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REVIEW

Pneumocystis Mating-Type Locus and Sexual Cycle during Infection

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SUMMARY *Pneumocystis* species colonize mammalian lungs and cause deadly pneumonia if the immune system of the host weakens. Each species presents a specificity for a single mammalian host species. *Pneumocystis jirovecii* infects humans and provokes pneumonia, which is among the most frequent invasive fungal infections. The lack of *in vitro* culture methods for these fungi complicates their study. Recently, high-throughput sequencing technologies followed by comparative genomics have allowed a better understanding of the mechanisms involved in the sexuality of *Pneumocystis* organisms. The structure of their mating-type locus corresponding to a fusion of two loci, Plus and Minus, and the concomitant expression of the three mating-type genes revealed that their mode of sexual reproduction is primarily homothallism. This mode is favored by microbial pathogens and involves a single self-compatible mating type that can enter into the sexual cycle on its own. *Pneumocystis* sexuality is obligatory within the host's lungs during pneumonia in adults, primary infection in children, and possibly colonization. This sexuality participates in cell proliferation, airborne transmission to new hosts, and probably antigenic variation, processes that are crucial to ensure the survival of the fungus. Thus, sexuality is central in the *Pneumocystis* life cycle. The obligate biotrophic parasitism with obligate sexuality of *Pneumocystis* is unique among fungi pathogenic to humans. *Pneumocystis* organisms are similar to the plant fungal obligate biotrophs that complete their entire life cycle within their hosts, including sex, and that are also difficult to grow *in vitro*.

KEYWORDS obligate parasite, obligate sexuality, *Pneumocystis*, Taphrinomycotina, opportunistic fungi, sexuality

INTRODUCTION

Pneumocystis species are fungi that colonize the lungs of mammals (1). They belong to the subphylum Taphrinomycotina of the ascomycetes, which also includes plant pathogens and commensals. Each *Pneumocystis* species presents specificity for one mammalian host species, although the strictness of this characteristic remains to be understood (2, 3). Should the host's immune system weaken, *Pneumocystis* organisms turn into pathogens that

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This review is dedicated to my dear wife Brigitte, who provides constant support throughout the years.

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cause deadly pneumonia. That caused by the species infecting humans, *Pneumocystis jirovecii*, is among the most frequent invasive fungal infections (4), including in HIV-negative children (5). The lack of a long-term *in vitro* culture methods for these fungi complicates their study. Infections of rats and mice by *Pneumocystis carinii* and *Pneumocystis murina*, respectively, are used as models of the infection in humans. *Pneumocystis* organisms proliferate extracellularly within the lumen of the host's lungs' alveoli. They are biotrophic parasites that acquire nutrients from living host cells (6, 7). Whole-genome sequencing followed by comparative genomics showed that *Pneumocystis* species miss enzymes to carry out several biosynthetic pathways, revealing that they are obligate parasites scavenging essential compounds from their host (8–11).

Researchers believed first that the life cycle of *Pneumocystis* organisms might be devoid of a sexual phase. Nevertheless, microscopic observation of synaptonemal complexes within *P. carinii* cells revealed that meiosis occurs (12, 13). Indeed, these structures are involved in the alignment of the homologous chromosomes and mediate crossover between them. Characterization of several genes involved in sexuality subsequently confirmed the occurrence of sex during infection (14–18). Accordingly, the life cycles proposed for *Pneumocystis* organisms typically include both asexual and sexual phases (19–21); the most updated one is shown in Fig. 1 (22). The haploid trophic forms would multiply asexually by binary fission and possibly by endogeny and would be involved in mating, which initiates the sexual phase. The latter culminates by the production of asci containing each of eight haploid ascospores that ensure dissemination by the air route and participate into proliferation within the host lungs. Thus, sexuality proved to be central in the *Pneumocystis* life cycle. Other features of the cell cycle shown in Fig. 1 are commented on the following sections.

Early studies have assessed the occurrence of sex during the *Pneumocystis* cell cycle, but the process remained obscure. Recently, the mechanisms of *Pneumocystis* sexuality began to be unraveled thanks to whole-genome analyses, transcriptomics, and comparative genomics. In this article, I summarize the present knowledge of *Pneumocystis* sexuality and point to open questions.

PNEUMOCYSTIS SEX-RELATED GENES

Eighty-three putative sex-related genes have been identified in the genome of each *Pneumocystis* species (14–18, 23–25) (Table 1). Their detection mainly relies on their homology with genes involved in the well-characterized sexuality of the close relative *Schizosaccharomyces pombe* (26). These genes are potentially involved in all processes of fungal sexuality: i.e., mating signaling, cell-cell fusion, karyogamy, meiosis, and mating-type (*MAT*) locus silencing and switching (24, 25). However, extensive rewiring of the *MAT* pathways is common among fungi (27, 28), and therefore, the presence or absence of specific orthologs might be insignificant. For example, the gene *mei3*, which is required for entry into meiosis in *S. pombe*, is absent in *Pneumocystis*, which implies that the latter species uses another way to carry out this step. Nevertheless, the set of sex-related genes present in the *Pneumocystis* genome is consistent with the occurrence of the processes that are integral to sexuality (i.e., karyogamy and meiosis), whereas other sexual processes may not take place. Indeed, the genes *tht1* and *dmc1* have conserved function among fungi (16, 29) and thus are signatures of karyogamy and meiosis, respectively (Table 1). The structure of the *Pneumocystis MAT* locus, which is discussed in the following section, provides insights into this issue.

