Evolution of Mating within the *Candida parapsilosis* Species Group†‡

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*Candida parapsilosis* and *Candida metapsilosis* are closely related to *Candida parapsilosis*, a major cause of infection in premature neonates. Mating has not been observed in these species. We show that ~190 isolates of *C. parapsilosis* contain only an *MTLa* idiomorph at the mating-type-like locus. Here, we describe the isolation and characterization of the *MTL* loci from *C. parapsilosis* and *C. metapsilosis*. Among 16 *C. orthopsilosis* isolates, 9 were homozygous for *MTLa*, 5 were homozygous for *MTLα*, and 2 were *MTLa/α* heterozygotes. The *C. orthopsilosis* isolates belonged to two divergent groups, as characterized by restriction patterns at *MTL*, which probably represent subspecies. We sequenced both idiomorphs from each group and showed that they are 95% identical and that the regulatory genes are intact. In contrast, 18 isolates of *C. metapsilosis* contain only *MTLa* idiomorphs. Our results suggest that the role of *MTL* in determining cell type is being eroded in the *C. parapsilosis* species complex. The population structure of *C. orthopsilosis* indicates that mating may occur. However, expression of genes in the mating signal transduction pathway does not respond to exposure to alpha factor. *C. parapsilosis* is also nonresponsive, even when the GTPase-activating protein gene SST2 is deleted. In addition, splicing of introns in *MTLa1* and *MTLa2* is defective in *C. orthopsilosis*. Mating is not detected. The alpha factor peptide, which is the same sequence in *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*, can induce a mating response in *Candida albicans*. It is therefore likely either that mating of *C. orthopsilosis* takes place under certain unidentified conditions or that the mating pathway has been adapted for other functions, such as cross-species communication.

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*Candida parapsilosis* is a nosocomial fungal pathogen, and infection is most common in neonates, transplant patients, and patients receiving parenteral nutrition (56). *Candida parapsilosis* is closely related to *Candida albicans* and is a member of the CTG clade (species in which CTG is translated as serine rather than leucine) (12, 22, 51). *C. parapsilosis* isolates historically have been categorized as group I, II, or III (24, 34, 39). However, molecular fingerprinting and mitochondrial genome architectures have shown that these groups correspond to three species now known as *C. parapsilosis* (formerly group I), *C. orthopsilosis* (group II), and *C. metapsilosis* (group III) (61, 69) (Fig. 1A).

The CTG clade contains a number of fully sexual haploid species, such as *Candida lusitaniae* and *Candida guilliermondii*, and a number of diploid species (e.g., *Candida tropicalis*, *Candida dubliniensis*), in which a fully sexual cycle has never been observed (6, 9, 10, 12, 37). In *C. albicans*, the discovery of a mating-type-like (*MTL*) locus that resembled the *Saccharomyces cerevisiae* MAT locus was the first indication that a sexual cycle may be present (29). There are two *MTL* idiomorphs in *C. albicans*. The *MTLa* idiomorph encodes a homeodomain protein, a1, and an HMG domain protein, a2 (11, 73). The *MTLa* idiomorph encodes a1 and a2 proteins; a1 is required for expression of α-specific mating genes (73). In a heterozygous diploid a/a cell, the α1/α2 heterodimeric complex represses expression of mating genes (73). Both idiomorphs carry alleles of a poly(A) polymerase gene (*PAP*), an oxysterol binding protein gene (*OBP*), and a phosphatidylinositol kinase gene (*PIK*), which were acquired early in the evolution of the CTG clade but have no known function in mating (12).

Discovery of the *MTL* loci was shortly followed by experimental evidence for mating between diploid cells of opposite mating types, although initially observed to occur at a very low frequency (30, 50). Subsequent studies showed that the mating frequency is greatly increased when cells undergo a phenotypic switch from white to opaque cells (52). The switch to opaque cells was first observed in the clinical isolate WO-1 by Slutsky et al. (67). Opaque cells are elongated and have “pimples” present on the cell surface. They also absorb the dye phloxine B, resulting in pink colonies, whereas white cells cannot take up the dye (4). The white/opaque switch is regulated by *WOR1*, whose expression is repressed by α1/α2 (28, 68, 78). *WOR1* regulates own expression and also expression of *EGF1*, *WOR2*, and *CZF1* (79). This circuit results in high levels of Efg1 in white cells and of Wor1 in opaque cells (79). The white/opaque switch is also regulated by many environmental stimuli, such as temperature (47), oxygen and carbon dioxide concentrations (27, 58), and genotoxic and oxidative stress (1). Opaque cells are more stable at low temperatures, which favors mating on the surface of the skin (21, 35). At body temperature, opaque-phase cells are stabilized under low oxygen and high carbon dioxide conditions (27).

