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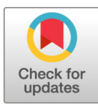

A Molecular Window into the Biology and Epidemiology of *Pneumocystis* spp.

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SUMMARY *Pneumocystis*, a unique atypical fungus with an elusive lifestyle, has had an important medical history. It came to prominence as an opportunistic pathogen that not only can cause life-threatening pneumonia in patients with HIV infection and other immunodeficiencies but also can colonize the lungs of healthy individuals from a very early age. The genus *Pneumocystis* includes a group of closely related but heterogeneous organisms that have a worldwide distribution, have been de-

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tected in multiple mammalian species, are highly host species specific, inhabit the lungs almost exclusively, and have never convincingly been cultured *in vitro*, making *Pneumocystis* a fascinating but difficult-to-study organism. Improved molecular biologic methodologies have opened a new window into the biology and epidemiology of *Pneumocystis*. Advances include an improved taxonomic classification, identification of an extremely reduced genome and concomitant inability to metabolize and grow independent of the host lungs, insights into its transmission mode, recognition of its widespread colonization in both immunocompetent and immunodeficient hosts, and utilization of strain variation to study drug resistance, epidemiology, and outbreaks of infection among transplant patients. This review summarizes these advances and also identifies some major questions and challenges that need to be addressed to better understand *Pneumocystis* biology and its relevance to clinical care.

KEYWORDS *Pneumocystis*, molecular biology, epidemiology, genome features, strain variation, transmission

INTRODUCTION

Pneumocystis is a ubiquitous unicellular fungus that is distributed worldwide. Initially believed to be a single protozoan species infecting a broad range of mammalian hosts, it has now been recognized as a genus of fungi comprising a group of highly diversified species with an apparently strict host species specificity. The species that infects humans is *Pneumocystis jirovecii*. In immunosuppressed individuals, especially those with untreated human immunodeficiency virus (HIV) infection, *Pneumocystis* can cause a severe, potentially fatal pneumonia, commonly known as *Pneumocystis* pneumonia (PCP, the preferred acronym in the *Pneumocystis* research community) and also occasionally referred to as *P. jirovecii* pneumonia (PJP) and pneumocystosis. Immunocompetent individuals can be infected by *P. jirovecii*, but this infection is usually asymptomatic or manifested only as a mild respiratory infection. In fact, based on serologic and PCR assays, most infants appear to have been infected by 1 year of age (1, 2).

Pneumocystis was first recognized in the mid-20th century as an important human pathogen causing epidemics of interstitial pneumonia in premature infants and malnourished children in Europe (3, 4). During the 1960s to 1970s, PCP was reported with increasing frequency for immunocompromised patients, especially cancer patients, as intensive chemotherapeutic or anti-inflammatory regimens were initially being developed. However, it was in the early 1980s that *Pneumocystis* rose to national prominence, when the occurrence of PCP (as well as Kaposi's sarcoma) in previously healthy young men led to the initial recognition of the AIDS epidemic (5). Prior to the widespread utilization of combination antiretroviral therapy (cART), there were >20,000 new cases of PCP per year in the United States alone (6). Although there have been dramatic declines in its incidence following the introduction of cART, PCP remains one of the most common and serious opportunistic infections in the HIV/AIDS population (7). PCP cases now occur primarily in persons who are unaware of their HIV infection or are not receiving cART therapy or PCP prophylaxis (8–10) and who have advanced immunosuppression, with CD4 counts of $<$100 cells/μl (11). Recently, the incidence of PCP has been growing in patients without HIV infection as a result of more widespread use of potent immunosuppressive agents, including various immunodepleting monoclonal antibodies (12, 13), and greater utilization of organ transplantation; multiple outbreaks have been reported for transplant patients, especially renal transplant patients, in the past 2 decades (14, 15). Increasingly, *Pneumocystis* colonization is also being identified in patients with a variety of pulmonary conditions, and it may be contributing to more rapid declines in pulmonary function (16).