THE MATING-TYPE LOCUS OF *PNEUMOCYSTIS*

The 83 *Pneumocystis* sex-related genes include three putative *MAT* genes, the genes that govern fungal sexuality by controlling cellular mating-type identity and that are central to this process (Table 1). Taking *S. pombe* sexuality as a model (26) (Fig. 2A), the *Pneumocystis matMc* gene encodes the transcription factor with a high-mobility group DNA-binding domain responsible for the differentiation into the cellular mating type Minus (M). The *Pneumocystis matPi* gene encodes the transcription factor with a homeobox DNA-binding domain that functions with the cofactor *matMi*, which is the third

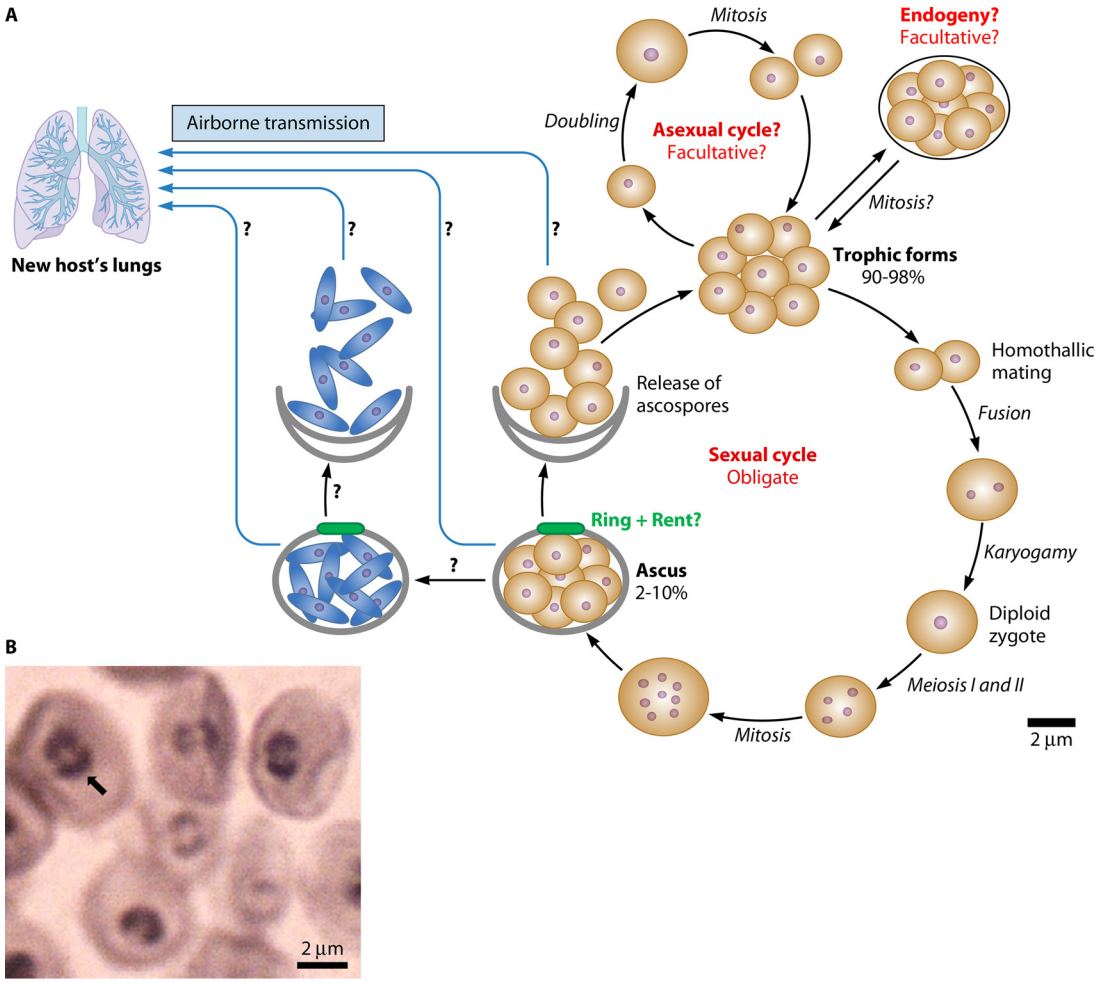

FIG 1 (A) Hypothetical life cycle of *Pneumocystis*. Violet dots represent nuclei. The question marks indicate events not supported or poorly supported by the data. Elongated ascospores that present a condensed cytoplasm are shown as blue spindle-shaped cells. No differences between the different *Pneumocystis* species were reported. (B) Structures like parentheses on *P. jirovecii* asci. The arrow points to this structure of a single ascus. It may correspond to the thickened ring observed in ascomycetes, in the center of which a rent is formed, allowing dehiscence (i.e., the release of the ascospores). Grocott's methenamine silver staining is shown. (Panels A and B are redrawn and reproduced from Fig. 1 and 2, respectively, of reference 22 under the terms of the Creative Commons Public Domain declaration [https://creativecommons.org/publicdomain/zero/1.0].)

Pneumocystis MAT gene identified. In *S. pombe*, *matPi* with *matMi* activates *mei3*, which derepresses meiosis in the zygote (Fig. 2A). In the absence of *mei3* in *Pneumocystis* species, the function of *matPi* with *matMi* remains to be understood. Neither an ortholog to the *S. pombe* transcription factor *matPc* responsible for differentiation into the mating type Plus (P) was identified in *Pneumocystis*, nor were any other types of *MAT* transcription factors (24, 30). Importantly, the identity of *Pneumocystis matMc* was confirmed experimentally by functional complementation restoring meiosis and sporulation of the *S. pombe matMc* mutant (30). These observations suggest that the three *MAT* genes identified are sufficient to trigger sexual mating and meiosis in *Pneumocystis* species, but that the mechanisms involved differ from those in *S. pombe*.

The three *MAT* genes identified are close to each other in the genome of each *Pneumocystis* species; they constitute the *MAT* locus (Fig. 3). An intergenic region of 100 to 300 bp separates *matMc* and *matMi* genes, which are divergently transcribed. The third *MAT* gene, *matPi*, is located approximately 8 kbp away, on the same strand as *matMc*. The upstream region of each *MAT* gene harbors a potential transcription promoter (30). The shared promoter region of *matMc* and *matMi* suggests a common regulation ensuring a tightly coordinated expression. Such an arrangement is a