Many of the genes required for mating in *S. cerevisiae* are conserved in the genomes of *C. albicans* and other species in the CTG clade (12, 14, 49). These include the diffusible mating pheromones, α-factor and α-factor. In *C. albicans*, α-factor pheromone is produced by *MTLa* cells and is sensed by the receptor protein Ste2 on *MTLa* cells (7, 42, 55). Conversely,
FIG. 1. Organization of MTL in C. orthopsilosis. (A) Phylogenetic relationship of the C. parapsilosis species group. The phylogenetic tree was inferred from an alignment of ITS sequences and was drawn using the PhyML package in SeaView (25). Sequences were obtained from GenBank (accession numbers AY391845.1, AJ635316.1, AJ698048.1, and AJ698049.1). (B) Organization of the MTLa idiomorphs (top) and MTLα idiomorphs (bottom). Genes are drawn approximately to scale. Sizes of the idiomorphs in type 2 isolates are shown. (C, top) The entire MTL idiomorphs were amplified from type 1 and type 2 isolates of C. orthopsilosis by using primers from within GAP1 and orf19.3202. The PCR products were digested with EcoRI. (Bottom) Amplification of MTLa-specific products (a lanes) and MTLα-specific products (b lanes). The order of the isolates in both panels are as follows. Type 1: lane 1, CP25; lane 2, CP47; lane 3, CP288; lane 4, CP354; lane 5, 942211K; lane 6, CP85; lane 7, CP124; lane 8, CP289; lane 9, J981224. Type 2: lane 10, CP269; lane 11, CP287; lane 12, CP296; lane 13, 90-125; lane 14, CP185; lane 15, CP331; lane 16, CP125; lane M1, 1-kb Plus ladder from Invitrogen. (D) Alignment of MTLa1 from type 1 and type 2 isolates of C. orthopsilosis, in comparison with the ortholog from C. albicans. The sequences were aligned using T-Coffee (54) and visualized with Boxshade.
a-factor is produced by MTLα cells, and MTLα cells respond via the receptor Ste3 (18). Exposure of opaque cells to mating pheromones results in shmoo formation and the expression of genes required for mating (7, 42, 55). Although white-phase cells do not generate shmoo, they do have a well-characterized response to α-factor. Daniels et al. (17) showed that α-factor induces adhesiveness and biofilm formation in white cells. Biofilms containing white-phase cells promoted chemotropism between the rarer opaque a/a and α/α cells and therefore enhanced mating. The white cell response to α-factor is mediated via the same pathway as the opaque response except for the final downstream targets (62, 76, 77). However, white/opaque switching is confined to the very closely related species C. albicans and C. dubliniensis and has no role in mating in other species in the CTG clade (62).

The mating signal transduction pathway is generally conserved in C. parapsilosis (12). However, MTLα is a pseudogene (46), and we show here that the majority (and possibly all) organization of MTL is served in C. albicans/orthopsilosis. We also show that the pseudogenization of MTLα is a recent event that is specific to P. parapsilosis but that the role of MTL appears to be degenerating in all the species. We also show that Candida species may communicate via α-factor and the mating signal transduction pathway. However, we did not observe mating in any of the C. parapsilosis group.

MATERIALS AND METHODS

Species and strains. The isolates used are listed in Table S1 of the supplemental material. The majority of isolates of C. parapsilosis were obtained from Portugal (A. Rodrigues [64]) and Hungary (A. Gacser). Some isolates originated from Italy (S. Senesi), Belgium, the United Kingdom, the United States, and Hong Kong.

Media and reagents. Alpha pheromones KPHWTTGYYYEPQ (C. parapsilosis) and GFRFTNFYGFPEF (C. albicans) were synthesized by CS Bio Co. Strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose; Formedium) prior to DNA extraction. For colony selection, 2% agar was used. Transformants were selected on YPD agar containing nourseothricin (Werner Bioagents, Jena, Germany). Mating experiments were carried out on various media (described in Table S3 of the supplemental material). Mating products were isolated on the appropriate dropout medium using either YPD broth without amino acids and ammonium sulfate, plus 2% dextrose, 2% agar, 0.5% ammonium sulfate, and 0.75% amino acid mix. Oligonucleotide primers used for PCR and other applications are listed in Table S2 of the supplemental material.

Identification of MTLα and MTLα idiophoroms from C. orthopsilosis and C. metapsilosis. A fosmid library was generated from C. orthopsilosis 90-125 (type 2) and C. metapsilosis ATCC 96143 by Agowa (Germany), and the ends of 2,000 clones were sequenced. One fosmid insert from each was sequenced in full, and these were predicted to contain the MTLα based on conserved synteny with C. parapsilosis and other Candida species. MTLα was isolated from C. orthopsilosis, and MTLα was from C. metapsilosis. A second MTLα idiophorom was amplified by PCR from C. orthopsilosis 3981224 (type 1) by using oligonucleotide primers BUT252 (from within an ortholog of opf79.2029) and BUT284 (3’ to GAPI) and a long-template PCR kit from Roche. MTLα idiophoroms were amplified from C. orthopsilosis CP289 (type 1) and CP185 (type 2) using primers BUT282 and DL04 (within GAPI).

Sixteen isolates of C. parapsilosis were categorized by digestion with EcoRI. The MTLα idiophoroms were amplified using primers BUT282 and DL04. Mating type was confirmed using primers specific for each subtype [MTLa1p(T1)FP and MTLα1p(T1)RP within MTLα1 type 1, CO3 and CO6 within MTLα1 type 1, MTLα1p(T2)FP and MTLα1p(T2)RP within MTLα1 type 2, and MTLα1(T2)FP(a) and MTLα1(T2)RP within MTLα1 type 2].

