

# Function of *Cryptococcus neoformans* KAR7 (SEC66) in Karyogamy during Unisexual and Opposite-Sex Mating

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The human basidiomycetous fungal pathogen *Cryptococcus neoformans* serves as a model fungus to study sexual development and produces infectious propagules, basidiospores, via the sexual cycle. Karyogamy is the process of nuclear fusion and an essential step to complete mating. Therefore, regulation of nuclear fusion is central to understanding sexual development of *C. neoformans*. However, our knowledge of karyogamy genes was limited. In this study, using a BLAST search with the *Saccharomyces cerevisiae* KAR genes, we identified five *C. neoformans* karyogamy gene orthologs: *CnKAR2*, *CnKAR3*, *CnKAR4*, *CnKAR7* (or *CnSEC66*), and *CnKAR8*. There are no apparent orthologs of the *S. cerevisiae* genes *ScKAR1*, *ScKAR5*, and *ScKar9* in *C. neoformans*. Karyogamy involves the congression of two nuclei followed by nuclear membrane fusion, which results in diploidization. *ScKar7* (or *ScSec66*) is known to be involved in nuclear membrane fusion. In *C. neoformans*, *kar7* mutants display significant defects in hyphal growth and basidiospore chain formation during both a- $\alpha$  opposite and  $\alpha$ - $\alpha$  unisexual reproduction. Fluorescent nuclear imaging revealed that during *kar7*  $\times$  *kar7* bilateral mutant matings, the nuclei congress but fail to fuse in the basidia. These results demonstrate that the *KAR7* gene plays an integral role in both opposite-sex and unisexual mating, indicating that proper control of nuclear dynamics is important. *CnKAR2* was found to be essential for viability, and its function in mating is not known. No apparent phenotypes were observed during mating of *kar3*, *kar4*, or *kar8* mutants, suggesting that the role of these genes may be dispensable for *C. neoformans* mating, which demonstrates a different evolutionary trajectory for the *KAR* genes in *C. neoformans* compared to those in *S. cerevisiae*.

*Cryptococcus neoformans* belongs to the Basidiomycota and is an opportunistic fungal pathogen causing meningoencephalitis in immunocompromised cohorts, especially patients with HIV/AIDS, and in some cases, in otherwise immunocompetent individuals (10). Basidiospores generated via sexual development are considered major infectious propagules (6, 18, 60). Therefore, investigation of the sexual development of this fungus is of importance to understand its global impact on human health resulting in more than 1,000,000 infections annually (47).

*C. neoformans* has two mating types, **a** and  $\alpha$ , and opposite-sex mating occurs when **a** and  $\alpha$  cells encounter and recognize each other (25–27), resulting in formation of mating hyphae and bulb-like basidia generated at the apex of aerial hyphae followed by spore chain formations (31, 63). The mating type locus of *C. neoformans* represents a bipolar mating system, in which the homeodomain and pheromone/pheromone receptor loci are genetically and physically linked, whereas in many other basidiomycetes, the two loci are unlinked and segregate independently to comprise tetrapolar mating systems (reviewed in reference 28). However, the mating type is unbalanced in natural populations, where  $\alpha$  mating types are predominant over **a** mating type isolates; hence, the rarity of mating type **a** poses a barrier to opposite-sex mating (reviewed in reference 28). Indeed, *C. neoformans* undergoes  $\alpha$ - $\alpha$  unisexual reproduction, which mimics opposite-sex mating morphologically but occurs during solo culture of  $\alpha$  isolates.

The molecular mechanism(s) underlying the increase in ploidy during mating is of central interest; in the case of unisexual reproduction, diploidization may occur “early” before the sexual hyphal growth phase, wherein diploidization could be a necessary condition for filamentation. In contrast, as seen in opposite-sex mating, diploidization may occur “late” in the basidia just prior to meiosis and production of spore progeny (Fig. 1A). A promising

avenue to understand this process involves the identification and characterization of karyogamy genes that enable nuclear fusion. In addition, although *C. neoformans* is predominantly a haploid organism, clinical and environmental diploid isolates are also found (12, 34). Thus, elucidation of the roles of karyogamy genes during mating will advance our knowledge about this ubiquitous fungal pathogen and its routes to diploidization and sexual reproduction.

Karyogamy (or nuclear fusion) during mating is well understood in the model yeast *Saccharomyces cerevisiae* (52). There are two distinct steps in karyogamy: nuclear congression and nuclear fusion. The first step involves the genes encoding Kar1, Kar3, Kar4, and Kar9 and serves to pair the nuclei close to one another (24, 40, 43, 44, 52). The second step, in which nuclear membranes fuse, involves the genes encoding Kar2, Kar5, Kar7/Sec66, and Kar8 (3, 7, 52, 54) (Fig. 1B). Kar1 is an essential protein involved in nuclear fusion during mating and also plays a role in mitosis for spindle pole body duplication. Kar1 localizes to the half-bridge of the spindle pole body (13, 53, 57). Kar3 is a minus-end-directed motor kinesin that functions during mitosis and meiosis and localizes to the spindle pole body (40, 42). Kar4 is a transcription factor induced in response to pheromone that functions in nuclear congression (23, 24). One of its targets is the *KAR3* gene (24). Kar9 is a cortical protein required for proper localization of the

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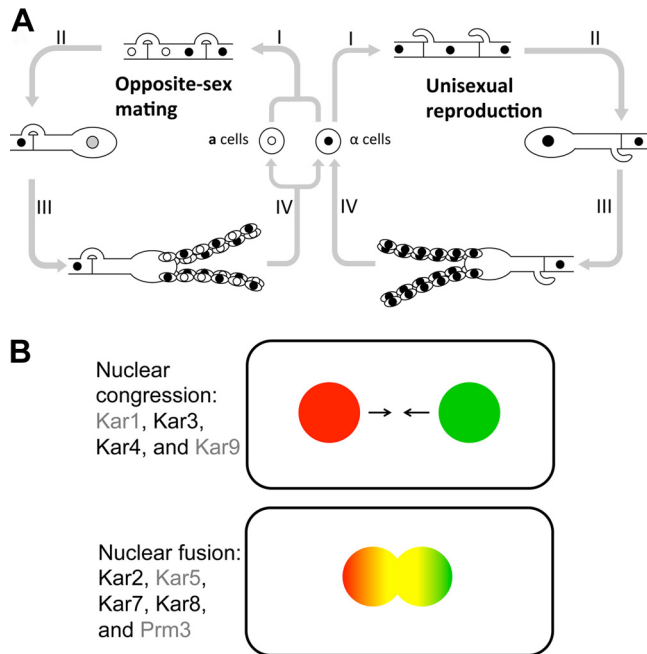
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**FIG 1** Illustration of two modes of *C. neoformans* sexual development and two distinct stages involved in karyogamy. (A) Opposite-sex mating. (I) Cells of two different mating types recognize each other and undergo cell-cell fusion to form dikaryotic hyphae. (II) At the apex of aerial hyphae, a basidium forms and karyogamy occurs, representing "late" diploidization. (III) Meiosis occurs, and four basidiospore chains are produced from the surface of the basidium by repeated rounds of mitosis of the postmeiotic nuclei. (IV) Progeny of two mating types disperse. (A) Unisexual reproduction. (I) Only  $\alpha$  mating type cells undergo hyphal growth, where "early" diploidization might contribute to filamentation. (II) At the apex of aerial hyphae, a basidium forms and "late" diploidization can occur. (III) Meiosis occurs, and four basidiospore chains are formed. (IV) Haploid progeny disperse. Thus, during unisexual reproduction, two different hypothetical timings for diploidization exist. The figure depicts the hypothesis of "late" diploidization. (B) Nuclear congression and nuclear fusion. First, two nuclei migrate toward each other (nuclear congression): Kar1, Kar3, Kar4, and Kar9 are involved in this process in *S. cerevisiae*. Second, nuclear membrane fusion occurs and Kar2, Kar5, Kar7, and Kar8 are involved in this process. Karyogamy components that are unique to *S. cerevisiae* compared to *C. neoformans* are shown in gray.

mitotic spindle and orienting cytoplasmic microtubules (43). Kar2 is a homolog of BiP/GRP78 (54) and an ATPase that functions as a molecular chaperone involved in the transport of proteins into the endoplasmic reticulum (ER) (51, 59). Kar5 is an ER membrane protein whose expression is regulated by pheromone (3, 7). Kar7 (or Sec66) is a subunit of the Sec63 complex that is involved in protein targeting and import into the ER (23, 65). Kar8 (or Jem1) is a DnaJ-like chaperone localized to the ER membrane and is known to interact genetically with KAR2 (46, 62).