TABLE 1 Sex-related genes identified in *Pneumocystis* species

Role	Gene name (alternate)	Gene ID			*S. pombe* putative ortholog		Reference(s)
		P. jirovecii	*P. carinii*	*P. murina*	Gene ID	Function	
Mating-type locus	*matMc*	T551_02162	T552_02831	PNEG_02275	SPBC1711.02	Expression of M-specific genes	22, 24, 25
	matPi	T551_02159	T552_02829	PNEG_02373	SPMTR.02	Expression of *mei3*, required for meiosis	24, 25
	matMi	Supercontig 9: 23185–23497+	Supercontig 13: 81309–81521+ (24) or 81264–81476+ (25)	Supercontig 13: 81097–81427+	SPBC1711.01c	Expression of *mei3*, required for meiosis	24, 25
Signal transmission	*ste12* (*fab1*)	T551_03275	T552_00248	PNEG_03088	SPBC3E7.01	Secretion of pheromones	24
	mam1	T551_03503	T552_00261	PNEG_03100	SPBC25B2.02c	Export of M-pheromone	24
	mam2	T551_00015	T552_02343	PNEG_03148	SPAC11H11.04	Response to P-pheromone	23–25
	map3 (*ste3*)	T551_02750	T552_00176	PNEG_03013	SPAC3F10.10c	Response to M-pheromone	15, 23–25
Signal transduction	*ste11*	T551_02014	T552_02504	PNEG_02134	SPBC32C12.02	Expression of *MAT* genes	14, 23, 24
	cdc42	T551_02552	T552_03168	PNEG_01785	SPAC110.03	Development of cell polarity	24, 25
	byr1	T551_02571	T552_03147	PNEG_01806	SPAC1D4.13	Regulation of sexual differentiation and conjugation	24
	byr2 (*ste8*)	T551_00958	T552_01502	PNEG_00717	SPBC1D7.05	Regulation of conjugation	24, 25
	gap1	T551_02909	T552_04103	PNEG_00245	SPBC646.12c	Regulation of cell morphogenesis during conjugation	24
	gpa1	T551_00018	T552_02341	PNEG_03151	SPBC24C6.06	Regulation of pheromone signaling	24
	ral2	T551_01782	T552_03378	PNEG_03376	SPBC21.05c	Regulation of pheromone signaling	24
	ras1	T551_03115	T552_00578	PNEG_00279	SPAC17H9.09c	Regulation of pheromone signaling	24
	shk1 (*ste20*)	T551_02174	T552_03023	PNEG_03507	SPBC1604.14c	Regulation of pheromone signaling	24, 25
	spk1 (*fus3*)	T551_02467	T552_02142	PNEG_03249	SPAC31G5.09c	Regulation of pheromone signaling	23–25
	tim10	T551_03530	T552_01369	PNEG_00015	SPAC232.03c	Regulation of pheromone signaling	25
	git11 (*ste18*)	T551_00557	T552_01736	PNEG_02719	SPBC215.04	Regulation of pheromone signaling	25
	git5 (*gpb1*)	T551_00090	T552_00915	PNEG_01572	SPBC32H8.07	Regulation of pheromone signaling	25
	ste4	T551_01873	T552_01857	PNEG_02045	SPAC1565.04c	Regulation of pheromone signaling	25
	ste6	T551_02045	T552_00858	PNEG_01634	SPCC1442.01	Positive regulation of pheromone signaling	24
	rgs1	T551_01456	T552_01691	PNEG_02767	SPAC23F3.12c	Negative regulation of pheromone signaling	24, 25
	scd1	T551_02407	T552_03331	PNEG_03360	SPAC16E8.09	Regulation of cell shape	24, 25
	scd2	T551_02328	T552_03072	PNEG_03558	SPAC23H10.07	Regulation of cell shape	24, 25
	zfs1 (*moc4*)	T551_01998	T552_01981	PNEG_01914	SPBC1718.07c	Regulation of cell shape	24
Signal regulation	*map1*	T551_01436	T552_02410	PNEG_00576	SPAC11E3.06	Expression of P-specific genes	24
	bob1 (*pfd5*)	T551_01168	T552_02444	PNEG_02075	SPBC215.02	Regulation of sexual differentiation	24
	cdc2	T551_01094	T552_00498	PNEG_00359	SPBC11B10.09	Negative regulation of mitotic to meiotic cycle	24
Mating process	*cwp1* (*ram2*)	T551_01863	T552_01846	PNEG_02057	SPAPB1A10.04c	Sexual pheromone maturation	25
	kex1	T551_02518	T552_01067	PNEG_01420	SPBC16G5.09	Sexual pheromone maturation	25
	kex2	T551_03487	T552_04260	PNEG_00129	SPAC23E12.09c	Sexual pheromone maturation	25
	dpp2	T551_03591	Not detected	Not detected	SPACUNK4.08	Sexual pheromone maturation	25
	iph1	T551_03640	T552_02967	PNEG_03454	SPACUNK4.12c	Sexual pheromone maturation	25
	rce1	T551_02384	T552_02990	PNEG_03476	SPAC1687.02	Sexual pheromone maturation	25
	ste24	T551_02959	T552_04168	PNEG_02341	SPAC3H1.05	Sexual pheromone maturation	25

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TABLE 1 (Continued)