Screening of MTL in C. parapsilosis isolates. Degenerate PCR was designed in an alignment of sequences from C. albicans, C. tropicalis, C. orthopsilosis, and C. metapsilosis by using iCODEHOP v10.8 and Biocldt v7.0 (http://www.mbio.ncsu.edu/biocldt/biocldt.html). Two sets of primers were designed. DMTLα1pF1B and DMTLα1pRI3 amplify a 500-bp fragment from MTLα1 from three of the test species (excluding C. tropicalis). DMTLα1pF3B (within PAPs) and DMTLα1pRI4 (within MTLα1) amplify a 750-bp fragment from all the test species. MTX3 and MTX6 (which amplify a fragment from within MTLα1 in C. parapsilosis [46]) were used as a positive control. All of a total of 120 clinic isolates of C. parapsilosis from Portugal, 63 isolates from three sites in Hungary, and 6 isolates from Germany, Italy, and Puerto Rico were screened (see Table S1 of the supplemental material). All isolates in which a putative MTLα-specific product was amplified were checked by sequencing the equivalent internal transcribed spacer (ITS) region (oligos ITS4 and ITS5; [see Table S2 of the supplemental material]). All were derived from species incorrectly identified as C. parapsilosis (see Table S1).

Screening of MTL in C. parapsilosis isolates. Degenerate PCR primers were designed from an alignment of MTLα2a sequences. Primers MTLα2D2F and MTLα2D2R amplify a fragment of approximately 260 bp from the test species. Eighteen isolates of C. parapsilosis were screened. Any amplified fragments were cloned in vector pCR4-TOPO (Invitrogen Life Technologies) and sequenced. None corresponded to MTLα sequences (data not shown).

Screening of gene deletions in C. parapsilosis and C. orthopsilosis. To delete SST2 (gapr910), a 502-bp upstream region was amplified by PCR from C. parapsilosis CLIB214 genomic DNA using GoTaQ DNA polymerase (Promega) and the oligonucleotide primers CpsSST2KO1 and CpsSST2KO2, which contain recognition sites for KpnI and Apal, respectively (see Table S2 in the supplemental material). A 501-bp downstream fragment from SST2 was also amplified using the primers CpSST2KO3 and CpSST2KO4, which contain recognition sites for SacII and SaeI. The PCR products were purified using a Qiagen PCR purification kit and ligated at either end of a SAT1 flipper cassette in pCD8 (20), generating plasmid pCPST2. The entire cassette plus flanking regions was excised with digestion with KpnI and SacI, gel purified, and transformed into C. parapsilosis CLIB214. Integration of the cassette at SST2 was confirmed by PCR using a primer 5’ to SST2 (CpsSST2KO5) and a primer from inside the cassette (BUT237) (1.7 kb). Intact SST2 alleles were identified by PCR with CpsSST2KO5 and CpsSST2KO6 (1.2 kb). Primers CpsSST2KO1 and CpsSST2KO4 were used to verify recycling of the cassette and deletion of the SST2 gene (1.0 kb). The same cassette was used to delete both SST2 alleles. The deletion was also confirmed by Southern blotting, using approximately 20 µg of RNase-treated genomic DNA digested with ScaI. A probe was amplified from C. parapsilosis CLIB214 genomic DNA by using primers SST2For and SST2Rev, which amplify a region upstream of SST1 (approximately 1 kb). Transformation of C. parapsilosis CLIB214 genomic DNA by using primers SST2For and SST2Rev, which amplify a region upstream of SST2. The probe was labeled, and Southern blotting was performed using the DIG High Prime labeling and DIG detection starter kit II (Roche).

A similar method was used to delete HIS1 and LEU2 from the C. orthopsilosis strains CP47 (MTLα) and CP289 (MTLα), respectively. Primers CoHIS1KO1/CoLEU2KO1 and CoHIS1KO2/CoLEU2KO7 were used to amplify an upstream region of either HIS1 or LEU2 (approximately 500 bp). Primers CoHIS1KO3/CoLEU2KO3 and CoHIS1KO4/CoLEU2KO4 were used to amplify a region downstream of either HIS1 or LEU2 (approximately 1 kb). The cassette at the HIS1 or LEU2 allele was confirmed by PCR using a primer 5’ to the gene (CoHIS1KO5/CoLEU2KO9) and a primer from inside the cassette (BUT237) (approximately 1.6 kb). To confirm the presence of intact HIS1 or LEU2 alleles, the primer pairs CoHIS1KO5/CoLEU2KO9 and CoHIS1KO6/CoLEU2KO6 were used (approximately 1.2 kb). Primers CoHIS1KO1/CoLEU2KO1 and CoHIS1KO4/CoLEU2KO4 were used to verify recycling of the cassette and deletion of the HIS1 and LEU2 genes (approximately 1.1 kb).

Transformation of C. parapsilosis and C. orthopsilosis and recycling of the SAT1 flipper cassette. Strains were transformed by electroporation as described previously with some modifications (20). After electroporation, 950 µl of fresh YPD was added immediately and the mixture was incubated at 30°C for 3 to 4 h. Following incubation cells were pelleted, washed once in 1 ml of water, and resuspended in 300 µl of water. A 100-µl aliquot was plated onto YPD plates supplemented with nourseothricin at a concentration of 200 µg ml⁻¹. Transformants were obtained following 48 h of incubation at 30°C. The cassette was recycled from positive transformants by growth overnight in YPM (1% yeast extract, 2% peptone, and 2% maltose). One hundred cells were plated onto YPD plates containing 10 µg ml⁻¹ of nourseothricin and incubated overnight at 30°C. Following incubation, a mixture of large and small colonies was visible on the plate. Small colonies were restreaked onto fresh YPD agar plates and checked for nourseothricin sensitivity. The second allele was deleted using the same protocol.