*Schizosaccharomyces pombe* Tht1, a Kar5 homolog, and Tht2 (meiotically upregulated gene, Mug22), are known to be involved in karyogamy (39, 58). In *Neurospora*, the UV-sensitive *uvs-3* mutant exhibits an arrest prior to karyogamy during ascus formation in the perithecius (49). In the pathogenic fungus *Candida albicans*, the KAR3 gene is required for karyogamy during mating (4). *C. albicans* KAR8 (JEM1) is also known to complement the *S. cerevisiae* *kar8* mutant to restore karyogamy (38). However, little is known about the roles of KAR genes in other fungi, including *C. neoformans*. In this study, we identified orthologs of karyogamy

genes in *C. neoformans* and explored their functions during opposite-sex and unisexual reproduction. We found that the KAR7/SEC66 gene is required for both types of sexual reproduction. *kar7* mutants exhibited defects in sexual spore formation, indicating that karyogamy occurs inside the basidium prior to meiosis and spore formation. We found that the KAR3, KAR4, and KAR8 genes are dispensable for mating in *C. neoformans*. KAR2 was found to be essential for viability, and its possible functions in mating remain to be studied. We also found that orthologs of KAR1, KAR5, and KAR9 are apparently missing from the *C. neoformans* genome, possibly reflecting an evolutionary trajectory for the mechanics of mating and nuclear fusion different from that of *S. cerevisiae*.

## MATERIALS AND METHODS

**Strains, media, and culture conditions.** The *C. neoformans* strains and plasmids used in this study are listed in Table 1. The strains were grown on yeast extract-peptone-dextrose (YPD) agar containing the appropriate drugs for marker gene selections. For mating, strains were cultured in liquid YPD medium overnight, washed with distilled water, and plated on V8 medium (pH 7) for serotype D and MS medium (64) for serotype A. Plasmid preparation and cloning were performed as described previously (55).

**Disruption of KAR genes.** *kar* null mutant strains were generated using disruption cassettes with neomycin (*NEO*) or nourseothricin (*NAT*) drug resistance markers flanked by  $\sim 1$ -kb sequences corresponding to the 5' and 3' noncoding flanking regions of each KAR gene as described previously (14). The primers used in this study are listed in Table S1 in the supplemental material. For example, to disrupt the KAR7 gene in the KN99a or KN99 $\alpha$  strain, the 5' region of the KAR7 gene was amplified with primers JOHE20336 and JOHE20337 from genomic DNA, the *NEO* or *NAT* drug resistance gene cassette was amplified with JOHE20338 and JOHE20339 from plasmid pNATSTM#209 or pJAF1 (17), and the 3' region was amplified with primers JOHE20340 and JOHE20341. The three DNA fragments obtained were combined and amplified with two internal nested primers, JOHE20342 and JOHE20343, and the final overlap PCR products were purified and precipitated onto 0.6- $\mu$ m gold particles (Bio-Rad, Hercules, CA). The wild-type strains then were transformed biolistically with the DNAs obtained. The bombarded cells were transferred onto YPD medium containing the appropriate selection drugs. Positive transformants were screened by PCR and Southern blotting. The disruption procedures for the serotype D KAR7 and other KAR genes are described in the Materials and Methods section in the supplemental material.

**Tagging Nop1 with mCherry or GFP.** The primers used in these experiments are listed in Table S1 in the supplemental material. The nucleolar protein Nop1 was used to monitor nuclear positioning during mating. The serotype A strain *NOP1* gene was replaced with *NOP1-mCherry* by homologous recombination with *NOP1-mCherry-NEO* as described previously (22). The mCherry-encoding gene was flanked by the 3' end of the *NOP1* gene open reading frame (ORF). Positive transformants were selected, and homologous recombination was confirmed by PCR. To tag the N terminus of Nop1 with green fluorescent protein (GFP), the *NOP1* gene was amplified with primers JOHE23127 and JOHE23128 for serotype A or primers JOHE23129 and JOHE23130 for serotype D and the resulting fragments were cloned into the pCR21-TOPO vector (Invitrogen, Carlsbad, CA). The resulting plasmids, pSL01 and pSL02 (Table 1), respectively, were digested with BglII, and the BglII *NOP1* fragments were purified and cloned into pCN19 digested with BamHI (48), producing plasmids pSL04-1 (serotype A) and pSL05-1 (serotype D), which express GFP-Nop1 under the constitutive histone H3 promoter. The wild-type and *kar7* mutant strains were transformed with the plasmids as described above, and each positive transformant was screened for the GFP nuclear signal.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid <sup>a</sup>	Serotype	Mating type	Genotype
<b>Strains</b>			
<i>Wild type</i>			
KN99 $\alpha$	A	$\alpha$	Wild type
KN99a	A	a	Wild type
XL280	D	$\alpha$	Wild type
JEC21	D	$\alpha$	Wild type
JEC20	D	a	Wild type
MN142.3	D	$\alpha$	Diploid, <i>ura5::NAT/ura5::NEO</i>
<i>kar7</i> mutants			
SL280 <sup>1</sup>	A	$\alpha$	KN99 $\alpha$ <i>kar7::NEO</i>
SL281 <sup>1</sup>	A	$\alpha$	KN99 $\alpha$ <i>kar7::NEO</i>
SL282 <sup>2</sup>	A	$\alpha$	KN99 $\alpha$ <i>kar7::NEO</i>
SL283 <sup>2</sup>	A	$\alpha$	KN99 $\alpha$ <i>kar7::NEO</i>
SL284 <sup>2</sup>	A	$\alpha$	KN99 $\alpha$ <i>kar7::NEO</i>
SL285 <sup>1</sup>	A	a	KN99a <i>kar7::NAT</i>
SL286 <sup>1</sup>	A	a	KN99a <i>kar7::NAT</i>
SL287 <sup>1</sup>	A	a	KN99a <i>kar7::NAT</i>
SL288 <sup>2</sup>	A	a	KN99a <i>kar7::NAT</i>
SL289 <sup>2</sup>	A	a	KN99a <i>kar7::NAT</i>
SL290 <sup>3</sup>	A	a	KN99a <i>kar7::NEO</i>
SL291 <sup>3</sup>	A	a	KN99a <i>kar7::NEO</i>
SL292 <sup>3</sup>	A	a	KN99a <i>kar7::NEO</i>
SL293 <sup>3</sup>	A	a	KN99a <i>kar7::NEO</i>
SL294 <sup>3</sup>	A	a	KN99a <i>kar7::NEO</i>
SL276 <sup>1</sup>	D	$\alpha$	XL280 <i>kar7::NEO</i>
SL277 <sup>1</sup>	D	$\alpha$	XL280 <i>kar7::NEO</i>
SL278 <sup>2</sup>	D	$\alpha$	XL280 <i>kar7::NEO</i>
SL279 <sup>2</sup>	D	$\alpha$	XL280 <i>kar7::NEO</i>
SL275	D	$\alpha$	JEC21 <i>kar7::NEO</i>
SL355 <sup>1</sup>	D	$\alpha$	MN142.3 <i>kar7::HYG/KAR7</i>
SL356 <sup>1</sup>	D	$\alpha$	MN142.3 <i>kar7::HYG/KAR7</i>
SL357 <sup>2</sup>	D	$\alpha$	MN142.3 <i>kar7::HYG/KAR7</i>
SL358 <sup>2</sup>	D	$\alpha$	MN142.3 <i>kar7::HYG/KAR7</i>
SL359 <sup>2</sup>	D	$\alpha$	MN142.3 <i>kar7::HYG/KAR7</i>
SL360 <sup>2</sup>	D	$\alpha$	MN142.3 <i>kar7::HYG/KAR7</i>
SL361 <sup>1</sup>	D	$\alpha$	SL355 <i>kar7::HYG/kar7::URA5 ura5::NAT/ura5::NEO</i>
SL362 <sup>2</sup>	D	$\alpha$	SL355 <i>kar7::HYG/kar7::URA5 ura5::NAT/ura5::NEO</i>
SL363 <sup>2</sup>	D	$\alpha$	SL355 <i>kar7::HYG/kar7::URA5 ura5::NAT/ura5::NEO</i>
SL364 <sup>3</sup>	D	$\alpha$	SL355 <i>kar7::HYG/kar7::URA5 ura5::NAT/ura5::NEO</i>
SL365 <sup>3</sup>	D	$\alpha$	SL355 <i>kar7::HYG/kar7::URA5 ura5::NAT/ura5::NEO</i>
<i>kar8</i> mutants			
SL270 <sup>1</sup>	D	$\alpha$	XL280 <i>kar8::NAT</i>
SL274 <sup>2</sup>	D	$\alpha$	XL280 <i>kar8::NAT</i>
SL271	D	a	JEC20 <i>kar8::NAT</i>
SL272 <sup>1</sup>	D	$\alpha$	JEC21 <i>kar8::NAT</i>
SL273 <sup>2</sup>	D	$\alpha$	JEC21 <i>kar8::NAT</i>
<i>kar3</i> mutants			
SL295 <sup>1</sup>	A	$\alpha$	KN99 $\alpha$ <i>kar3::NEO</i>
SL296 <sup>2</sup>	A	$\alpha$	KN99 $\alpha$ <i>kar3::NEO</i>
SL297 <sup>1</sup>	A	a	KN99a <i>kar3::NAT</i>
SL298 <sup>2</sup>	A	a	KN99a <i>kar3::NAT</i>
<i>kar4</i> mutants			
SL366 <sup>1</sup>	D	$\alpha$	Diploid MN142.3 <i>kar4::HYG/KAR4 ura5::NAT/ura5::NEO</i>
SL367 <sup>1</sup>	D	$\alpha$	Diploid MN142.3 <i>kar4::HYG/KAR4 ura5::NAT/ura5::NEO</i>
SL368 <sup>2</sup>	D	$\alpha$	Diploid MN142.3 <i>kar4::HYG/KAR4 ura5::NAT/ura5::NEO</i>
SL372 <sup>1</sup>	D	$\alpha$	Haploid progeny of SL366 <i>kar4::HYG</i>
SL373 <sup>2</sup>	D	$\alpha$	Haploid progeny of SL366 <i>kar4::HYG</i>