Role	Gene name (alternate)	Gene ID			*S. pombe* putative ortholog		Reference(s)
		P. jirovecii	*P. carinii*	*P. murina*	Gene ID	Function	
	cpp1 (*ram1*)	T551_02704	T552_03212	PNEG_01739	SPAC17G6.04c	Sexual pheromone maturation	25
	mam4 (*ste14*)	T551_02897	T552_01163	PNEG_00234	SPAC10F6.12c	Sexual pheromone maturation	25
	pmd1	T551_03503	T552_00261	PNEG_03100	SPCC663.03	Sexual pheromone export	25
Cell-cell fusion	*fus1*	T551_03421	T552_02692	PNEG_02523	SPAC20G4.02c	Cytoplasmic membrane fusion	24
	prm1	T551_03460	T552_00209	PNEG_03049	SPAP7G5.03	Cytoplasmic membrane fusion	24
	cfr1	T551_03398	T552_02419	PNEG_00567	SPAC6G9.12	Cytoplasmic membrane fusion	24
Karyogamy	*tht1* (*kar5*)	T551_03047	T552_03361	PNEG_03392	SPAC13C5.03	Nuclear membrane fusion	25
	pkl1 (*kar3*)	T551_01305	T552_02382	PNEG_02951	SPAC3A11.14c	Nuclear membrane fusion	25
	mal3 (*bim1*)	T551_00265	T552_00117	PNEG_01219	SPAC18G6.15	Nuclear membrane fusion	25
	bip1 (*kar2*)	T551_03062	T552_03346	PNEG_03406	SPAC23A12.15c	Nuclear membrane fusion	25
Meiosis	*dmc1*	T551_00146	T552_01043	PNEG_01443	SPAC8E11.03c	Meiotic recombination	16, 24, 25
	rad51 (*rhp51*)	T551_03070	T552_03387	PNEG_03414	SPAC644.14c	Meiotic strand invasion and exchange	18, 24, 25
	mei2	T551_03162	T552_02431	PNEG_02898	SPAC27D7.03c	Commitment to meiosis	17, 24
	ran1 (*pat1*)	T551_02982	T552_00659	PNEG_01031	SPBC19C2.05	Repression of sexual conjugation	24
	srw1 (*ste9*)	T551_00377	T552_01429	PNEG_00790	SPAC144.13c	Regulation of meiotic metaphase	24
	rec12 (*spo11*)	T551_00124	T552_00948	PNEG_01539	SPAC17A5.11	Initiation of meiotic recombination	25
	mcp7 (*mnd1*)	T551_02965	T552_02592	PNEG_02325	SPAC13A11.03	Recombinase	25
	hop1	T551_02693	T552_03234	PNEG_01729	SPBC1718.02	Meiotic chromosome synapsis	25
	meu13 (*hop2*)	T551_02448	T552_02162	PNEG_03219	SPAC232.15	Meiotic chromosome pairing	25
	rec8	T551_00216	T552_02404	PNEG_00582	SPBC29A10.14	Meiotic sister chromatid cohesion	25
MAT locus silencing	*chp2*	T551_03054	T552_03354	PNEG_03399	SPBC16C6.10	Heterochromatin assembly	24
	cul4 (*pcu4*)	T551_01564	T552_00671	PNEG_01020	SPAC3A11.08	Heterochromatin assembly	24
	hip1 (*hir1*)	T551_01243	T552_02514	PNEG_02145	SPBC31F10.13c	Heterochromatin assembly	24
	hpc2	T551_02364	T552_02145	PNEG_03236	SPBC947.08c	Heterochromatin assembly	24
	hrk1	T551_01486	T552_01661	PNEG_02797	SPAC23C4.03	Heterochromatin assembly	24
	hrp3	T551_02019	T552_02498	PNEG_02128	SPAC3G6.01	Heterochromatin assembly	24
	mit1	T551_02073	T552_02028	PNEG_01867	SPBP35G2.10	Heterochromatin assembly	24
	pip1 (*rbx1*)	T551_00033	T552_03469	PNEG_01300	SPAC23H4.18c	Heterochromatin assembly	24
	pob3	T551_03410	T552_01102	PNEG_01383	SPBC609.05	Heterochromatin assembly	24
	psc3	T551_02039	T552_00863	PNEG_01628	SPAC17H9.20	Heterochromatin assembly	24, 25
	rhp6 (*ubc2*)	T551_01736	T552_02893	PNEG_02338	SPAC18B11.07c	Heterochromatin assembly	24
	spt6	T551_02570	T552_03148	PNEG_01805	SPAC1F7.01c	Heterochromatin assembly	24
	clr4	T551_00331	T552_02811	PNEG_02256	SPBC428.08c	Histone methyltransferase	24
	raf1	T551_01972	T552_01956	PNEG_01941	SPCC613.12c	Histone methyltransferase	
	lid2	T551_00473	T552_00980	PNEG_01507	SPBP19A11.06	Histone demethylase	24
	pmt3	T551_01148	T552_02465	PNEG_02095	SPBC365.06	Histone demethylase	24
	tra1	T551_00450	T552_01000	PNEG_01486	SPBP16F5.03c	Histone acetyltransferase	24
	clr6	T551_00446	T552_01004	PNEG_01482	SPBC36.05c	Histone deacetylase	24
	phd1 (*hos2*)	T551_00611	T552_02792	PNEG_02422	SPAC3G9.07c	Histone deacetylase	24

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TABLE 1 (Continued)

Role	Gene name (alternate)	Gene ID			*S. pombe* putative ortholog		Reference(s)
		P. jirovecii	*P. carinii*	*P. murina*	Gene ID	Function	
MAT locus switching	*hsk1*	T551_01602	T552_02102	PNEG_03279	SPBC776.12c	Imprinting	24
	swi3	T551_01272	T552_02542	PNEG_02174	SPBC30D10.04	Imprinting, conversion	24
	msh2	T551_02326	T552_02183	PNEG_03198	SPBC19G7.01c	Conversion	24, 25
	rad16 (*rad1*)	T551_00218	T552_02406	PNEG_00580	SPCC970.01	Conversion	24, 25
	swi1	T551_01097	T552_00501	PNEG_00356	SPBC216.06c	Conversion	24
	swi10	T551_02136	T552_01836	PNEG_01115	SPBC4F6.15c	Conversion	24
	swi5	T551_02707	T552_03209	PNEG_01744	SPBC409.03	Switching	24
	rad22	T551_01250	T552_02521	PNEG_02152	SPAC30D11.10	Switching	24, 25