Shmoo formation. Opaque-phase cells of C. albicans CA12 (st2 deletion) and RBY1220 (bar1 deletion) were grown overnight at 25°C. Cultures were then
resuspended to an $A_{600}$ of 0.2 in fresh SpiderM medium supplemented with 10% dimethyl sulfoxide (DMSO). C. albicans α-factor (10 μg ml$^{-1}$), or C. parapsilosis α-factor (10 μg ml$^{-1}$) and incubated for 6 h at 25°C with shaking. From each culture, 500 μl was centrifuged, washed in 1 ml of water, resuspended in 100 μl of calcifluor white (1 mg ml$^{-1}$ in 10 mM NaOH), and incubated at room temperature for 10 min. Images were obtained using a ColorView II camera (Soft Imaging Systems) mounted on an Olympus BX40 fluorescence microscope with analYSIS software.

**Mating assays.** Control mating experiments were carried out using both white- and opaque-phase cells of C. albicans RBY1118 (MTLα his1/1 leu2/2) and RBY1180 (MTLα/a arg4/a) (65). Strains were cross-streaked on SpiderM agar plates and incubated for 3 days at room temperature. The cultures were replica plated to minimal medium lacking histidine, leucine, and arginine and incubated for several days at room temperature to observe mating products. Similar experiments were carried out using C. parapsilosis CDH4 (MTLα/a his1/1) and C. parapsilosis CDU1 (MTLα/a ura3/1a3). C. orthopsilosis LH11 (MTLα/a his1/1) and C. orthopsilosis LHL1 (MTLα/a leu2/2) and other combinations were also crossed (see Table S3 in the supplemental material). Cells of opposite mating type of C. orthopsilosis (2x10$^6$) were also mixed and incubated under various conditions before plating on selective media. No mating products were detected. Mating experiments were carried out at various temperatures and multiple media (see Table S3). Cells were incubated from 4 to 12 days prior to plating on selective agar.

**Analysis and extraction of RNA.** C. parapsilosis CLIB214, C. parapsilosis LHS1 (stt2α), and C. orthopsilosis CP47 were grown in SpiderM overnight at 30°C with shaking. Opaque cells of C. albicans CA12 (stt2α) and RBY1220 (bar1α) were incubated in the same medium overnight at 25°C. All cultures were diluted to an $A_{600}$ of 0.2 in fresh SpiderM medium prior to incubation at the appropriate temperature. C. albicans strains were incubated at 25°C (three biological replicates). C. parapsilosis and C. orthopsilosis strains were incubated at 25°C (two biological replicates), 30°C (three biological replicates), and 37°C (two biological replicates). Cultures were grown for 2 h followed by addition of synthetica alpha factor from C. parapsilosis and C. albicans at a final concentration of 10 ng ml$^{-1}$ or DMSO and incubated for a further 4 h at the appropriate temperature. Following incubation, cells were pelleted, resuspended in 100 μl of RNAlater (Ambion), and stored at −80°C. RNA was extracted using a RiboPure yeast kit from Ambion. A supplemental treatment with DNase I was carried out to ensure that there was no DNA contamination. To generate cDNA, 2 μg of DNase I-treated RNA was incubated at 70°C for 10 min with 0.1 μg of oligo(dT) in a final volume of 5 μl and then chilled on ice. A 15-μl cocktail containing 4 μl of 5× reverse transcription (RT) reaction buffer (Promega), 1 μl 10 mM deoxy-nucleoside triphosphates, 1 μl RNasin (40 U μl$^{-1}$, Promega), and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U μl$^{-1}$; Promega) was then added. The mixture was incubated at 37°C for 1 h followed by 95°C for 2 min. Quantitative RT-PCR was carried out on an Agilent Technologies Stratagene Mx3005P system using Brilliant II SYBR green PCR Low Rox master mix (608080) as per the manufacturer’s instructions. Two technical replicates were used for each sample. Cycling conditions consisted of 1 cycle at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final cycle of 95°C for 1 min was followed by melting curve analysis performed at 55°C to 95°C (temperature transition, 0.2°C steps) with stepwise fluorescence detection. Primers used for analysis are listed in Table S2 of the supplemental material. Relative expression changes were identified using the $\Delta C_T$ method, in relation to the expression of ACT1.

**Analysis of RNA splicing.** C. parapsilosis CLIB214, C. parapsilosis type 1 strains (5 MTLa, 1 MTLa/a, and 3 MTLa/a/α), C. orthopsilosis type 2 strains (4 MTLa, 1 MTLa/a, and 2 MTLa/a/α), and C. metapsilosis (strains CP59, CP86, CP92, CP286, ATCC 96143, and ATCC 96144) were grown overnight in SpiderM at 30°C. The cultures were diluted to an $A_{600}$ of 0.2 in fresh SpiderM medium and incubated at 25°C or 30°C with shaking until an $A_{600}$ of 1.0 was reached. RNA was extracted as described above. Both cDNA and genomic DNA were used as templates for PCR amplification, using GoTaQ (Promega). The primers used are listed in Table S2 of the supplemental material.

**Nucleotide sequence accession numbers.** Sequences of the MTLa idiomorphs were deposited in GenBank and assigned accession numbers as follows: HQ696678 (MTLa); ATCC 96143), HQ696679 (MTLa; 398122), HQ696680 (MTLa; Cp289), HQ696681 (MTLa; 90-125), and HQ696682 (MTLa; Cp185).