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid <sup>a</sup>	Serotype	Mating type	Genotype
<i>kar2</i> mutants			
SL369 <sup>1</sup>	D	α	Diploid MN142.3 <i>kar2::HYG/KAR, ura5::NAT/ura5::NEO</i>
SL370 <sup>1</sup>	D	α	Diploid MN142.3 <i>kar2::HYG/KAR2 ura5::NAT/ura5::NEO</i>
SL371 <sup>2</sup>	D	α	Diploid MN142.3 <i>kar2::HYG/KAR2 ura5::NAT/ura5::NEO</i>
Strains with fluorescent Nop1			
SL305	A	α	KN99α with <i>NOP1-mCherry</i> (NEO)
SL306	A	α	KN99α with <i>NOP1-mCherry</i> (NEO)
SL307	A	α	KN99α with <i>NOP1-mCherry</i> (NEO)
SL321	A	a	KN99a with <i>GFP-NOP1</i> (NAT)
SL322	A	a	KN99a with <i>GFP-NOP1</i> (NAT)
SL323	A	a	KN99a with <i>GFP-NOP1</i> (NAT)
SL324	A	a	KN99a with <i>GFP-NOP1</i> (NAT)
SL308	A	a	SL285 ( <i>kar7</i> ) <i>NOP1-mCherry</i> (NEO)
SL309	A	a	SL285 ( <i>kar7</i> ) <i>NOP1-mCherry</i> (NEO)
SL310	A	a	SL285 ( <i>kar7</i> ) <i>NOP1-mCherry</i> (NEO)
SL311	A	a	SL285 ( <i>kar7</i> ) <i>NOP1-mCherry</i> (NEO)
SL312	A	a	SL285 ( <i>kar7</i> ) <i>NOP1-mCherry</i> (NEO)
SL313	A	a	SL285 ( <i>kar7</i> ) <i>NOP1-mCherry</i> (NEO)
SL316	A	a	SL290 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL317	A	a	SL290 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL318	A	a	SL290 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL319	A	a	SL290 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL347	A	α	SL280 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL348	A	α	SL280 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL349	A	α	SL280 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL350	A	α	SL280 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL338	D	α	XL280 <i>GFP-NOP1</i> (NAT)
SL339	D	α	XL280 <i>GFP-NOP1</i> (NAT)
SL340	D	α	XL280 <i>GFP-NOP1</i> (NAT)
SL341	D	α	XL280 <i>GFP-NOP1</i> (NAT)
SL342	D	α	XL280 <i>GFP-NOP1</i> (NAT)
SL380	A	α	SL295 ( <i>kar3</i> ) <i>GFP-NOP1</i> (NAT)
SL381	A	α	SL295 ( <i>kar3</i> ) <i>GFP-NOP1</i> (NAT)
SL383	A	α	SL296 ( <i>kar3</i> ) <i>GFP-NOP1</i> (NAT)
SL385	A	α	SL296 ( <i>kar3</i> ) <i>GFP-NOP1</i> (NAT)
SL374	D	α	SL277 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL375	D	α	SL277 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL376	D	α	SL277 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
SL377	D	α	SL277 ( <i>kar7</i> ) <i>GFP-NOP1</i> (NAT)
Plasmids			
pSL01			Amp <sup>r</sup> Kan <sup>r</sup> <i>NOP1</i> fragment in pCR21-TOPO for N-terminal GFP tagging with pCN19
pSL02			Amp <sup>r</sup> Kan <sup>r</sup> <i>NOP1</i> fragment in pCR21-TOPO for N-terminal GFP tagging with pCN19
pSL04-02			Amp <sup>r</sup> BglII fragment of <i>NOP1</i> from pSL01 in pCN19/BamHI, vector for GFP-Nop1 for serotype A
pSL05-1			Amp <sup>r</sup> BglII fragment of <i>NOP1</i> from pSL02 in pCN19/BamHI, vector for GFP-Nop1 for serotype D
pCN19			Amp <sup>r</sup> plasmid harboring <i>GFP</i> under histone H3 promoter

<sup>a</sup> Each superscript 1, 2, or 3 indicates a group of independent transformants or progeny.

**Microscopy.** Colony morphology and mating hyphae were observed by using a Nikon Eclipse E400 microscope equipped with a Nikon DXM1200F camera. Initial fluorescent signal screening was performed by using a Zeiss Axioskop 2 Plus with an AxioCam MRm camera (Carl Zeiss, Inc., Thornwood, NY). Further nuclear positioning analyses during mating were performed by using the Zeiss Axio Observer Z1 microscope system (Carl Zeiss, Inc., Thornwood, NY) equipped with an Opto-electronically motorized XY stage, Pecon XL S1 incubator, and Coolsnap ES2 high-resolution charge-coupled device (CCD) camera (Photometrics,

Inc., Huntington Beach, CA) in the Duke University Light Microscopy Core Facility (LMCF).

For scanning electron microscopy (SEM), the edges of mating colonies were washed with 0.1 M Na cacodylate buffer (pH 6.8), and 1-mm<sup>3</sup> blocks of mating areas were excised and incubated in fixation buffer at 4°C. Samples were then rinsed in cold 0.1 M Na cacodylate buffer three times, postfixed in 2% osmium tetroxide in 0.1 M Na cacodylate buffer for 2.5 h at 4°C, critical point dried, and sputter coated before being viewed by SEM.