Symptoms of PCP are nonspecific and include fever, nonproductive cough, shortness of breath, chest pain, and fatigue. Physical examination also gives nonspecific findings, and pulmonary auscultation is usually normal. Chest radiographs usually

display diffuse interstitial or alveolar infiltrates but are normal in some cases. Note that PCP can present with a broad range of radiographic patterns, including asymmetrical or lobar infiltrates, nodules, cavities, pleural effusions, and pneumothorax (17). Arterial blood gases may show hypoxemia that can be brought on or exacerbated by exercise. While most HIV-infected patients have CD4 cell counts of <200 cells/μl (18, 19) prior to the development of PCP, the CD4 count is a less reliable predictor of risk in the non-HIV-infected population (20, 21).

Although the lungs are the primary site of infection or colonization, rare cases of extrapulmonary involvement of *Pneumocystis* have been reported for humans, as extensively reviewed by Ng et al. (22). Commonly involved extrapulmonary sites include the eyes, ears, lymph nodes, liver, spleen, and bone marrow, while systemic dissemination has also been reported. More than half of the patients with these cases had known concurrent PCP.

As *Pneumocystis* cannot reliably be cultured *in vitro*, diagnosis of PCP has relied primarily on the microscopic detection of organisms in respiratory specimens, such as bronchoalveolar lavage (BAL) fluid, induced sputum, and lung biopsy specimens, after chemical staining (e.g., using methenamine silver, toluidine blue O, or Diff-Quik methods) (23–26) or immunofluorescence staining, which can be performed using commercially available anti-*Pneumocystis* monoclonal antibodies. The latter is more sensitive and specific than colorimetric staining (27–29). Serologic tests to detect anti-*P. jirovecii* antibodies have not proven useful clinically for establishing a diagnosis of PCP or for assessing prognosis (30–35). Detection of β-1,3-glucan, a component of the cell wall, in serum or BAL fluid appears to be sensitive but not specific (23, 24, 36–38). In recent years, multiple PCR assays have been developed for detection of *P. jirovecii*, most commonly utilizing primers for the mitochondrial large-subunit rRNA gene (mtLSU) or the multicopy major surface glycoprotein (*msg*) gene family (39–46), and these assays appear to be 10 to 100 times more sensitive than microscopic detection of stained organisms. Several commercially available diagnostic PCR kits are approved for clinical use in a number of countries, primarily in Europe (47–50); none are currently FDA approved for use in the United States.

The first-line drug for treatment of PCP is the combination of trimethoprim and sulfamethoxazole (TMP-SMX), which can be administered orally or intravenously (51). This combination is highly effective and generally well tolerated. For patients who cannot tolerate this regimen or for whom it fails, alternative therapies include clindamycin-primaquine, dapsone-trimethoprim, intravenous pentamidine, and atovaquone. Prophylaxis with TMP-SMX, dapsone, atovaquone, and aerosol pentamidine is effective at preventing PCP in at-risk populations. The widespread use of TMP-SMX has raised concerns about the development of drug resistance by *P. jirovecii* (52–58).

This review summarizes recent advances in the biology and epidemiology of *Pneumocystis*, with a focus on its taxonomic classification, atypical fungal nature, genome features, life cycle, strain variation, and transmission.

BIOLOGY OF *PNEUMOCYSTIS*

Atypical Fungal Nature

Although *Pneumocystis* was originally classified as a protozoan, it is now unequivocally recognized as a fungus based on overwhelming genomic evidence and phylogenic analyses, as described in detail below (see "Species and Taxonomy"). Such a classification is also supported by studies of cell wall composition and metabolic pathways. However, despite many common characteristics, *Pneumocystis* is often regarded as an atypical fungus, with substantial differences from other fungi. The knowledge of the atypical nature of *Pneumocystis* is evolving gradually with advances in *Pneumocystis* research. Earlier reviews on this topic can be found elsewhere (59, 60).