**RESULTS**

Identification of MTLa idiomorphs from C. orthopsilosis and C. metapsilosis. MTLa idiomorphs were first identified by constructing fosmid libraries using genomic DNA from C. orthopsilosis 90-125 and C. metapsilosis ATCC 96143. The fosmids were screened by sequencing the ends of the inserts and searching for similarities to the MTL region of other Candida species. An MTLa idiomorph was identified from C. orthopsilosis (Fig. 1) and an MTLa idiomorph from C. metapsilosis (Fig. 2).

To help identify isolates containing the opposite mating type, we designed oligonucleotides from unique regions outside the idiomorphs and amplified a region of approximately 10 kb for 16 C. orthopsilosis isolates. Restriction analysis of this region revealed several different patterns from individual isolates (Fig. 1C). The isolates fell into two groups, labeled type 1 and type 2 (Fig. 1C). We sequenced entire MTLa and MTLa idiomorphs from a representative of each type.

Unlike C. parapsilosis, where the MTLa1 region is a pseudogene and no MTLa idiomorphs have been identified (46), the MTLa and MTLa idiomorphs from C. orthopsilosis are all intact and resemble the structures in C. albicans and C. tropicalis (12). Each idiomorph encodes two regulatory genes (α1 and α2 in MTLa and α1 and α2 in MTLa) and idiomorph specific alleles of Pik, Pap, and Obp, which have no known role in mating. The MTLa idiomorph in the type 2 isolate is 9,078 bp long, and the MTLa idiomorph is slightly longer, at 9,492 bp. The type 1 idiomorphs are similar in size; however, we could not determine the exact size, because we did not sequence across the left junction of the type 1 MTLa idiomorph. Interestingly, the left-hand junction of MTLa lies directly between the stop codons of MTLa2 and of the adjacent gene, Gap1, in both type 1 and type 2 isolates.

The two MTLa idiomorphs and the two MTLa idiomorphs from C. orthopsilosis are 95% identical in DNA sequence (data not shown). However, at the protein level the regulatory proteins range in similarity from 80% (Mtlα1) (Fig. 1D) to 93% (Mtlα1) (Fig. 2B), whereas the Pik, Pap, and Obp proteins are much more similar (97 to 99%) (data not shown).

The differences between the idiomorphs of the same mating type suggest that isolates characterized as C. orthopsilosis may belong to more than one species. Sequence analysis of the ITS confirmed that these isolates were correctly identified as C. orthopsilosis but that there are at least two divergent groups within these species (see Fig. S1 in the supplemental material), equivalent to C. orthopsilosis type 1 and C. orthopsilosis type 2 (Fig. 1). Allele-specific oligonucleotides designed to amplify parts of MTLa1 and MTLa1 showed that each type includes isolates with only MTLa idiomorphs, only MTLa idiomorphs, or both MTLa and MTLa idiomorphs (Fig. 1C).

In contrast to C. orthopsilosis, amplification of the MTL region from all 18 isolates of C. metapsilosis yielded fragments with identical restriction patterns (Fig. 2). We could not detect any evidence of the presence of MTLa genes elsewhere in the genome, based on degenerate PCR (data not shown). We therefore assume that all the isolates tested contain only MTLa idiomorphs, similar to the sequenced isolate. The organization of MTLa in C. metapsilosis is identical to that of C. orthopsilosis, except for the presence of a repeated motif in Mtlα1 in C. metapsilosis that is not well conserved in the other species (Fig. 2B). In contrast, we detected only MTLa idiomorphs in 189 isolates of C. parapsilosis, based on degenerate PCR designed to amplify the region between PApα and
C. orthopsilosis isolates do not mate. We found approximately equal numbers of MTLa and MTLα isolates of C. orthopsilosis (for both types), with one heterozygous (MTLα/MTLα) isolate of each type (Fig. 1). This population structure suggests that individual isolates may be mating and undergoing some form of sexual reproduction. We therefore attempted to find evidence of mating products, by using a his1 knockout generated in a type 1 MTLa isolate (CP47) and a leu2 knockout generated in an MTLα isolate (CP289) of the same type (Fig. 3A). Attempted crosses were performed under several conditions (see Table S3 in the supplemental material); however, no His+/Leu+ prototrophs were identified. Mating between MTLa and MTLα isolates of C. albicans was clearly detectable, in both opaque- and white-phase cells (Fig. 3B). Because it has been reported that identical idiomorphs of C. albicans can mate from cells of the opposite mating type results in induction of expression of the pheromone receptor and of other genes in the mitogen-activated protein kinase pathway (7, 32, 45, 55). We identified the /H9251-factor pheromone gene from C. parapsilosis and from genome surveys of C. orthopsilosis 90-125 and C. metapsilosis ATCC 96143 (Fig. 4). After processing, the C. parapsilosis gene is predicted to encode two copies of a 13-amino-acid peptide and one copy of a 14-amino-acid peptide. C. orthopsilosis encodes one copy each of a 13- and a 14-amino-acid peptide. The gene sequence from C. metapsilosis is not complete, but it encodes at least three copies of a 13-amino-acid peptide and one copy of a 14-amino-acid peptide. The predicted mature peptide sequences are identical in the three species (Fig. 4). Based on similarities with S. cerevisiae and C. albicans, the 13-amino-acid peptide (KPHWTTYGYYEPQ) is likely to be biologically active (7, 55), and this one was chemically synthesized. However, the addition of this factor exogenously did not induce expression of STE2 (the