TABLE 2 Karyogamy genes in *S. cerevisiae* and *C. neoformans* serotypes A and D

Function	Karyogamy gene in:				Description
	<i>S. cerevisiae</i>	<i>C. neoformans</i>			
		Designation	Serotype A	Serotype D	
Nuclear congression	<i>KAR1</i>	None	None	None	Kar1 protein localizes to the half-bridge of the spindle pole body
	<i>KAR3</i>	<i>CnKAR3</i>	CNAG_05752	CNF02260	Kar3 is a minus-end-directed microtubule motor that localizes to the spindle pole body.
	<i>KAR4</i>	<i>CnKAR4</i>	CNAG_04487	CNI00070	Kar4 is a transcription factor required for gene regulation in response to pheromones, and expression of <i>KAR3</i> and <i>CIK1</i> .
	<i>KAR9</i>	None	None	None	Kar9 is a karyogamy protein required for correct positioning of the mitotic spindle.
Nuclear membrane fusion	<i>KAR2</i>	<i>CnKAR2</i>	CNAG_06443	CNN01680	Kar2 is an ATPase involved in protein import into the ER and functions as a chaperone to mediate protein folding in the ER.
	<i>KAR5</i>	None	None	None	The Kar5 protein is required for nuclear membrane fusion during karyogamy and is regulated by pheromone.
	<i>KAR7</i>	<i>CnKAR7</i>	CNAG_01647	CNC01600	Kar7 or Sec66 is a nonessential subunit of the Sec63 complex (Sec63p, Sec62p, Sec66p, and Sec72p) and together with the Sec61 complex, Kar2p/BiP, and Lhs1p forms a channel competent for SRP-dependent protein translocation.
	<i>KAR8</i>	<i>CnKAR8</i>	CNAG_01347	CND04620	Kar8 or Jem1 is a DnaJ-like chaperone required for nuclear membrane fusion during mating; a null mutant is viable.

## RESULTS

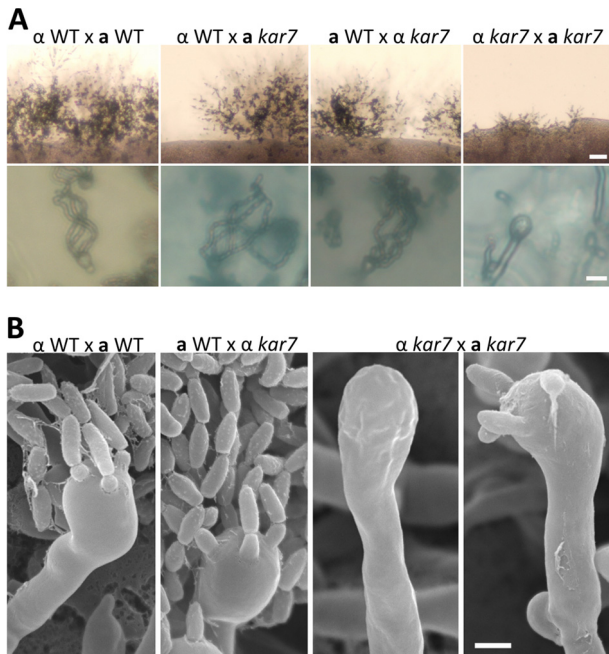
**Identification of *KAR* genes in *Cryptococcus neoformans*.** To elucidate the roles of the karyogamy genes, we identified *KAR* gene orthologs in the *C. neoformans* serotype A ([http://www.broadinstitute.org/annotation/genome/cryptococcus\\_neoformans/MultiHome.html](http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html)) and D ([http://www-sequence.stanford.edu/group/C\\_neoformans/](http://www-sequence.stanford.edu/group/C_neoformans/)) genomes (21, 36). *S. cerevisiae* *KAR* genes and protein sequences were used to identify *C. neoformans* *KAR* gene (*CnKAR*) orthologs in *C. neoformans* serotype A and serotype D. In the BLASTp analyses (1), proteins with reciprocal best hits were considered orthologs for each Kar protein. A summary of the *C. neoformans* *KAR* genes (*KAR2*, *KAR3*, *KAR4*, *KAR7*, and *KAR8*) is listed in Table 2. Interestingly, there were no orthologs of *ScKAR1*, *ScKAR5*, and *ScKAR9* in either the *C. neoformans* serotype A or D genomes, where our analyses resulted in no apparent BLAST hits. The related *C. gattii* genomes (VGI strain WM276 and VGII strain R265) ([http://www.broadinstitute.org/annotation/genome/cryptococcus\\_neoformans\\_b/GenomeStats.html](http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b/GenomeStats.html)) also lack genes encoding these four Kar protein homologs (see Table S1 in the supplemental material) (16).

**The *C. neoformans* *KAR7* ortholog is required for opposite-sex mating.** To assess the role of the *KAR7* gene in opposite-sex mating, the *C. neoformans* *KAR7* gene was replaced with the neomycin (*NEO*) or nourseothricin (*NAT*) drug resistance cassettes in strains of both opposite mating types, as described in Materials and Methods. Two congenic serotype A strains of opposite mating type, KN99a and KN99 $\alpha$ , were employed to assess the roles of *KAR7*. We obtained independent *KAR7* disruption mutants from separate transformations for each mating type (Table 1).

To test mating ability, unilateral and bilateral mutant crosses (together with wild-type bilateral control crosses) were performed on MS mating media (see Materials and Methods). In wild-type crosses, dikaryotic mating hyphae formed and eventually produced aerial hyphae, which in response to an unknown signal form bulb-like basidia at their apices. Four chains of basidiospores then emerged from the surface of each basidium. In unilateral

crosses,  $\alpha$  wild-type  $\times$  a *kar7* or  $\alpha$  *kar7*  $\times$  a wild-type, the overall level of mating hyphae was reduced compared to those observed in bilateral wild-type crosses (Fig. 2A). However, we were still able to observe mating hyphae as well as basidia decorated with four spore chains, indicating that one wild-type allele of *KAR7* from the wild-type mating partner is sufficient for sexual development to progress at a wild-type or near-wild-type level. In contrast, in bilateral  $\alpha$  *kar7*  $\times$  a *kar7* mutant crosses, a significantly reduced level of mating hyphae was observed (Fig. 2A). Although aerial hyphae were formed followed by basidium formation, no obvious basidiospore chains were observed (bald basidia). In some exceptional cases, a single basidiospore from one or each chain formation center was attached to a basidium without progressing to form mature spore progeny (Fig. 2B). These results indicate that Kar7 is required for the formation of spore chains and also plays a role in hypha formation at the early stages of sexual development during opposite-sex mating.

**Nuclear localization during wild-type and *kar7* mutant opposite-sex matings.** Monitoring nuclear positioning provides a way to elucidate the role of karyogamy genes during mating. To assess this in live cells, we fused the Nop1 nucleolar protein with mCherry (C-terminus tagged) or GFP (N-terminus tagged). In KN99 $\alpha$  cells, the *NOP1-mCherry* gene was introduced into the native *NOP1* gene and homologous recombination was confirmed by PCR (data not shown). In KN99a cells, the *GFP-NOP1* gene was ectopically integrated (Table 1). Wild-type a and  $\alpha$  strains expressing either Nop1-mCherry or GFP-Nop1, respectively, were crossed and their nuclear positions were monitored during the mating process. In wild-type crosses, we observed that the mCherry and GFP signals are largely superimposed, indicating that cell-cell cytosolic fusion occurs during opposite-sex mating, allowing mixing of the nuclear signals in the dikaryon, and thus the nucleus positioning to be observed is only for opposite-sex mating (Fig. 3). Microscopic observations with live cell matings were conducted, and representative nuclear positions during wild-type opposite-sex



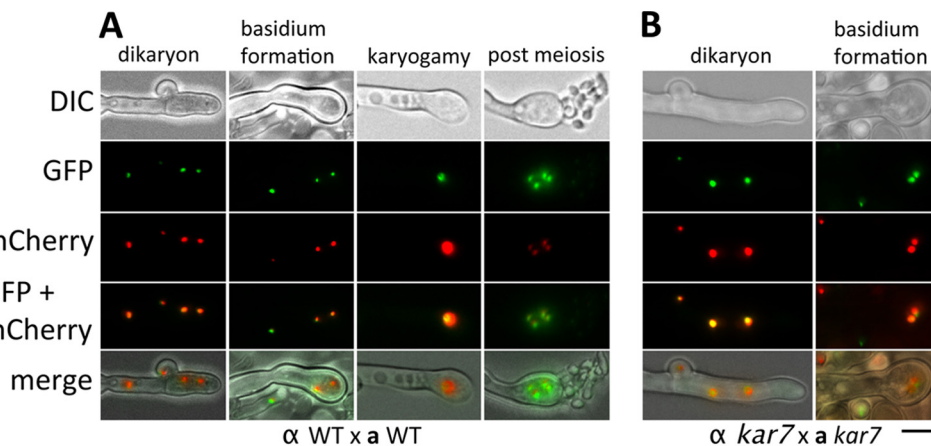
**FIG 2** *KAR7* is required for opposite-sex mating in *C. neoformans*. (A) Wild-type (WT) mating produces abundant dikaryotic hyphae decorated with fused clamp connections, basidia, and chains of spores. A unilateral mating between wild-type and *kar7* mutant strains also produces mating hyphae that are less abundant than those in wild-type crosses. In the bilateral *kar7* × *kar7* mutant crosses, few hyphae were observed. (B) For both wild-type × wild-type and wild-type × *kar7* crosses, four complete chains of basidiospores were observed; however, in the bilateral *kar7* × *kar7* mutant crosses, bald basidia lacking any spores or occasionally basidia with only a single immature spore attached were observed. Scale bars = 100 μm for the upper row and 10 μm for the bottom row in panel A and 5 μm for panel B.