Following its reclassification as a fungus in the late 1980s (61, 62), considerable efforts were made to test the anti-*Pneumocystis* activities of classical antifungal drugs, including azoles, such as fluconazole, which targets ergosterol synthesis, and amphotericin B, which binds to ergosterol in the cell membrane (63–65). Unexpectedly,

Pneumocystis was found to be resistant to these drugs, presumably due to its absence of ergosterol, the major sterol in the cell membranes of most fungi (64, 66). Instead of ergosterol, cholesterol was found to be the major sterol in *Pneumocystis* (64, 67, 68), though the metabolic pathways for both sterols remained poorly understood until the recent sequencing of *Pneumocystis* genomes. Based on genome analysis, *Pneumocystis* lacks several key enzyme genes involved in ergosterol biosynthesis (69). In addition, a homolog of Dhcr24 (24-dehydrocholesterol reductase; EC 1.3.1.72), which is necessary for cholesterol biosynthesis in mammals, is missing in both human and rodent *Pneumocystis* spp. It has been hypothesized that *Pneumocystis* may sequester cholesterol from its host (67, 69, 70).

Another major atypical feature of *Pneumocystis* is the pleomorphic shape and fragile cell wall of the trophic form, in contrast to the rigid cell wall of typical fungi. It has long been known that fungal cell walls are rich in glycoproteins, mannans, glucans, chitin, and chitosan. Early studies found that the *Pneumocystis* cell wall contains abundant glycoproteins and, in cysts only, β-glucans (71–73); however, other components were not well characterized until recently. Genome analysis in combination with experimental validation (described in greater detail below) has revealed that constituents characteristic of cell walls in other fungi, including outer chain N-mannans (the high-mannose residues of glycoproteins) and chitin, are absent in *Pneumocystis* (69). Thus, at present, *Pneumocystis* is the only fungus lacking chitin in its cell wall. Consistent with earlier studies, *Pneumocystis* has all the enzymes required for biosynthesis and degradation of β-1,3-glucan and β-1,6-glucan (69) but has lost genes found in many other fungi that are required for biosynthesis and degradation of α-glucan.

The third atypical feature of *Pneumocystis* is the inability of researchers to propagate the organism *in vitro*, despite extensive efforts that have utilized fungal culture media and other culture systems, including coculture with mammalian cells. Whole-genome analysis has provided some possible insight into this. Compared to those of other fungi, *Pneumocystis* has a highly compact genome, with a consequent loss of many biological pathways (69), which potentially renders *Pneumocystis* highly dependent on the host to complement these losses, as discussed in detail below. This suggests that successful culture will depend on a better understanding of the specific nutrients that need to be supplemented and the mechanism through which *Pneumocystis* acquires these nutrients. It is plausible that *Pneumocystis* cannot be cultured axenically but will require a feeding cell layer to complement certain as-yet-undefined biologic processes.

Finally, another unique feature of *Pneumocystis*, which is related to its host dependence, is its adaptation to and possible coevolution with its host species; as a consequence, each *Pneumocystis* species appears to exclusively infect one host species and is unable to infect a different host species (74). This is distinctly different from the case for many other pathogenic fungi that can inhabit diverse environmental niches and can also infect different host species. No apparent clues to this specificity have been identified in genome analyses or other studies.

Species and Taxonomy

Although *Pneumocystis* has been found in nearly every mammalian species examined, to date, mainly using PCR-based methods, only a limited number of *Pneumocystis* species have formally been classified and named at the species level according to the International Code of Botanical Nomenclature rules; these are *P. jirovecii* (infecting humans and named in honor of Otto Jirovec) (75), *P. carinii* (infecting rats and named in honor of Antonio Carini) (76), *P. murina* (infecting mice) (77), *P. wakefieldiae* (infecting rats and named in honor of Ann Wakefield) (78), and *P. oryctolagi* (infecting rabbits) (79). *Pneumocystis* organisms identified from other mammals have usually been named using a trinomial system of special form (*formae speciales*) names associated with host genera following the nomenclature system recommended in 1994 by the *Pneumocystis* workshop (80). For example, the *Pneumocystis* sp. infecting rhesus monkeys (*Macaca mulatta*) is referred to as *Pneumocystis carinii* f. sp. *macacae* (81).