**FIG. 2.** (A) Amplification of the MTL locus from C. metapsilosis. A 13.2-kb fragment spanning the MTL locus was amplified from 18 isolates of C. metapsilosis by using oligos BUT287 and BUT289 from within the GAP1 and orf19.3202 genes. The PCR fragments were digested with EcoRV (two strains are not shown). The restriction patterns are almost identical, with only minor differences, suggesting that all isolates contain only one MTL idiomorph (MTLα). Lane M, 1-kb Plus ladder (Invitrogen); lane 2, CP61; lane 3, CP86; lane 4, CP87; lane 5, CP88; lane 6, CP92; lane 7, CP178; lane 8, CP231; lane 9, CP261, lane 10, ATCC95143; lane 11, ATCC95144; lane 12, CP5; lane 13, CP271; lane 14, CP286; lane 15, CP376; lane 16, CP397. (B) Alignment of Mtl1 sequences, obtained using the methods described in the legend for Fig. 1. An additional repeated motif in the C. metapsilosis sequence is indicated by the dark line above the sequence.

MTLα1 (see Fig. S2 and Table S1 in the supplemental material).

**C. orthopsilosis isolates do not mate.** We found approximately equal numbers of MTLa and MTLα isolates of C. orthopsilosis (for both types), with one heterozygous (MTLα/MTLα) isolate of each type (Fig. 1). This population structure suggests that individual isolates may be mating and undergoing some form of sexual reproduction. We therefore attempted to find evidence of mating products, by using a his1 knockout generated in a type 1 MTLα isolate (CP47) and a leu2 knockout generated in an MTLα isolate (CP289) of the same type (Fig. 3A). Attempted crosses were performed under several conditions (see Table S3 in the supplemental material); however, no His+/Leu+ prototrophs were identified. Mating between MTLα and MTLα isolates of C. albicans was clearly detectable, in both opaque- and white-phase cells (Fig. 3B). Because it has been reported that identical idiomorphs of C. albicans can mate (same-sex or homothallic mating) (3) and that C. albicans can mate with C. dublinensis (57), we also looked for mating products between C. orthopsilosis or C. parapsilosis and opaque cells of C. albicans, between two MATa strains of C. parapsilosis, and between C. orthopsilosis and C. albicans. However, none was identified (see Table S3).

**Analysis of the mating signal transduction pathway in C. orthopsilosis.** In an attempt to identify the molecular basis of the defect in mating in C. orthopsilosis, we investigated the response of the mating signal transduction pathway. In both S. cerevisiae and C. albicans, exposure of cells to mating pheromone from cells of the opposite mating type results in induction of expression of the pheromone receptor and of other genes in the mitogen-activated protein kinase pathway (7, 32, 45, 55). We identified the /H9251-factor pheromone gene from C. parapsilosis and from genome surveys of C. orthopsilosis 90-125 and C. metapsilosis ATCC 96143 (Fig. 4). After processing, the C. parapsilosis gene is predicted to encode two copies of a 13-amino-acid peptide and one copy of a 14-amino-acid peptide. C. orthopsilosis encodes one copy each of a 13- and a 14-amino-acid peptide. The gene sequence from C. parapsilosis is not complete, but it encodes at least three copies of a 13-amino-acid peptide and one copy of a 14-amino-acid peptide. The predicted mature peptide sequences are identical in the three species (Fig. 4). Based on similarities with S. cerevisiae and C. albicans, the 13-amino-acid peptide (KPHWTTYGYYEPQ) is likely to be biologically active (7, 55), and this one was chemically synthesized. However, the addition of this factor exogenously did not induce expression of STE2 (the
predicted pheromone receptor), STE4 (a component of the heterotrimeric G protein required for signaling), or CPH1 (a transcription factor at the end of the mating pathway) in either C. orthopsilosis or C. parapsilosis (Fig. 5A). Deleting SST2, a GTPase-activating protein, renders both S. cerevisiae and C. albicans more sensitive to the effects of α-factor (13, 19). We therefore knocked out SST2 in an MTLa isolate of C. parapsilosis, generating strain LHS1 (see Fig. S3 in the supplemental material). However, once again, adding exogenous α-factor does not result in induction of expression of STE2 (Fig. 5A).

To test if the synthetic α-factor from the C. parapsilosis group was functional, we tested its effects on C. albicans (Fig. 5B and C). We used C. albicans with a knockout of either stt2 (18) or bar1 (65) to increase sensitivity. The C. parapsilosis α-factor induced expression of the pheromone receptor STE2 and the transcription factor CPH1 in opaque cells of both stt2 and bar1 knockouts of C. albicans (Fig. 5B). Opaque cells also formed mating projections (or shmoos) when exposed to α-factor from either C. albicans or C. parapsilosis/C. orthopsilosis/C. metapsilosis (Fig. 5C). Our observations are supported by very recent reports that α-factor from C. parapsilosis (and other Candida species) induces same-sex mating and adherence in C. albicans (2).

**Defective splicing in MTL in C. orthopsilosis.** Three of the regulatory genes (al1, a2, and a2) at the MTL in Candida species contain introns. Intron position and structure are generally conserved. We investigated splicing of the regulatory genes in C. orthopsilosis and C. metapsilosis. MTLa2 is efficiently spliced in C. orthopsilosis, both in α/α homozygotes and in α/a heterozygotes, and also in C. metapsilosis α/α homozygotes (Fig. 6B and C). However, MTLa2 was not spliced at all in 11 isolates of C. orthopsilosis tested (from both type 1 and type 2), even when exogenous alpha factor was added (Fig. 6B shows results for one isolate). MTLal, which contains two introns, is inefficiently spliced at intron 2. In contrast, the introns in MTLa1 are efficiently spliced in C. parapsilosis (Fig. 6A) (46). C. parapsilosis does not have an intact MTLa2 gene (46).