mating are presented in Fig. 3A as follows. (i) In dikaryotic (1N + 1N) hyphae, a nucleus can be observed moving through the clamp connection from the apical hyphal compartment to the subapical hyphal cell, (ii) at the apical hyphal tip, a basidium

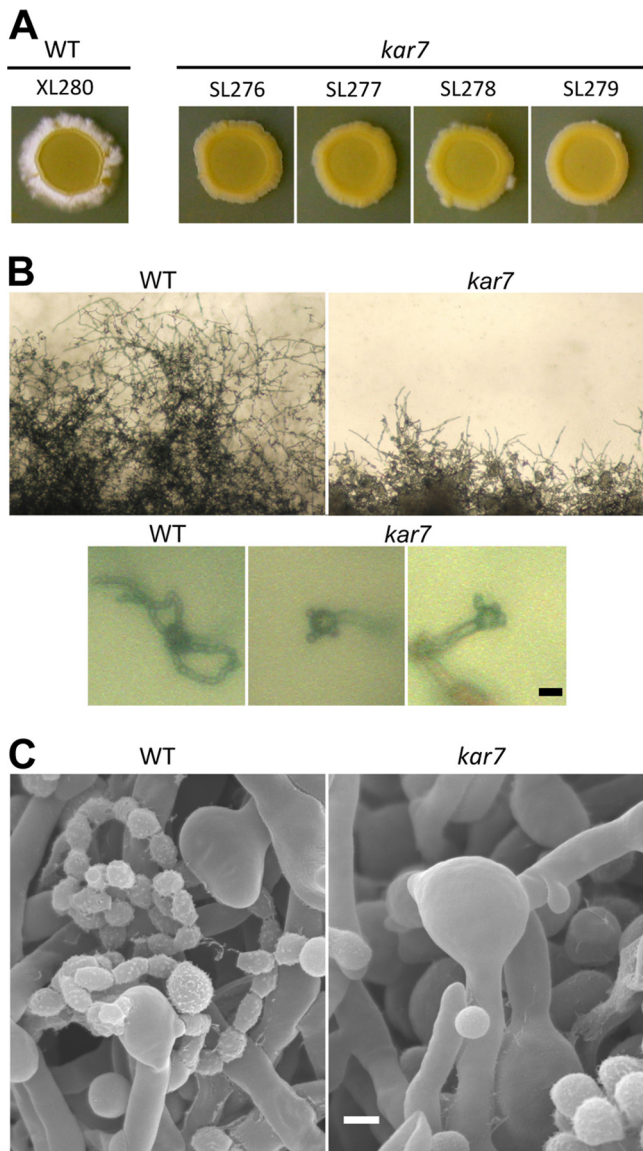
formed, wherein the two nuclei congress, and (iii) fusion of two nuclei occurs, followed by (iv) meiosis.

*C. neoformans* forms holobasidia (single-celled basidia), unlike the closely aligned species *Cryptococcus heveanensis*, where phragmobasidia (basidia divided into four cells by septa) are formed (41). We also found that karyogamy could occur in the subapical area of hyphae of the basidium as well as more apically within the basidium proper, as seen in *Pisolithus microcarpus* (9) (Fig. 3; see Fig. S1 in the supplemental material). In bilateral α *kar7* × α *kar7* mutant crosses, although we were able to observe early stages of nuclear dynamics, including nuclear migration through a clamp connection and nuclear congression in the basidia, no apparent nuclear fusion was observed, which likely results in the observed failure to enter into meiosis or form basidiospore chains (Fig. 3B). These results indicate that the *KAR7* gene is required for karyogamy in the basidia to complete later steps in the mating process, including nuclear fusion to form the diploid, entry into meiosis, and production of sexual spore progeny in *C. neoformans*.

***KAR7* is necessary for unisexual reproduction.** It is of interest to establish whether *KAR7* is also involved in unisexual mating in *C. neoformans*. Same-sex mating under laboratory conditions is not yet known in serotype A; therefore, we employed the serotype D α strain, XL280, which displays robust unisexual reproduction/same-sex mating (30–32). The *KAR7* locus ORF was replaced with a NEO resistance cassette by biolistic transformation and homologous recombination, as described above for the serotype A strains. The wild type and the independently derived *kar7* mutants were obtained and cultured individually to undergo unisexual mating on V8 medium (pH 7) in the dark. In solo culture, the wild-type strain XL280 produced hyphae, basidia, and basidiospores at the edge of the colonies (unisexual reproduction), whereas the independent *kar7* mutants all exhibited a severe defect in producing unisexual hyphae (Fig. 4A) compared to the wild type. These findings indicate a role for *Kar7* in the early stages of hypha formation during same-sex mating (Fig. 1). Further analyses found that the *kar7* mutants can produce hyphae and basidia but are defective in basidiospore chain formation, indicating that



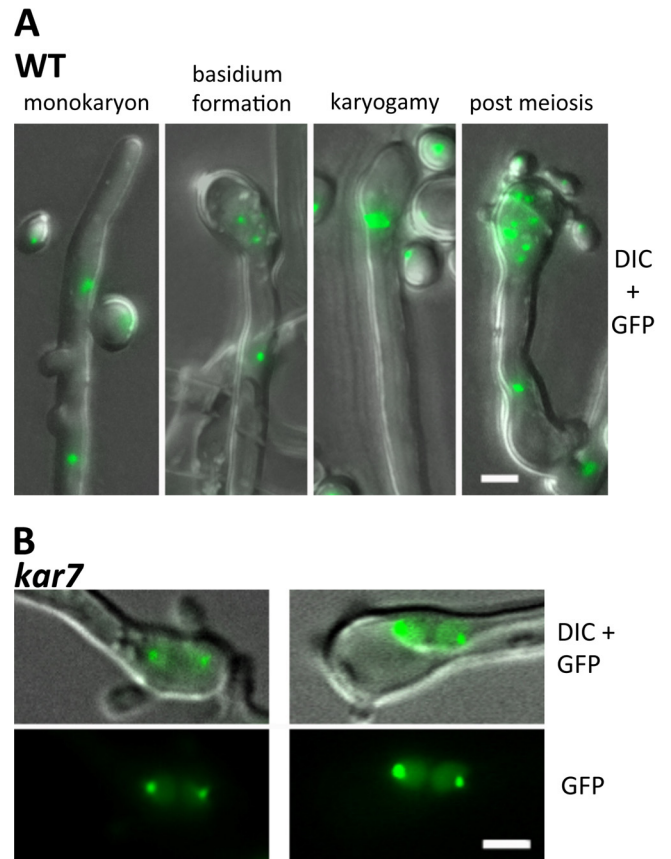
**FIG 3** Nuclear positioning during mating in the wild type (A) and *kar7* mutant (B). DIC, differential interference contrast. α mating type cells producing Nop1-GFP and α mating type cells producing Nop1-mCherry were cocultured on MS mating medium. The two fluorescent signals were observed to merge, indicating that cytosolic fusion occurs during the process of α-α opposite-sex mating. The two nuclei remain separated in the dikaryotic hyphae. In the basidium, the two nuclei migrate toward each other and karyogamy occurs, resulting in diploidization. Meiosis follows, and chains of basidiospores are produced (A). In the crosses between two *kar7* mutants, the dikaryotic hyphae produced are similar to those in the wild type; however, in the mutant basidia, the two congressed nuclei do not undergo fusion, resulting in a failure to proceed into meiosis or produce spore progeny (B). Scale bar = 5 μm.



