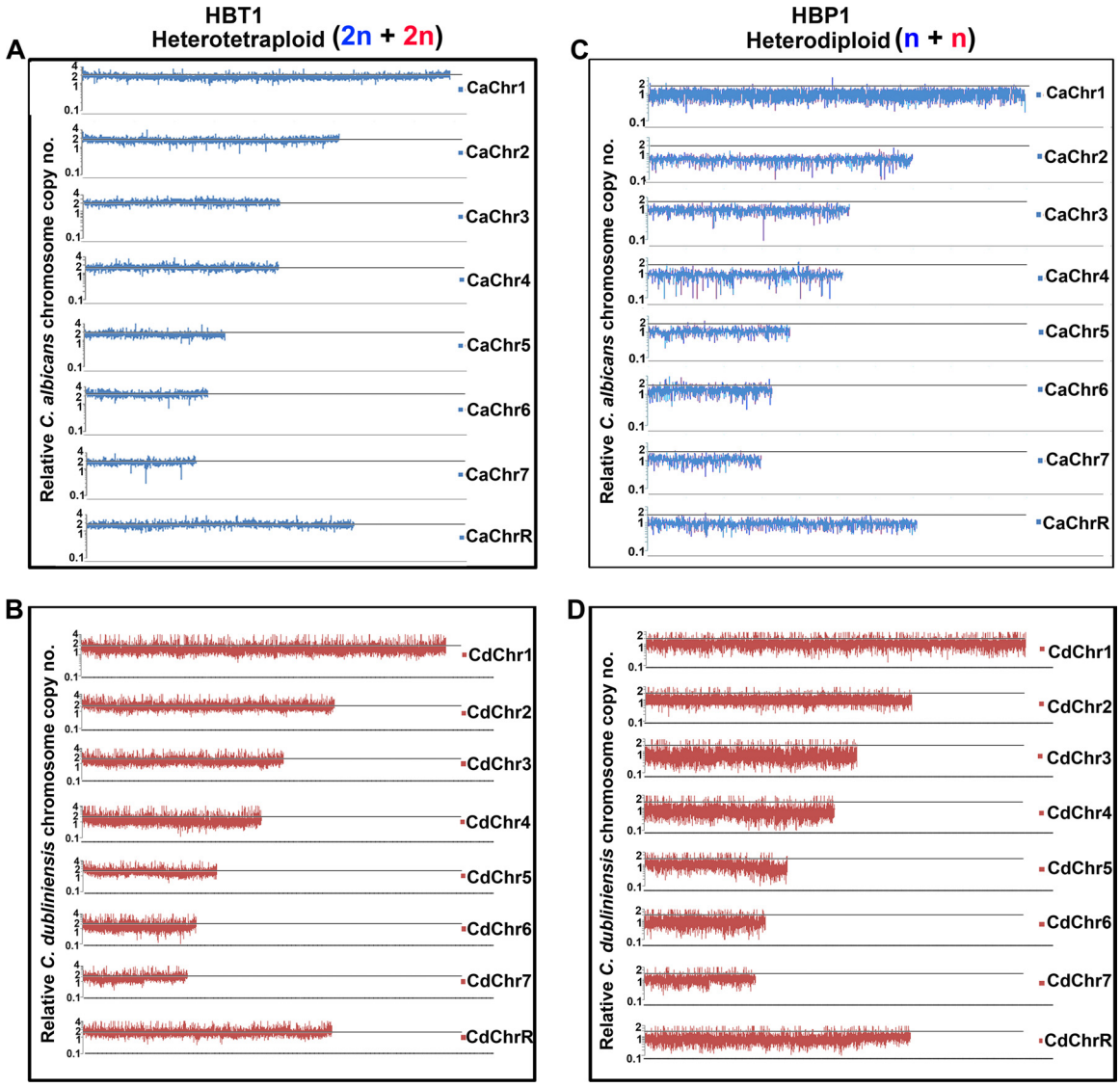


**FIG 5** Comparative genome hybridization analysis of the heterotetraploid and the heterodiploid progeny strains. aCGH analysis shows the relative copy number of each *C. albicans* (blue lines) and *C. dubliniensis* (red lines) chromosome in the heterotetraploid (HBT1) and heterodiploid (HBP1) hybrid strains. The copy number of each probe on a given chromosome was determined as described in the Materials and Methods in the supplemental material and plotted on the *y* axis against its position on the *x* axis. On the basis of the homology of the probes to the *C. albicans* sequence, the probes specific to *C. dubliniensis* were plotted as represented on chromosomes of the *C. albicans* genome. The relative *C. albicans* and *C. dubliniensis* chromosome copy number in HBT1 (A and B) and HBP1 (C and D) are shown.