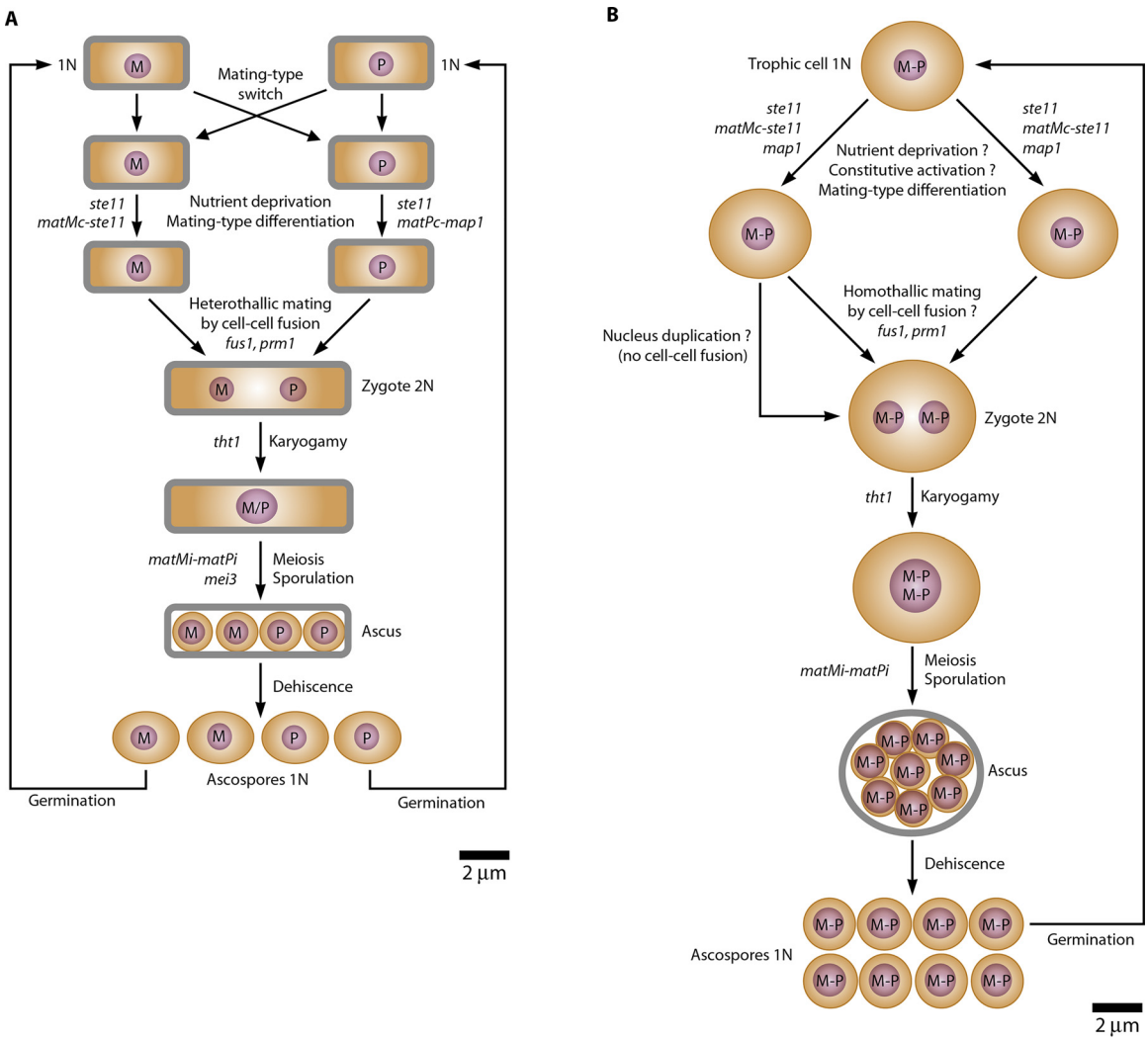

FIG 2 Schematic sexual cycles of *Schizosaccharomyces pombe* (A) and *Pneumocystis* species (B). The genes involved in the regulation of steps are indicated. The circles filled in violet represent the nuclei. M, minus *MAT* locus; P, plus *MAT* locus; N, number of chromosomes of a haploid set. (A) Secondary homothallism of *S. pombe* involving mating-type switch and heterothallic mating by cell-cell fusion. The haploid and diploid mitotic cycles by fission are not shown. P/M, active P and M *MAT* loci on different chromosomes. (B) Hypothetical primary homothallism of *Pneumocystis* species. The question marks indicate events that are not demonstrated. The occurrence or not of homothallic mating by cell-cell fusion remains to be determined. The involvement of the genes mentioned is presumed but not established. The genes *matPc* and *mei3* are absent in *Pneumocystis* species (see the text). The haploid mitotic cycles shown in Fig. 1 are not shown (asexual cycle and endogeny). P-M, fused P and M *MAT* loci on the same chromosome.

hallmark of most active genes that could structure chromatin and nascent mRNAs for subsequent regulation, providing fine-tuned expression (31–33). The arrangement with divergent transcription is frequent among fungal *MAT* loci, including in the ascomycetous models *Saccharomyces cerevisiae* and *S. pombe* (Fig. 3), as well as in basidiomycetes (34). The common regulation of *matMc* and *matMi* is likely to be ensured by the *Pneumocystis* ortholog to the *S. pombe* transcription factor Ste11. In *S. pombe*, this regulation relies on the single recognition motif TTTCTTTGTT, which is present close to the middle of the intergenic region (26, 35). In *Pneumocystis*, the recognition motif, or part of it, could be the CCTTG sequence that resembles it, which is conserved in *P. jirovecii* and *P. carinii* at the same location. The latter hypothesis is also based on the observation that this motif is duplicated in about 50% of the *P. jirovecii* isolates, which suggests its importance (24). In *P. murina*, the motif CCTGT or CCGTT might be involved. Consistent with its potential important role, *ste11* is expressed during pneumonia in rats as the second most abundant transcript (23). The latter observation is

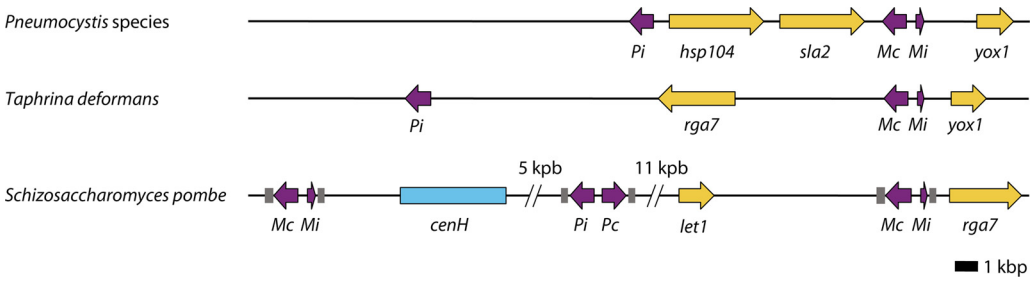

FIG 3 Schematic representation of *MAT* loci present in the subphylum Taphrinomycotina. *MAT* genes are shown in violet, other relevant genes in yellow, and the centromere-homologous sequence *cenH* in blue. The chromosomes carrying the genes are shown as black lines. The drawings are roughly to scale. The primary homothallic species *Pneumocystis* and *Taphrina deformans* each harbor a single *MAT* locus that includes three genes. The secondary homothallic species *S. pombe* harbors three *MAT* loci (or cassettes), each including two genes. The two *MAT* loci flanking *cenH* are silenced, whereas the third locus is active (here, for example, the M mating type). The *cis*-acting sequence motifs H1, H2, and H3, involved in mating-type switching and flanking each *MAT* locus, are shown in dark gray. The *MAT* locus is located on supercontigs (scaffolds) 9, 13, and 13, respectively, of the *P. jirovecii*, *P. carinii*, and *P. murina* genome assemblies (25). (Each scaffold presumably corresponds to one chromosome.)

also compatible with Ste11 participating in conjunction with MatMc in the activation of the M-specific genes, as observed in *S. pombe*.