**FIG 4** *KAR7* is necessary to complete unisexual reproduction in *C. neoformans*. The edges of colonies of wild-type (WT) strain XL280 exhibit abundant monokaryotic hyphae; however, two independent sets of *kar7* mutants (the first and second ones are independent from the third and fourth from left to right) produced considerably less hyphae (A). The wild type produces four intact chains of basidiospores, whereas in the *kar7* mutant, unisexual reproduction was arrested (possibly before meiosis), resulting in no spore progeny production. Images in panels B and C show *kar7* mutant basidia with a single attached immature spore. Scale bars = 10  $\mu\text{m}$  for panel B and 5  $\mu\text{m}$  for panel C.

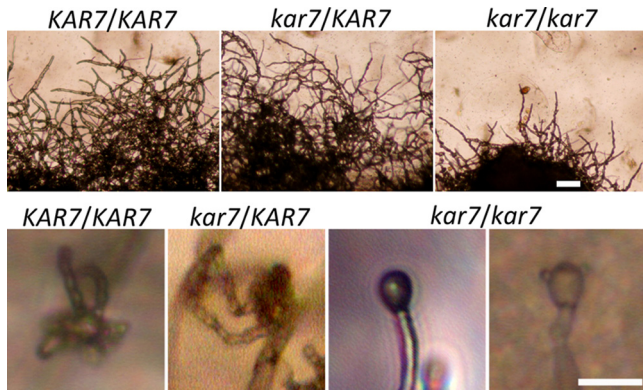
*Kar7* is also required during same-sex mating to complete karyogamy, meiosis, and spore progeny production, similar to its role in  $\alpha$ - $\alpha$  opposite sex mating (Fig. 4B and C). Scanning electron microscopy analysis further verified that bald basidia, or in some cases basidia decorated with only one or two immature basidiospores, were formed without completing sexual reproduction. These results indicate that *Kar7* function is necessary for same-sex as well as opposite-sex mating.

**Nuclear positioning during same-sex mating.** To observe nuclear positions during unisexual mating, we integrated the GFP-Nop1-encoding gene ectopically into the genomes of the wild-



**FIG 5** Nuclear positions during unisexual reproduction in the wild type and *kar7* mutant. Nuclei were visualized with the GFP-Nop1 protein. (A) In the wild-type XL280 strain, sexual hyphae are monokaryotic with unfused clamp connections. Two nuclei congress in the basidia and undergo karyogamy, meiosis occurs, and basidiospores are produced. DIC, differential interference contrast. (B) In contrast, in the *kar7* mutants, the two nuclei congress but do not undergo karyogamy, resulting in a failure to produce the diploid nucleus or undergo meiosis, and consequently, no spore chains are produced. Scale bars = 5  $\mu\text{m}$ .

type XL280 and *kar7* mutant strains. GFP signal-positive strains were chosen and grown on V8 medium (pH 7) to stimulate same-sex mating. Nuclear positions during mating were monitored (Fig. 5). Based on the GFP-Nop1 signal, the same-sex mating hyphae are monokaryotic (1N) and the clamp connections are unfused (see the monokaryon panel in Fig. 5A). This is in contrast to opposite-sex mating (Fig. 3A), during which dikaryotic hyphae are produced with two separate fluorescent Nop1 signals that were observed in a single hyphal compartment and one labeled nucleus moves through clamp cells that then fuse. At the terminal same-sex mating hyphal compartment, a nucleus underwent mitosis to generate a transient dikaryotic stage (1N + 1N), and karyogamy then occurred inside the basidia or subapical hyphae (Fig. 5; see Fig. S1 in the supplemental material). Meiosis and complete basidiospore chain formation then followed (Fig. 5A). However, in the *kar7* mutant same-sex mating assays, two separate nuclei were observed that persisted in the basidia and did not fuse. As a result, diploidization did not occur, causing a failure to enter into meiosis or form basidiospore chains (Fig. 5B). This observation leads us to conclude that diploidization during XL280 same-sex mating can occur in the basidia as a late step in the pathway and that these



**FIG 6** Unisexual reproduction of a diploid *kar7/kar7* strain. In the diploid strain, both alleles of the *KAR7* gene were disrupted. The wild type and the heterozygous *KAR7/kar7::HYG* mutant undergo complete same-sex mating, including abundant sexual hypha formation and basidium formation followed by spore chain production. However, *kar7::HYG/kar7::URA5* mutants display mating defects, as seen in the haploid *kar7* mutants: for example, less abundant sexual hyphae formation and bald or single spore-attached basidium formation as a consequence of a failure to enter into meiosis or produce spore chains. Scale bars = 100  $\mu\text{m}$  for the upper row and 10  $\mu\text{m}$  for the bottom row.

steps (karyogamy and meiosis) may be necessary for nuclei to be packaged into spores. However, we also observed that in the serotype D strain JEC21, the *kar7* mutant was completely unable to initiate filamentation, indicating that karyogamy and diploidization can also occur as an early stage required for unisexual mating hyphal development (see Fig. S2 in the supplemental material).

**Diploid *kar7/kar7* mutants are defective in unisexual reproduction.** Our observation that *kar7* mutants failed to complete unisexual reproduction prompted us to test the impact of *kar7* in an  $\alpha/\alpha$  diploid strain. If diploidization in the basidia is essential to complete mating, and the sole role of Kar7 is to promote karyogamy and diploidization, then *kar7/kar7* diploid mutants might bypass the normal requirement for Kar7 for diploidization and complete unisexual reproduction. On the other hand, if the *kar7/kar7* diploid mutants are still defective in unisexual mating, it would suggest that Kar7 plays an additional role beyond karyogamy/diploidization during same-sex mating.

To test these hypotheses, we constructed an  $\alpha/\alpha$  diploid derivative of strain XL280. First, the *URA5* locus was replaced with either the *NEO* or *NAT* resistance cassette. The resulting haploid strains, XL280 *ura5::NEO* and XL280 *ura5::NAT*, were then fused to produce the *ura5::NEO/ura5::NAT* strain MN142.3 (Table 1). The ploidy of this isolate was confirmed to be diploid by fluorescence-activated cell sorter (FACS) and nucleus staining with DAPI (4',6-diamidino-2-phenylindole) (data not shown). The MN142.3  $\alpha/\alpha$  diploid strain exhibits robust same-sex mating (Fig. 6), as expected based on previous studies (31). We disrupted both alleles of the *KAR7* gene in strain MN142.3 by gene replacement with the hygromycin resistance gene cassette and the wild-type *URA5* gene (Table 1) as markers. The mutants were confirmed by Southern blotting (see Fig. S3 in the supplemental material). The heterozygous *kar7::HYG/KAR7* mutants displayed wild-type unisexual hyphal growth and basidiospore chain formation (Fig. 6). However, interestingly, independently derived homozygous *kar7/kar7* diploid mutants displayed reduced filamentation and defects in basidiospore chain formation (Fig. 6). This result suggests that Kar7 may play an additional role(s) during mating beyond its role

in karyogamy and diploidization. As a note, it is also formally possible that Kar7 functions early in some cells/hyphae and late in others such that there is a mixed culture of haploid and diploid monokaryotic hyphae produced. Nuclear position monitoring will provide further insight into the distinct roles of Kar7 during these developmental processes.