**DISCUSSION**

Species within the Candida (CTG) clade fall into two related groups. One consists of predominantly haploid species (Candida guilliermondii, Candida lusitaniae, Debaryomyces hansenii, and Pichia stipitis), in which mating and meiosis is intact, or relatively intact (9, 10, 37). The sister group contains species such as C. albicans, C. dubliniensis, C. tropicalis, C. parapsilosis, and Lodderomyces elongisporus, for which all described isolates are diploid, and, for most, sexual structures have not been observed. Potential ascospores of L. elongisporus have been described (44, 59, 75), but these have not been studied in detail. C. albicans and C. dubliniensis undergo a parasexual cycle; mating between diploid cells with opposite mating types leads to the formation of a tetraploid cell (30, 50). Chromosomes are gradually lost in a random process (5), and recombination between homologous chromosomes occurs in a Spol1-dependent manner (23). Mating only occurs in cells that have undergone a developmental switch from white to opaque forms (reviewed in reference 48). This raises the question of whether mating and meiosis were lost in the entire
revealed that the ability to undergo meiosis (60).

C. lusitaniae mal complex formation and crossover inference (12). However, pathway, as well as some proteins associated with synaptone- and lineage-specific losses (12). For example, meiosis, although there have been some species-specific and most have retained the core genes required for mating and induction under very specific conditions.

Most) of the species retain the ability to undergo sexual reproduction (Fig. 4). Identification of alpha factor pheromone from the C. parapsilosis species group. The pre-pro-alpha factor sequences were aligned using T-Coffee (54) and manually edited using SeaView (25). The arrows indicate the putative sites (KR) cleaved by the Kex2 orthologs (16). The adjacent repeating X-Ala sequences are predicted to be removed by orthologs of Ste13 (31), leaving the mature peptide (indicated with a solid black line). The peptide from C. tropicalis is likely to be longer than those from the other species (indicated with a broken line). The C. metapsilosis sequence is not complete and was obtained from a sequence survey. C. par, C. parapsilosis; C. ortho, C. orthopsilosis; C. meta, C. metapsilosis; C. alb, C. albicans; C. dub, C. dubliniensis; C. trop, C. tropicalis.

diploid Candida clade and regained in some format in an ancestor of C. albicans and C. dubliniensis, or whether all (or most) of the species retain the ability to undergo sexual reproduction under very specific conditions.

An analysis of gene loss in Candida species suggests that most have retained the core genes required for mating and meiosis, although there have been some species-specific and lineage-specific losses (12). For example, C. guilliermondii and C. lusitaniae have lost the Dmc1-dependent recombination pathway, as well as some proteins associated with synaptone- mal complex formation and crossover inference (12). However, C. lusitaniae (and probably C. guilliermondii also) has retained the ability to undergo meiosis (60).

The first description of the C. parapsilosis MTL locus revealed that MTLa2 is a pseudogene (46), and this was supported by analysis of seven other isolates and by whole-genome sequencing of a clinical isolate (12). The population structure of the C. orthopsilosis MTLa1 is a recent event in C. parapsilosis, because the gene is intact in C. orthopsilosis. It is therefore tempting to speculate that the loss of a mating partner (MTLa) and the degeneration of the MTLa idiomorph may have led to the global spread of a clone with increased virulence.

The population structure of the C. orthopsilosis isolates (approximately equal proportions of MATa and MATα idiomorphs) suggests that mating is occurring. However, we cannot observe this in the laboratory. It is possible that we have not yet identified the ideal conditions; for example, it took several years to correlate mating efficiency in C. albicans with the formation of opaque-phase cells (52). However, even white-phase cells of C. albicans mate at a low frequency that is easily detectable (Fig. 3B). We therefore believe that if mating were occurring in C. orthopsilosis, even at very low frequencies, we would be able to detect it by using crosses of auxotrophic strains (Fig. 3B). We have tested only one pair of C. orthopsilosis isolates in direct mating assays. However, we saw no mating response (shmoo formation, growth arrest, or transcriptional induction) in any of the isolates examined.

It is possible that loss of mating in C. orthopsilosis is also a recent event and is correlated with deficiencies in splicing of the regulatory genes at the MTLa idiomorph (Fig. 6). Inefficient splicing of MTLa1 and MTLa2 was observed in all available sequenced isolate was also 25 to 70 times lower than in other Candida genomes (12). Polymorphisms are more frequent in C. orthopsilosis and C. metapsilosis (26, 70, 71). This suggests that C. parapsilosis may have undergone a population bottleneck and that all global isolates are closely related. C. parapsilosis is much more commonly isolated from clinical samples than either C. metapsilosis or C. orthopsilosis (15, 43, 66). Our analysis shows that the degeneration of MTLa1 is a recent event in C. parapsilosis, because the gene is intact in C. orthopsilosis. It is therefore tempting to speculate that the loss of a mating partner (MTLa) and the degeneration of the MTLa idiomorph may have led to the global spread of a clone with increased virulence.