Two genes are generally conserved close to the *MAT* locus among ascomycetes: *sla2* and *apn2* or *dic1* (36, 37). The *Pneumocystis MAT* locus harbors only *sla2*, between *matPi* and *matMc*, together with the *hsp104* gene (Fig. 3). The *Pneumocystis MAT* locus presents only a limited synteny with that of the other members of the Taphrinomycotina subphylum: i.e., only one gene in common with the close relative *Taphrina deformans* (*yox1*) and none in common with *S. pombe* (24) (Fig. 3). The *MAT* loci of the two latter relatives do not harbor the *sla2* gene and present only one gene conserved (*rga7*). This lack of synteny suggests important evolutionary distances among members of the subphylum Taphrinomycotina. The gene *sla2* encodes the adaptor linking actin to clathrin involved in endocytosis and the cytoskeleton. As in *S. pombe*, it might be essential under most conditions in *Pneumocystis*. This essentiality might prevent deletions and ensure the stability of the *Pneumocystis MAT* locus. Such a phenomenon is also postulated for the gene *let1*, which is present between the *S. pombe* active and silent *MAT* loci (26, 38) (Fig. 3).

The structure and content of the *Pneumocystis MAT* locus suggest a specific mode of sexual reproduction, which is discussed in the following section.

MODE OF SEXUAL REPRODUCTION OF *PNEUMOCYSTIS* SPECIES

The presence of genes involved in the differentiation of both P and M mating types (P and M genes) in the *Pneumocystis MAT* locus is incompatible with heterothallism because this mode of sexual reproduction implies that the P and M genes are in different genomes (39). Secondary homothallism is also unlikely because this mode involves more than one *MAT* locus: i.e., two or three, with one active and the other(s) silenced. Moreover, the latter mode requires genetic elements for switching the active *MAT* locus and silencing the others that are absent in *Pneumocystis* genomes (*cis*-acting sequence motifs of H, proximity to telomere or centromere-like repeats) (24, 30) (Fig. 3). Homothallism resulting from unidirectional mating-type switching (40) is also unlikely because (i) it requires indirect or direct repeats for the deletion and reconstitution of one *MAT* locus, and (ii) it implies a mixture of different *MAT* loci in each cell population, which is not observed in *Pneumocystis*. The presence of both M and P genes in the *Pneumocystis MAT* locus is consistent with a fusion of two *MAT* loci, M and P, that were present in an ancestor, followed by the loss of one *MAT* gene (*matPc*). Such a scenario has previously been proposed to account for fused *MAT* loci of other fungi (41). These observations suggest that the sexual mode of reproduction of *Pneumocystis* species is primary homothallism, the mode that involves a single self-compatible mating type that can engage on its own into the sexual phase.

The hypothesis of primary homothallism is supported by the presence of the same *MAT* locus in all *P. jirovecii* DNA samples (30). It is further supported by the finding that

TABLE 2 Results from reverse transcriptase PCR amplification of *Pneumocystis* transcripts from bronchoalveolar lavage fluid samples from 10 patients with *Pneumocystis* pneumonia and from infected mouse lungs[a]

	PCR result for:					
		MAT transcription factors			Pheromone receptors	
cDNA source	β-Tubulin[b]	*matMc*	*matMi*	*matPi*	*mam2*	*map3*
P. jirovecii patient no.:						
1	+	+	+	+	+	+
2	−	−	−	−	−	−
3	+	−	+	−	+	−
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	−	−	+	+	−
7	+	+	+	+	+	+
8	+	−	−	+	−	−
9	−	−	−	−	−	−
11	+	+	+	+	−	+
P. murina	+	+	+	+	+	+

[a]+, positive PCR result; −, negative PCR result. (Adapted from reference 47.)

[b]Amplification of the β-tubulin transcripts was used as a control (30). It assessed adequate reverse transcriptase PCR by the absence of the intron in these PCR products. This control suggested that the negative PCR results obtained in these experiments are due to RNA degradation. The latter may have occurred during the uncontrolled period between collection of the samples from the patients and their arrival in our laboratory.

all three *MAT* genes are expressed concomitantly during pneumonia in both humans and mice (Table 2). Such coexpression is expected because expression of both P and M genes is generally required for successful initiation of the sexual cycle in primary homothallic species (39). This finding is particularly relevant in mice because infections are thought to be caused by a single *P. murina* strain, strongly suggesting expression from the same *MAT* locus. On the other hand, infections in humans are most often, if not always, polyclonal (42), which leaves the possibility that the coinfecting *P. jirovecii* strains may differ in the expression of their *MAT* genes. It must be stressed that, although primary homothallism of *Pneumocystis* appears almost certain, one cannot totally exclude a new mode of sexual reproduction involving previously unknown mechanisms. Indeed, fungi present a myriad of different mechanisms to trigger sexuality (39), and new ones could be discovered in the future. The definitive ascertainment of the mode of sexual reproduction of *Pneumocystis* organisms may require their culturing *in vitro*.

Primary homothallism is also observed in other human-pathogenic fungi: e.g., *Candida albicans* (43) and *Cryptococcus neoformans* (44). Moreover, sexual systems resembling homothallism are probably used by human-pathogenic protozoans: i.e., *Toxoplasma*, *Giardia*, *Trypanosoma*, and *Leishmania* (45). This suggests that this mode of sexual reproduction is advantageous for microbial pathogens. The reason hypothesized is that it alleviates the need to find a compatible mating partner in the restricted niches of the host body, while still providing the benefits of sex (increase in genetic diversity and virulence and elimination of deleterious mutations) (45, 46).

The mechanisms involved in primary homothallism in fungi are poorly known compared to those of secondary homothallism and heterothallism. In addition, they probably vary considerably according to the fungal species and rewiring of the pathways. The mechanisms of sexuality in action in *Pneumocystis* species are discussed in the following section.