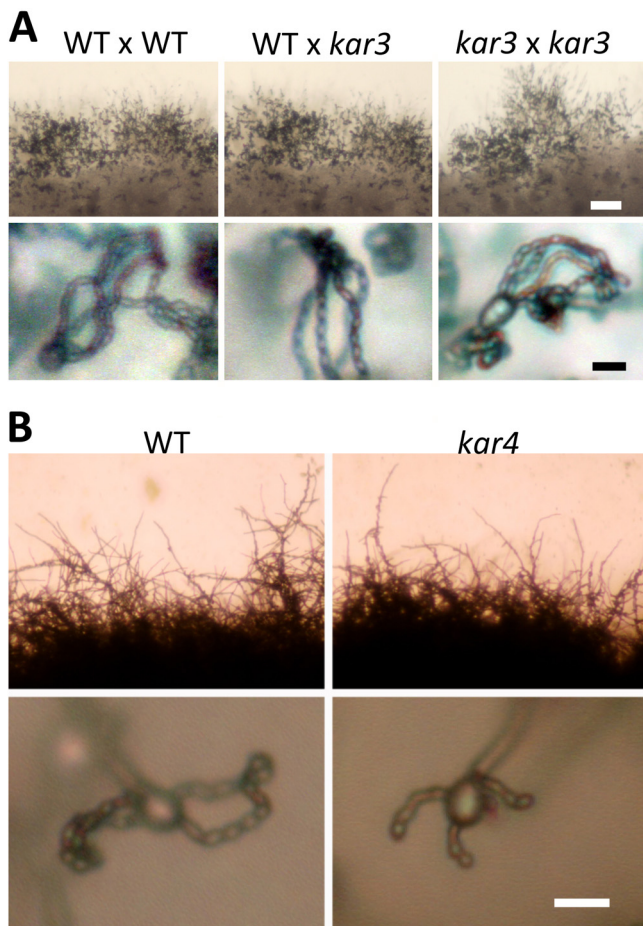
***KAR2* is essential for viability, but its role in *C. neoformans* mating is unknown.** Exhaustive trials to disrupt the *KAR2* gene in *C. neoformans* were unsuccessful. Thus, we tested if the *KAR2* gene is essential for viability. One *KAR2* allele from the diploid strain MN142.3 was replaced with the *HYG* drug resistance cassette. We obtained a heterozygous *kar2::HYG/KAR2* diploid isolate that underwent unisexual reproduction, from which spore-derived progeny were analyzed based on hygromycin resistance. Of 12 haploid progeny, none were *kar2* mutants ( $n = 12$ ;  $P = 0.0005$ ), indicating *KAR2* is essential. We did not observe any hyphal morphogenesis defect of the heterozygous *kar2/KAR2* mutant strains.

***KAR3*, *KAR4*, and *KAR8* are dispensable during mating.** The functions of three other *KAR* genes (*KAR3*, *KAR4*, and *KAR8*) during mating were assessed. The *KAR3* gene was disrupted in the *C. neoformans* serotype A strain pair KN99 $\alpha$  and KN99a. *kar3::NAT* and *kar3::NEO* mutants crossed unilaterally (mutant  $\times$  wild type) or bilaterally (mutant  $\times$  mutant) underwent opposite-sex mating successfully (Fig. 7A). Because Kar3 is a microtubule motor protein involved in nuclear congression in *S. cerevisiae* (Fig. 1 and Table 2), we tested if there is any nuclear distance difference in mating hyphae between the wild type and  $\alpha$  *kar3*  $\times$  a *kar3* crosses. To monitor nuclei,  $\alpha$  mating type *kar3* mutants (SL295 and SL296) were transformed with the GFP-Nop1 plasmid (pSL04-1), and the resulting  $\alpha$  *kar3* strains expressing GFP-Nop1 (Table 1) were crossed with a *kar3* mutants. The distance between two nuclei in one dikaryotic hyphal compartment was not significantly different between wild-type and  $\alpha$  *kar3*  $\times$  a *kar3* matings ( $5.57 \pm 0.47$   $\mu\text{m}$  versus  $5.53 \pm 0.76$   $\mu\text{m}$ , respectively) (see Fig. S4 in the supplemental material). These results indicate that Kar3 is not necessary to complete the mating process or govern nuclear dynamics in the dikaryotic hyphae.

We initially speculated that the *C. neoformans* *KAR4* might be essential because gene disruption attempts were not successful, even though the *kar4* mutant is viable in *S. cerevisiae*. However, we did obtain viable *kar4* mutants through a diploid unisexual reproduction approach (see above and Materials and Methods). One *KAR4* allele in the diploid strain MN142.3 was replaced with the hygromycin resistance gene, and the resulting heterozygous *kar4/KAR4* strains underwent hyphal morphogenesis and sporulation on V8 medium (pH 7) in the dark. Spores were dissected and we recovered 7 *kar4::HYG* haploid progeny out of 13 progeny analyzed. Based on PCR, these *kar4* mutant progeny lack the ORF of the wild-type gene (data not shown). The ploidy of the progeny was confirmed to be haploid, based on FACS analysis of cells stained with propidium iodide (see Fig. S5 in the supplemental material), indicating that the *KAR4* gene is not essential. However, *kar4* mutants did not exhibit any unisexual mating defects (Fig. 7B) compared to the wild type, indicating that *KAR4* is also dispensable for unisexual mating.

The *KAR8* gene encoding a putative protein chaperone in the ER membrane was replaced with the *NEO* or *NAT* cassette in the congenic serotype D strains JEC21 $\alpha$  and JEC20a (Table 1). In the unilateral *kar8*  $\times$  wild-type and bilateral *kar8*  $\times$  *kar8* mutant crosses, we found that disruption of *KAR8* did not result in



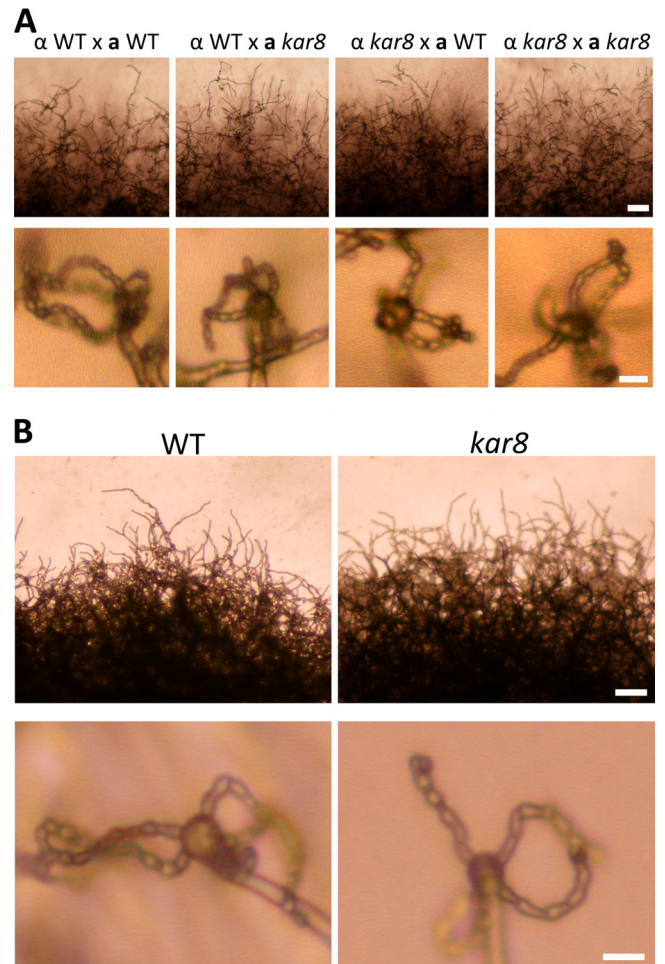


**FIG 7** *KAR3* and *KAR4* are dispensable for mating in *C. neoformans*. (A) Serotype A *kar3* mutants complete opposite-sex mating without any marked defects observed in either unilateral or bilateral mutant crosses. (B) Unisexual reproduction of *kar4* mutants produces four chains of basidiospores. V8 (pH 7) and MS media for serotypes D and A, respectively, were used for mating and incubated at room temperature in the dark for 3 weeks before observation. Scale bars = 50  $\mu\text{m}$  for the upper row and 10  $\mu\text{m}$  for the bottom row in panel A and 10  $\mu\text{m}$  for panel B.

mating defects, wherein the mutants were able to complete mating and produce spore progeny (Fig. 8A). We also tested the role of Kar8 in same-sex mating. The XL280 strain was used, and the *KAR8* locus was replaced with the *NAT* marker gene. The *kar8::NAT* mutant exhibited morphologically complete same-sex mating similar to that of the wild-type parental strain (Fig. 8B). These results indicate that Kar8 is dispensable for both opposite-sex and unisexual mating.