lence potential of the intra- and interlineage tetraploids (CAT1, CAT2, and CAT3) was comparable to that of their wild types at this dosage (Fig. 7; see Table S4 in the supplemental material). Their progeny strains also showed a 100% mortality rate by a maximum of 2 weeks. Thus, the difference in ploidy did not seem to have any significant effect on the virulence potential of *C. albicans*. The virulence of interspecies tetraploid strain HBT1 (*C. albicans* and *C. dubliniensis*) was also similar to that of the intraspecies counterparts at this dosage in the animals. However, differences between the two were observed at a lower dosage. When $5 \times 10^5$ cells of HBT1 were injected into mice, they did not kill the animals, whereas the same dosage of *C. albicans* tetraploids did. This indicated that the virulence of the heterotetraploid hybrid carrying two sets of *C. albicans* chromosomes was less than that of the homotetraploid *C. albicans* strain (Fig. 7C; see Table S4 in the supplemental material). The virulence levels of the *URA3* integrated homodiploid and heterodiploid progeny strains were also compared (see Table S4 in the supplemental material). While the *C. albicans* hybrid progeny showed different levels of virulence in mice, the heterodiploid progeny set of strains from this lineage had, remarkably, become avirulent.

## DISCUSSION

*C. albicans* and *C. dubliniensis* are mostly obligate diploid yeasts. A haploid derivative of *C. albicans* or *C. dubliniensis* could not be generated by a sexual or parasexual process. In order to achieve this, we first adapted a method to generate both intra- and interlineage *C. albicans* tetraploid strains by somatic hybridization. In-