MECHANISMS INVOLVED IN THE PRIMARY HOMOTHALLISM OF *PNEUMOCYSTIS* SPECIES

The absence of the transcription factor *matPc*, responsible in *S. pombe* for the differentiation into the cellular mating type P, suggests that *Pneumocystis* species are unisexual, involving a single mating type, M, as observed in other fungi (*C. albicans* and *C. neoformans*) (43, 44). However, analysis of the pheromone receptors suggested that the *P. jirovecii* trophic forms are of both mating types at the same time (47). Indeed, the genes *mam2* and *map3*,

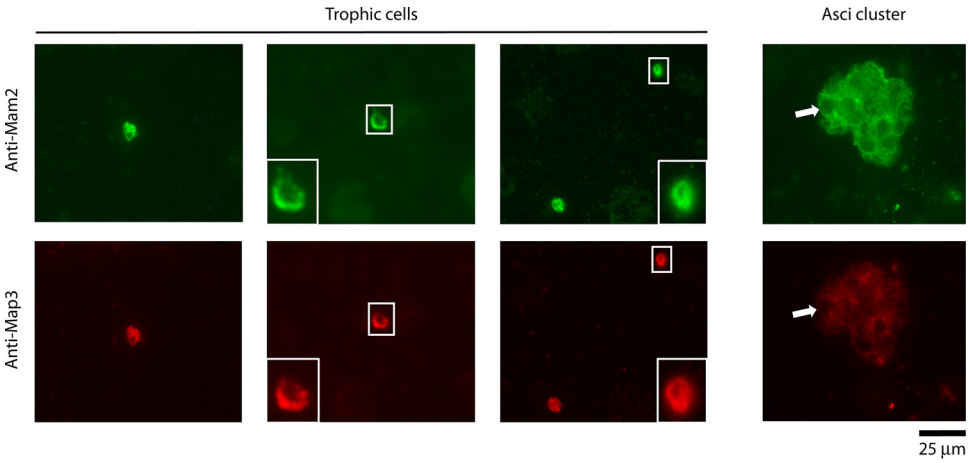

FIG 4 Indirect immunofluorescence microscopic analysis of Mam2 and Map3 pheromone receptors on *P. jirovecii* cells from a bronchoalveolar lavage fluid sample of a patient with *Pneumocystis* pneumonia. Shown is costaining with anti-Mam2 and anti-Map3 (pheromone receptors M and P, respectively). The presumed trophic cells in the small white squares are shown enlarged in the insets at the left or right bottom corners of the images, and a single spherical cell corresponding to an ascus within the cluster is indicated by the white arrows. A fluorescein isothiocyanate (FITC) filter (green) was used to visualize Mam2 staining (Alexa Fluor 488), and a tetramethyl rhodamine isocyanate (TRITC) filter (red) was used to visualize Map3 staining (Alexa Fluor 594). Bar, 25 μm. (Reproduced from reference 47.)

encoding, respectively, the receptors M and P, are present in every *Pneumocystis* genome (24, 25) and are both expressed during pneumonia in rats, mice, and humans (24, 25, 47) (Table 1). The *P. murina* infection is again particularly relevant because it strongly suggests that both receptors are expressed from the same genome, and not from different coinfecting strains. Immunostaining revealed that *P. jirovecii* trophic forms expose at their surface both receptors M and P at the same time (Fig. 4). Consequently, they could excrete both pheromones M and P, but this could not be investigated so far because the encoding genes could not be identified due to their significant divergence (24, 25). In *S. pombe*, the transcription factor Map1 forms a heterodimer with MatPc, which is responsible for the activation of the P-specific genes (26). An ortholog of *map1* is present in *Pneumocystis* and could be involved in the differentiation into the mating type P despite the absence of *matPc*.

The receptors M and P present at the cell surface of *Pneumocystis* trophic cells could be involved in the recognition of the mating partners. Being identical, these cells might mate randomly by cell-cell fusion within host's lung alveoli. However, this is not necessarily the case because cell-cell fusion can be replaced by nucleus duplication in homothallism, and the pheromone receptor systems play other roles in some primary homothallic fungi (48–50). For example, the close relative *T. deformans* does not rely on cell-cell fusion despite the fact that it harbors one pheromone receptor (24). Electron microscopy studies have presented few images of two *Pneumocystis* trophic cells with connected cytoplasmic membranes that could correspond to cell-cell fusion events (22). Moreover, plasmogamy during mating and cytokinesis during mitosis cannot be distinguished morphologically. Similarly, the connected nuclear membranes observed on some images may correspond to karyogamy or karyokinesis. Indeed, *Pneumocystis* might have a closed mitosis with the nuclear membrane present throughout the cell cycle as in most fungi, rather than an open one as in many basidiomycetes. *Pneumocystis* harbors potential orthologs of the *S. pombe* genes *fus1* and *prm1* (24). (Note that *prm1* is also present in *P. jirovecii* [our unpublished data].) These genes are essential to cell-cell fusion during mating in *S. pombe* (51). However, they are also present in *T. deformans* (24). Thus, the occurrence of cell-cell fusion during *Pneumocystis* sexuality remains an open question.

Heteroplasmy of mitochondria has been reported in *Pneumocystis* based on the presence of more alleles of the mitochondrial markers than of the nuclear ones (42). Although it cannot be excluded that it results from a higher frequency of mutations in

these organelles, this heteroplasmy might result from biparental inheritance of mitochondria that could be generated by cell-cell fusion during sexuality. This would fit that no studies reported cells other than the trophic cells and asci that could participate in anisogamy (22), one of the phenomena that can lead to uniparental inheritance of mitochondria. It is possible that the *Pneumocystis* mitochondrial heteroplasmy reflects frequent cell-cell fusions, which would be coherent with sexuality being necessary and possibly the preponderant mechanism of proliferation (22). This would imply a system of homoplasmy control weaker than that generally present in fungi (39), although persistent heteroplasmy in fungi has been reported (52).

Splicing variants corresponding to intron retention have been observed among the transcripts of the pheromone receptors (47, 53). These variants might be involved in the regulation of expression and associated with specific stages of the cell cycle. The timing of expression of the *Pneumocystis* sex genes is discussed in the following section.

OCCURRENCE OF *PNEUMOCYSTIS* SEXUAL CYCLE

The sexual cycle of many fungi, including *S. cerevisiae* and *S. pombe*, is triggered by deprivation of essential nutrients, such as a fermentable carbon source or nitrogen, or by stress (26, 54). As far as *Pneumocystis* is concerned, expression of *MAT* and other sex-related genes has been observed during pneumonia in humans, mice, and rats (15, 23, 25, 30, 47) (Table 2). It is plausible that *Pneumocystis* sexuality occurs when the host alveoli are filled with fungal cells (i.e., when pneumonia is overt), because this stage may correspond to an exhaustion of the nutriments as well as marked stress (23). This hypothesis would be consistent with the activation of sexuality upon treatment of *P. murina* with echinocandins, inhibiting growth and provoking stress (55). The sensitivity of the trophic forms to echinocandins might result from a link between asexual and sexual cycles through regulatory factors or from the presence of small amounts of 1,3-β glucan in their wall (22). However, sexuality may also take place in the lungs of colonized individuals as they are a source of *P. jirovecii* in a cluster of nosocomial cases (56), which implies airborne transmission by asci (Fig. 1A). The alveoli might not be filled in the latter situation because of the lower fungal load present in colonized humans (56). Thus, one cannot exclude that *Pneumocystis* sexuality is constitutively induced during growth—possibly by the stress resulting from the action of the host immune system. This hypothesis would fit that this sexuality might be the preponderant mechanism of proliferation. It would also be consistent with the fact that *Pneumocystis* sexuality proved to be obligatory within host lungs, a characteristic that is discussed in the following section.