## DISCUSSION

*C. neoformans* is thought to be predominantly a haploid organism. However, both clinical and environmental diploid isolates also occur and represent  $\sim 10\%$  of isolates analyzed (34). One central question is how are diploids in general formed? There are two possibilities: (i) endoreplication and (ii) pathways involving karyogamy. Endoreplication can produce diploids with homozygous alleles, and some known  $\alpha\text{AA}\alpha$ ,  $\text{aAAa}$ ,  $\alpha\text{DD}\alpha$ ,  $\text{aDDa}$ , isolates might have arisen via this process (12, 29, 33). However, other diploids are clearly heterozygous, including  $\alpha\text{AAa}$ ,  $\alpha\text{DDa}$ ,



**FIG 8** *KAR8* is not required for opposite- or same-sex mating. (A) Serotype D *kar8* mutants exhibit no apparent defects in sexual development during  $\text{a}\alpha$  opposite-sex mating. (B) An XL280-derived *kar8* mutant also displays no apparent mating defects. V8 medium (pH 7) was used for mating and incubated at room temperature in the dark for 3 weeks before observation. Scale bars = 100  $\mu\text{m}$  for the upper row and 5  $\mu\text{m}$  for the bottom row in both panels A and B.

and also  $\alpha\text{ADa}$ ,  $\text{aAD}\alpha$ , and  $\alpha\text{AD}\alpha$  hybrid isolates (12, 34, 35). In these cases, endoreplication is not sufficient to explain their occurrence, and cell-cell and nuclear fusion (karyogamy) are an alternative route to explain the existence of these heterozygous diploids. Karyogamy in basidiomycetes is associated with mating. However, a question remaining unanswered is when diploidization occurs during opposite- or same-sex mating—i.e., early karyogamy before hyphal formation or late karyogamy just prior to meiosis. While karyogamy during opposite-sex mating is typically modeled as occurring late, under laboratory conditions, two haploid cells can undergo cell-cell and nuclear fusion at 37°C, which is how diploids are constructed and isolated in the laboratory (19, 20). Interestingly,  $\text{a}/\alpha$  diploid strains exhibit thermal dimorphism, in which the diploid cell grows as yeast at 37°C, whereas at 24°C, the diploid cell undergoes filamentation to produce monokaryotic hyphae and complete sexual reproduction (56). This is an example of an “early” karyogamy event occurring during opposite-sex mating (Fig. 1). By analogy, karyogamy could also occur early, late, or both early and late, during unisexual reproduction. Examination of karyogamy genes during opposite-

sex and unisexual mating can further provide insight to understand when and how diploidization occurs in *C. neoformans*.

Although the mechanics of karyogamy are well established in *S. cerevisiae* (52), the roles of karyogamy genes are less well understood in basidiomycetes, including *C. neoformans*. In this study, we found that Kar7/Sec66 plays a key role in completion of both opposite-sex and unisexual mating. Mutants lacking this protein exhibit a failure of karyogamy in *C. neoformans* during both types of mating. Therefore, the *KAR7* gene is essential for diploidization. In the analysis of unisexual mating of the *kar7* mutants, the mating process was arrested at the basidium stage without karyogamy (Fig. 2 to 5). Thus, in the XL280 strain, diploidization can occur late in the basidium just prior to meiosis. However, we cannot exclude the possibility that the *KAR7* gene functions through processes in addition to karyogamy during mating. Our study with diploids homozygous for the *kar7* mutation revealed that diploidy itself is not sufficient to overcome the requirement for *KAR7* for meiotic entry and basidiospore chain formation (Fig. 6). The underlying role in this case remains to be elucidated. In addition, in the other serotype D strain analyzed (JEC21), a *kar7* mutant exhibited a complete defect in unisexual filamentation (see Fig. S2 in the supplemental material). In this case, early karyogamy and diploidization could be a prerequisite step for early hyphal growth. This hypothesis is supported by the findings that (i) diploid  $\alpha/\alpha$  strains are more filamentous than haploid  $\alpha$  strains, and (ii) blastospores emerging from the hyphae of the haploid strain JEC21 can be diploid (31).

Kar7/Sec66 is a component of the Sec63 complex that functions in protein translocation into the ER lumen (23, 65). The exact mechanistic role of the Kar7 protein in karyogamy is unknown: the target proteins for Kar7 and the Sec63 complex that are translocated into the ER and those in the ER transported to cytosol by the Kar7 and Sec63 complex remain to be identified. One possible function of Kar7 is that it is involved in transport of proteins to initiate and complete nuclear fusion and, potentially, the transport of proteins associated with triggering meiosis. We also observed that the *kar7* mutants exhibit some detrimental phenotypes during vegetative yeast growth (see Fig. S6 in the supplemental material), which indicates that the protein trafficking function may affect growth. It is also possible that Kar7 has a karyogamy-specific function other than protein trafficking in the ER membrane (45).

Among the *KAR* genes involved in nuclear congression, the *C. neoformans* genome lacks *KAR1* and *KAR9* genes, and, more interestingly, our study found that the *KAR3* and *KAR4* genes are not essential for sexual reproduction (Fig. 7), although in *S. cerevisiae*, both *KAR3* and *KAR4* are essential for karyogamy and mating (13, 23, 24, 40), and in *C. albicans*, *KAR3* is required to complete sexual development (4). It is of interest that the *C. neoformans* Kar3 and Kar4 proteins appear to play no role in mating and therefore are dispensable for karyogamy. *C. neoformans* is a basidiomycete, and its mating is characterized by an extended dikaryotic hyphal stage, wherein two parental nuclei are paired but unfused in a single hyphal compartment. Nuclear fusion (karyogamy) occurs inside the basidia at the apex of the aerial hypha (2, 11). It is possible that nuclear congression is a less relevant step in *C. neoformans* mating during which the dikaryotic stage is prolonged. In ascomycetes, including *S. cerevisiae*, the dikaryotic stage is transient and fleeting during mating, and thus karyogamy genes that function in nuclear congression are of substantial consequence during mating to ensure the rapidity of the

process. This phenotypic difference in nuclear positions during mating and the timing of nuclear fusion may impact the different evolutionary trajectories of the *KAR* genes involved in nuclear migration in the two phyla.

Among the two *KAR* genes involved in nuclear membrane fusion, *C. neoformans* *KAR8* was found to be dispensable for sexual reproduction (Fig. 8). Kar8 is a DnaJ-like protein anchored to the ER membrane, where it functions together with Hsp70. In *S. cerevisiae*, a *kar8* deletion mutant displays defects in karyogamy (46). However, in *C. neoformans*, only *KAR7* was found to be essential for *C. neoformans* mating, indicating a differential adaptation of the *KAR* genes in this fungus. For example, *S. cerevisiae* karyogamy genes, including *KAR4* and *KAR5*, are regulated by pheromone (Table 2); however, in *C. neoformans*, the pheromone signal is involved in the initial cell-cell fusion step (and subsequent clamp cell fusion events), which is separated from karyogamy by the extended dikaryotic hyphal stage (see Fig. S7 in the supplemental material).

Our finding that some *KAR* genes are dispensable for sexual reproduction in *C. neoformans* indicates that other novel proteins may be involved in *C. neoformans* karyogamy. Future work that analyzes mating defects, especially in mutants arrested at the basidium formation step without further progression into meiosis and sporulation, has the potential to identify novel genes involved in karyogamy in *C. neoformans*. Kar2 is an ATPase involved in protein trafficking in the ER, and in *C. neoformans*, *KAR2* is essential as in *S. cerevisiae*. The possible roles of *KAR2* in *C. neoformans* nuclear dynamics remain to be elucidated.

It is curious that some of the genes one would expect to be involved in karyogamy and sexual reproduction don't seem to play such a role in *C. neoformans*. This may indicate that karyogamy is more plastic than one might have anticipated. Analogies are found in meiosis in other fungi and insects. The *Candida lusitanae* genome is missing many key meiotic genes, but it still undergoes meiosis during sexual reproduction (8, 50). *Caenorhabditis elegans*, *Drosophila melanogaster*, *Neurospora crassa*, *Ustilago maydis*, and *C. lusitanae* all lack the meiosis-specific Dmc1 protein that functions in DNA double-strand-break repair during meiosis in other species, and yet all still successfully complete meiosis (5, 15, 61). In addition, in *Schizosaccharomyces pombe* synaptonemal-complex component protein genes are missing, and instead LinE elements are involved in chromosomal pairing and migration (37).

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