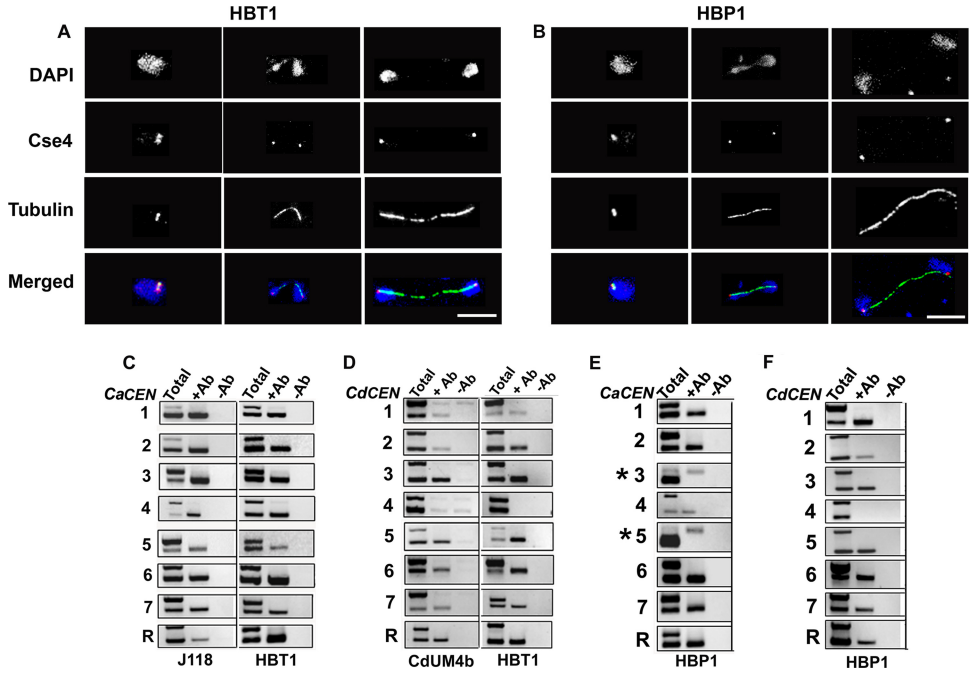


**FIG 6** The centromeric histone Cse4 binds to centromeres in both the heterotetraploid and heterodiploid hybrids. (A and B) Fixed cells of the indicated strains were stained with DAPI, anti-Cse4, or anti-tubulin antibodies. The corresponding spindle structures are shown by coimmunostaining with antitubulin antibodies. Bars, 10 μM. (C to F) ChIP assays were performed with anti-Ca/CdCse4 antibodies in the indicated strains. The DNA fractions obtained from the ChIP assays were analyzed by use of *C. albicans*-specific (Ca*CEN1* to Ca*CENR* [rows 1 to 7 and R, respectively]) (C and E) or *C. dubliniensis*-specific (Cd*CEN1* to Cd*CENR* [rows 1 to 7 and R, respectively]) (D and F) centromere primers from each of the 8 chromosomes of *C. albicans* or *C. dubliniensis*. A noncentromeric locus (Ca*LEU2* or Cd*LEU2*) was used as a negative control (top bands in each lane except in Ca*CEN3* and Ca*CEN5* in panel E, marked by asterisks); the bottom bands represent the ChIP DNA fractions representing *CEN* regions in total DNA (Total), DNA immunoprecipitated with anti-Cse4 antibodies (+Ab), and only beads without antibody (−Ab).

duction of chromosome loss generated stable diploid progeny *C. albicans* strains. We applied this strategy to develop a heterotetraploid strain, a hybrid of diploid *C. albicans* and *C. dubliniensis*. Surprisingly, induction of chromosome loss in this strain generated a major class of stable progeny strains that were heterodiploid, presumably carrying a haploid set of chromosomes of each species. aCGH analysis confirmed the haploidization of the chromosome content in the heterodiploid species. The hybrid strains maintained the centromere identity and segregated chromosomes stably through many generations. We demonstrated in this study that a *C. albicans* or *C. dubliniensis* strain can exist with only a haploid genome content of each species, as in a hybrid.

Having created such stable strains that differ in chromosome content and the ploidy state, we further examined the biological fitness of these strains using virulence as a measurable attribute. Using a murine model of systemic infection, we concluded that a strain carrying a diploid set of chromosomes of *C. albicans* or *C. dubliniensis* is almost always virulent either as an individual species or as a hybrid (the heterotetraploid strain), whereas heterodiploid progeny hybrids from this lineage carrying a haploid set of chromosomes of each species are avirulent. The loss of virulence in heterodiploid strains observed in this study could be due to loss of a functional allele of a virulence gene of each species. An alternative explanation could be a requirement for a diploid genome

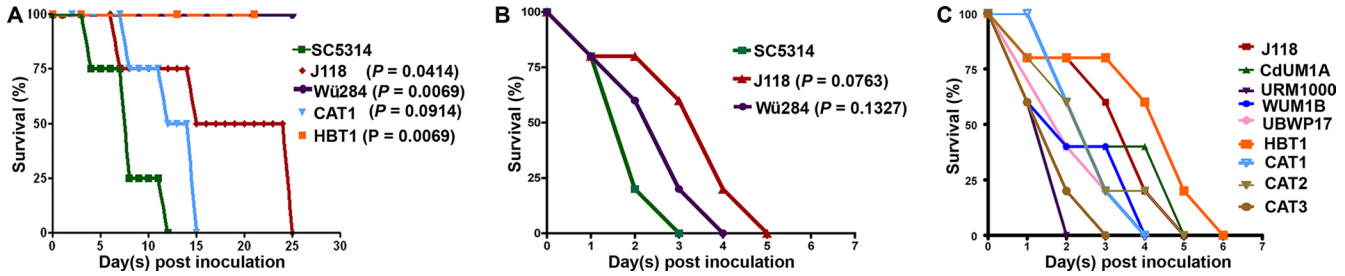


**FIG 7** Survival curves of female BALB/c mice intravenously challenged with various *Candida* strains. Female BALB/c mice were infected with the *Candida* strains as described in Materials and Methods. (A). Fifty microliters of saline containing $5 \times 10^5$ cells of SC5314, J118, Wü284, CAT1, or HBT1 was injected into the mice. (B) Saline containing $5 \times 10^6$ cells of each of SC5314, J118, and Wü284 was intravenously injected into the mouse tail vein. All strains (except the wild types) used for the virulence assay were heterozygous for *URA3* ($n = 5$). (C) Cells ($5 \times 10^6$) of each of the homo- and heterotetraploid strains were injected intravenously into the tail veins of the mice, and the virulence was compared with that of the parent strains.

content to maintain the dosage of genes of each species required for virulence. Subsequently, we examined other measurable fitness parameters in these strains, such as morphogenetic switching and growth under various conditions. While the heterotetraploid strains and most heterodiploid progeny strains showed no defects in switching, some heterodiploid strains showed a *C. dubliniensis*-like nonswitching phenotype. At 42°C, *C. dubliniensis* strains did not grow, but all the hybrid strains could proliferate without any significant delay.