OBLIGATE *PNEUMOCYSTIS* SEXUALITY WITHIN HOST LUNGS DURING PNEUMONIA

Two facts strongly suggest that *Pneumocystis* sexuality is obligatory within host lungs during pneumonia: the *MAT* and sex-related genes are concomitantly expressed, and asci are always present. Asci are observed in all pneumonia, so that their staining has been used for decades to diagnose the disease (Fig. 1B). Only a few infections with a reduced proportion of asci have been reported, and only under particular conditions: an athymic host (57), immunity reconstitution (58), and prophylaxis breakthrough (59). Asci are also observed in all primary infections that occur during the first 2 years of life (60, 61). Obligate sexuality is consistent with (i) asci and/or ascospores being the airborne infectious particles responsible for transmission between hosts because this ensures survival (62, 63) and (ii) its necessity for proliferation by the release of ascospores within the host lungs (22). On the other hand, the data gathered so far suggest that the asexual phase of proliferation by mitosis and possibly endogeny might be facultative (22) (Fig. 1A). It could be activated under certain peculiar conditions or at early stages of the infection and might be capable of latency, ensuring survival upon growth inhibition (62).

The obligate nature of *Pneumocystis* sexuality may also ensure the antigenic variation of these fungi, a system crucial for colonization and thus survival. Indeed, these pathogens dedicate about 8% of their genomes to a subtelomeric superfamily of genes encoding six families

of major surface glycoproteins (25, 64–67). The antigenic variation relies on recombinations creating continuously new mosaic genes, as well as on mutually exclusive expression of a single gene out of approximately 80 genes of the family encoding the most abundant glycoprotein at the cell surface (family I, also named A1). Recombinations among each of the six families and the exchange of the expressed gene of family I probably occur when all subtelomeres are close to each other: i.e., when they are clustered as a "bouquet" at the nuclear membrane during the meiotic prophase (68). Thus, antigenic variation probably requires sexuality to occur.

Although multicellular organisms cannot be compared easily with microbes, it is striking that mammals and plants, but only few fungi, share obligate sexuality during their life cycles (39). *Pneumocystis* is similar to the plant fungal obligate biotrophs that complete their entire life cycles within their hosts, including sex (6). Thus, *Pneumocystis* is an animal parasite resembling plant parasites that, consistently, has nutritional requirements observed in both these types of parasites (7, 10). Moreover, the reluctance of *Pneumocystis* organisms to sustain axenic growth *in vitro* is consistent with that of the fungal obligate biotrophs infecting plants (6). The *Pneumocystis* lifestyle differs from that of other human fungal pathogens that are necrotrophs obtaining nutriments from killed host cells with a facultative sexuality (*Candida*, *Aspergillus*, and dermatophytes).

The relationship between *Pneumocystis* organisms and their hosts fits the concept of "compatibility" used in the fungal plant pathogen field (6, 23). The latter consists of a relationship between an adapted biotrophic fungal pathogen and a susceptible host where there is complementation, but which may eventually lead to the development of the disease. Each *Pneumocystis* genome is only approximately 8 Mbp, which is among the smallest and most compact fungal genomes (69, 70). This compaction results not only from the loss of essential pathways but also from the presence of a single copy of the ribosomal DNA. Genome compaction is also observed in fungal pathogens adapted to host waxy surface of plants or fruits (69, 71). Like the surface of plants, the epithelial cells' surface in mammalian lungs may constitute an extreme environment that imposes restrictions on the parasites, such as nutrient limitation.

The obligate sexuality of *Pneumocystis* organisms probably has a great importance in their evolution. The selection of the homothallic mode for this sexuality during evolution is discussed in the following section.

POSSIBLE EVOLUTION OF *PNEUMOCYSTIS* SEXUALITY

The ancestral mode of sexual reproduction of fungi remains to be determined: homo- versus heterothallism (39). Primary homothallism has often been found to result from recombination events between heterothallic partners (39, 72). This possibility is compatible with the structure of the *Pneumocystis MAT* locus because it includes both P and M genes. The species *T. deformans* is putatively the closest known relative of *Pneumocystis* (9, 10, 25) and harbors a *MAT* locus similar to that of *Pneumocystis* (24) (Fig. 3). Thus, a heterothallic ancestor may have generated these two primary homothallic genera. On the other hand, this ancestor would have also evolved into the secondary homothallic relative *S. pombe* by subsequent acquisition of a supplementary *MAT* locus as well as the switching and silencing mechanisms (72). This putative scenario suggests that the acquisition of obligate biotrophy on mammals (*Pneumocystis*) or plants (*Taphrina*) involved the selection of primary homothallic strains about 100 million years ago (73). This would be consistent with the belief that primary homothallism is advantageous for microbial pathogens. At least for *Pneumocystis*, acquisition of primary homothallism would have ensured maintenance of sex, which is essential for its survival. This evolutionary hypothesis could be challenged by the characterization of the *MAT* locus and the mode of sexual reproduction of other related fungi.

CONCLUSIONS

Pneumocystis is unique among fungi pathogenic to humans. It differs by its obligate and biotrophic parasitism, its transmissibility between host individuals, and its obligate

sexuality within the host's lungs. The latter appears essential because it would ensure proliferation, dissemination, and possibly antigenic variation, processes that are all required for the survival of the fungus. Figure 2B shows the hypothetical sexual cycle of *Pneumocystis* that can be derived from the observations made so far. Importantly, whether or not cell-cell fusion occurs remains to be determined. The understanding of the mechanisms of this sexuality and its implications for *Pneumocystis* genetic diversity and evolution deserves further study.

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