MTLa1 and MTLa2 were observed in all available
isolates of both subtypes of *C. orthopsilosis*, but not of *C. parapsilosis* or *C. albicans*. Similar defects in splicing of *MTL*α1 were identified in *Candida glabrata*, which is also apparently incapable of mating (53). Both mating types are commonly found in the *C. glabrata* population, and MATaα cells are insensitive to treatment with alpha factor (53). It is possible that splicing in both *C. orthopsilosis* and *C. glabrata* is regulated under conditions (not yet known), which allow mating. Intriguingly, *L. elongisporus*, once thought to represent the sexual form of *C. parapsilosis*, has completely lost the *MTL* locus (12). Same-sex (homothallic) mating may be occurring in this species, but it remains to be characterized. Our analyses suggest that the mating idiomorphs are degenerating in the *C. parapsilosis* group, either through complete loss of the *MTL*α1 group, either through complete loss of the *MTL*α1 or *C. orthopsilosis*, but not by DMSO. Cultures were grown in SpiderM medium for 6 h with shaking at 25°C. Bar, 2 μm.

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**FIG. 5.** Transcriptional response to alpha factor treatment. (A and B) *C. parapsilosis* CLIB214 (wild type) and its isogenic *sst2* knockout *C. parapsilosis* LHS1, *C. orthopsilosis* CP47 (A) and opaque *C. albicans* CA12 (*sst2Δ*) and *C. albicans* RBY1220 (bar1Δ) (B) were grown in SpiderM medium at 25°C in the presence or absence of synthetic alpha factor from *C. parapsilosis* (CpAlpha) or *C. albicans* (CaAlpha) (10 μg ml⁻¹) or in the presence of DMSO. Expression levels of orthologous *STE2*, *STE4*, and *CPH1* genes were determined using quantitative RT-PCR. Expression is shown relative to that of the species-specific *ACT1* gene. The graphs show the expression levels relative to *ACT1* for two biological replicates (*C. parapsilosis* and *C. orthopsilosis*) or three biological replicates (*C. albicans*) plus the standard deviations. (C) Shmoo formation is induced in opaque *C. albicans* CA12 (*sst2Δ*) and RBY1220 (bar1Δ) cells grown the presence of α-factor (10 μg ml⁻¹) from *C. albicans* or *C. parapsilosis*, but not by DMSO. Cultures were grown in SpiderM medium for 6 h with shaking at 25°C. Bar, 2 μm.

The mating process is distinct from the means of regulating mating type. However, we have been unable to detect mating in any of the *C. parapsilosis* group, which raises the question as to why the signal transduction pathways have been conserved intact. One possibility is that they are also required for other biological functions. For example, in *C. albicans*, the mating pathway is involved in biofilm formation, and mating pheromone induces cohesiveness of white cells (17, 63, 64). However, this white cell response is mediated via a region of the alpha-pheromone receptor that is found only in *C. albicans* and *C. dubliniensis* (9, 77). We also do not observe any induction of biofilm formation in the *C. parapsilosis* group following treatment with pheromone (data not shown). It is, however, very interesting that *C. albicans* responds to synthetic alpha factor derived from *C. parapsilosis*/*C. orthopsilosis*/*C. metapsilosis*. This raises the possibility that *Candida* species have adapted the mating pathway as a mechanism of signaling between species. To date, however, the only response we can identify is from *C. albicans*. We also note that the predicted α-factor sequences are identical in *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* (Fig. 4). This is somewhat surprising, considering that the equivalent peptides from *C. albicans* and *C. dubliniensis* (which are much more closely related than *C. parapsilosis* and *C. orthopsilosis*) differ by 1 amino acid. The species in the *C. parapsilosis* group may therefore be communicating in ways that we cannot yet measure.

Our analysis demonstrated that the *C. parapsilosis* species group is more complex than initially realized. Isolates identified as *C. orthopsilosis* fall into two types, distinguished by the analysis of the structure of the *MTL* idiomorph. Sequencing of the ITS region of the ribosomal DNA (see Fig. S1 in the supplemental material) also places most isolates into two groups (with >99% identity), although at least one isolate (*C. orthopsilosis* 90-125) differs from both. Tay et al. (72) used RAPD and ITS sequencing to place *C. orthopsilosis* isolates
among type 1 isolates. Other studies have also identified heterogeneity differences between the P2 group of Tay et al. (72) and our type 2 isolates (data not shown). However, there are different to both. The P3 grouping is identical in ITS sequence

FIG. 6. Splicing of MTLa2, MTLa1, and MTLa2 in C. parapsilosis CLJ2B24 (A), C. orthopsilosis 981224 (type 1) (B), and C. metapsilosis ATCC 96143, ATCC 96144 and CP5 (C). The oligo combinations were used in a PCR amplification using genomic DNA (D lanes) and cDNA (C lanes) as templates. Controls without reverse transcriptase were also used (N). The sizes of the predicted spliced and unspliced products are indicated below the gel. Lane M, 1-kb ladder (Promega).

into two groups (P2 and P3), again with one isolate that was different to both. The P3 grouping is identical in ITS sequence to our type 2 isolates (data not shown). However, there are differences between the P2 group of Tay et al. (72) and our type 1 isolates. Other studies have also identified heterogeneity among C. orthopsilosis isolates (70, 74). We suggest that C. orthopsilosis isolates can be divided into at least two subspecies, represented by type 1 and type 2 in this study. The C. orthopsilosis type strain (ATCC 96139) belongs to the type 1 group (see Fig. S1 in the supplemental material). However, the variation in ITS sequences (for example, of C. orthopsilosis 90-125, and as reported by Tay et al. [72]) suggests that the number of subspecies may be even greater. This should be taken into account when analyzing phenotypic diversity, such as that for drug sensitivity.

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