This report describes several striking observations. First, there was a high propensity among the heterodiploid progeny strains to lose an entire haploid set of chromosomes from a species. Centromeres are clustered in *C. albicans* (2, 23, 30, 31) and *C. dubliniensis* (19). The 5-FOA selection performed in this study probably enriches a population of cells where centromere clustering is somehow compromised, leading to the loss of the *URA3*-containing chromosome. It has previously been shown that *S. cerevisiae* chromosomes physically interact with each other through similarly clustered centromeres (32). Assuming that an analogous interchromosomal interaction through clustered centromeres exists in *Candida*, loss of *URA3*-containing chromosome 2 might have made the cell prone to lose the chromosomes attached to it. Thus, although the mechanism of such a highly biased pattern of chromosome loss is uncertain, this finding suggests that loss of a single chromosome perhaps predisposes the genome to lose a complete haploid set of chromosomes. Hence, parasexual chromosome loss is more of a concerted rather than a random phenomenon (11). Based on the PCR-based analysis using the *CEN*-specific primers, we also found that some progeny strains lack both homologs of one of the two species. The frequency of loss of both homologous chromosomes of one species is also biased. These results suggest that cross-species complementation of an entire chromosome is possible for some chromosomes of each species.

Second, the heterodiploid hybrid progeny were as stable as the diploid *C. albicans* or *C. dubliniensis* strains, in terms of growth potential or viability, as the dosage of most genes was probably compensated for by the homologous genes of the other species. However, there was variation in ploidy between the intra- and interlineage homodiploid progeny population. This was probably due to the genome instability of the parent strains, which has already been documented (33). In terms of their virulence properties, 1 out of 10 progeny strains of CAT3 tested on mice did not kill the animals but the animals showed signs of chronic infection, while another strain took a longer time than the intralineage ones to show 100% virulence. On the contrary, the $Ura^+$ interspecies progeny strains remained avirulent even after *URA3* integration. Since all these strains grew well at 37°C, the reason for the reduced virulence of the two progeny strains described above (CAT3 derivatives) could be due to homozygosis of a mutant virulence-causing gene.

Third, the centromere identity of the chromosomes of each species is maintained in these hybrid strains. The hybrid condition did not reposition the centromeres, even though neocentromeres have a high propensity to be formed in *C. albicans* chromosomes (34). ChIP analysis revealed that the centromere location of chromosome 4 changed between two *C. dubliniensis* strains, Cd36 (19) and CdUM4b. It should be noted here that this type of centromere repositioning has been demonstrated previously (35) and may have implications in the evolution of the centromere location during speciation. Identification of the altered centromere location in chromosome 4 of *C. dubliniensis* strain CdUM4b can provide us with new insight into centromere repositioning in yeasts.

Finally, to our knowledge, this is the first demonstration of the centromere identity of a species being maintained even in an interspecies hybrid genomic context, suggesting that the chromosome-specific centromere formation of each species can be maintained even in the presence of two different Cse4 proteins in the hybrid strains.

A recent study demonstrated that *C. albicans* can exist in the haploid state. Even though the haploid state seems to be unstable, under a controlled condition, the haploid *C. albicans* strain can propagate and even mate (22). However, in the absence of meiosis, the process that triggers haploidization of this largely obligate diploid organism is still an enigma. We can expand the prospects of this discovery and explore if a haploid strain of either species can be generated in a strategized manner or not. It has been shown that haploid fertile *Arabidopsis thaliana* plants can be developed by manipulating the centromere-specific histone, CENH3 (36). A similar strategy of deleting CenH3 (Cse4) of one species in the heterodiploid hybrid *Candida* strain that we created in this study may result in uniparental chromosome loss to generate a pure haploid *C. albicans* or *C. dubliniensis* strain in future.

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ERRATUM

# A Stable Hybrid Containing Haploid Genomes of Two Obligate Diploid *Candida* Species

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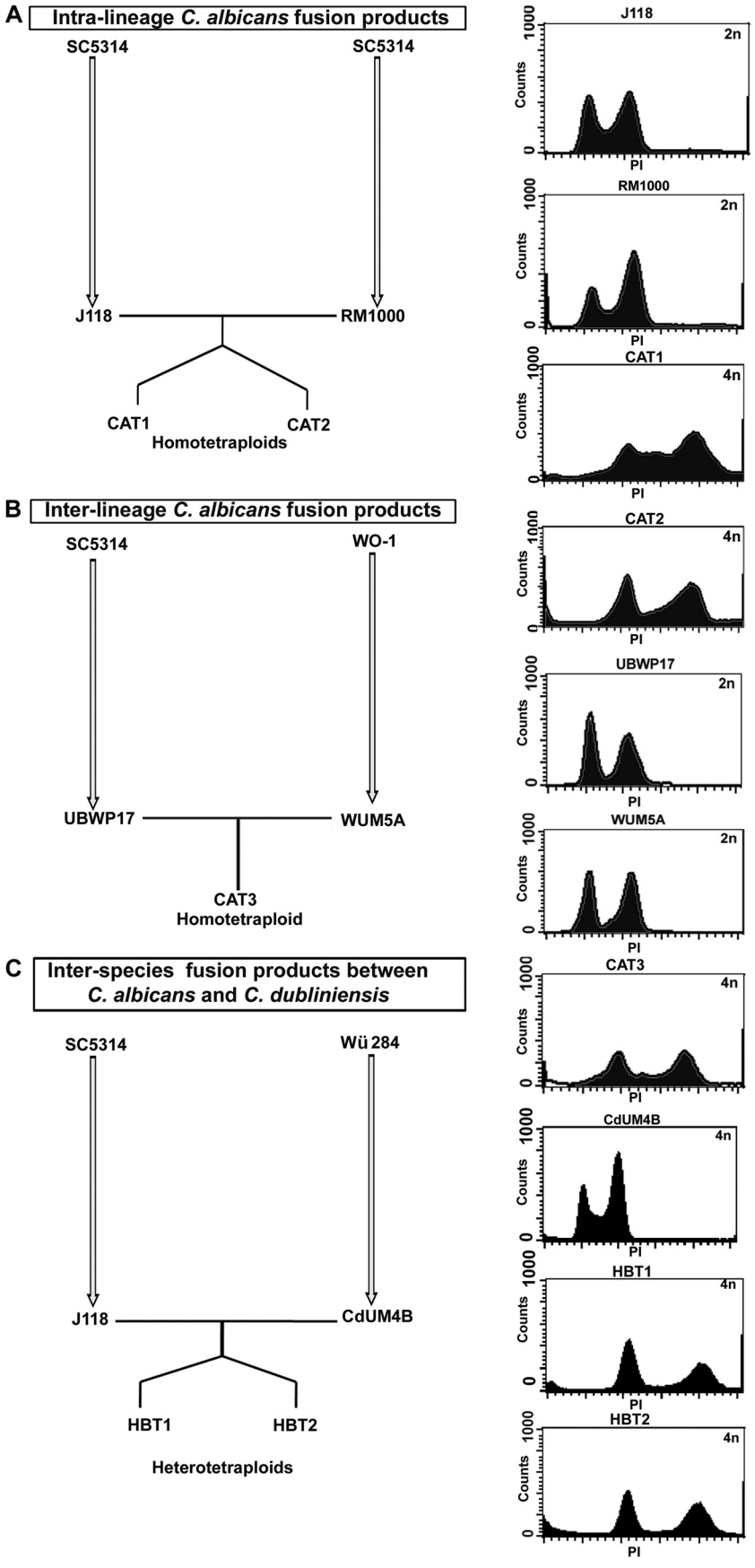
Volume 12, no. 8, p 1061–1071, 2013. The name of strain CdUM4b should read as CdUM4B throughout the paper and the supplemental material.

Page 1063: Table 1, the genotype of strain CdUM1A should be *ura3*Δ*1*::*MPA*$^{r}$*-FLIP/URA3* and the genotype of strain CdUM4B should be *ura3*Δ*1*::*FRT/ura3*Δ*2*::*FRT*.

Page 1064: Figure 2, panels B and C are incorrect. The correct figure should appear as shown below.


doi:10.1128/EC.00204-13


A
Intra-lineage C. albicans fusion products
SC5314
SC5314
J118
RM1000
CAT1
CAT2
Homotetraploids
B
Inter-lineage C. albicans fusion products
SC5314
WO-1
UBWP17
WUM5A
CAT3
Homotetraploid
C
Inter-species fusion products between C. albicans and C. dubliniensis
SC5314
Wü 284
J118
CdUM4B
HBT1
HBT2
Heterotetraploids
J118
2n
RM1000
2n
CAT1
4n
CAT2
4n
UBWP17
2n
WUM5A
2n
CAT3
4n
CdUM4B
4n
HBT1
4n
HBT2
4n
Counts
1000
0
PI