Whether the organisms detected in macaques, bats, horses, dogs, ferrets, and other host species represent unique *Pneumocystis* species remains to be clarified, but these observations support the notion that these organisms are highly ubiquitous and that each mammalian species is infected by at least one *Pneumocystis* species. Interestingly, based on single-locus PCR studies, wild rats (82), macaques (83, 84), bats (85), and dogs (86) are often infected by more than one *Pneumocystis* population, with sequence divergence approaching or exceeding interspecies levels, raising the question of whether these animals may be infected with more than one distinct species.

To complicate our understanding of this enigmatic organism, the taxonomic classification of *Pneumocystis* has undergone dramatic changes since its discovery. Initially, *Pneumocystis* was widely believed to be a protozoan based on some morphological features and drug sensitivity. It was not until 1988 that 16S rRNA gene analysis strongly suggested *Pneumocystis* to be a fungus (61). This finding has been confirmed unequivocally by all subsequent molecular phylogenetic analyses. Nevertheless, its taxonomic position within the fungal kingdom has varied in different studies depending on the genetic loci and fungal species involved (reviewed in reference 75). Most studies based on single-gene data sets and common fungal species have placed *Pneumocystis* into the Ascomycota phylum, with *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae* often being the closest relative (61, 62, 87, 88), while there are rare reports of placement in the Basidiomycota (89) or another phylum (90). An improved taxonomic classification gradually emerged synchronously with the increasing recognition of an early-diverging Ascomycota lineage, initially (in 1994) named Archiascomycetes (91) and subsequently renamed Taphrinomycotina (92), as a monophyletic subphylum in parallel with Saccharomycotina and Pezizomycotina (93). Currently, Taphrinomycotina includes seven highly heterogeneous genera: *Pneumocystis*, *Schizosaccharomyces*, *Taphrina*, *Saitoella*, *Neolecta*, *Protomyces*, and *Archaeorhizomyces* (93–96). Over the last decade, growing phylogenetic analyses based on multigene and genome-wide (phylogenomic) data sets have strongly supported the grouping of *Pneumocystis* within Taphrinomycotina as a monophyletic subphylum (69, 93, 95–103). However, its position within this subphylum remains unclear. A few phylogenomic studies have shown that *Pneumocystis* is closer to *Taphrina* than to *Schizosaccharomyces*, with strong bootstrap support (69, 97), but this relationship was not confirmed in other phylogenomic studies including different gene sets and/or different Taphrinomycotina members (95, 100, 102, 103). Figure 1 shows a phylogenomic tree inferred from 248 single-copy core orthologs among sequenced species representing all known genera of Taphrinomycotina. In this tree, the *Pneumocystis* genus and the *Schizosaccharomyces* genus cluster as sister groups basal to the group formed by *Taphrina* and *Protomyces*; our understanding of their interrelationships remains dynamic and can change as additional species are included. In order to define the precise relationship of *Pneumocystis* with other Taphrinomycotina members, it may be necessary to use additional integrated phylogenomic analyses (104) and broader taxon sampling of Taphrinomycotina.

Phylogenetic relationships within the *Pneumocystis* genus have also been studied extensively, mainly utilizing single-gene data sets. All studies have consistently shown that all known *Pneumocystis* species form a monophyletic group, while the relationships between different *Pneumocystis* species vary between studies, depending on the genes and species used. For the three most extensively studied species, it is very clear that the two species infecting rodents, *P. carinii* and *P. murina*, are phylogenetically closer to each other than to *P. jirovecii*, as determined by single- and multiple-gene analyses (77, 105–107) as well as by phylogenomic analyses using full mitochondrial and nuclear genome data sets (69, 99).

Phylogenetic analysis has extended our understanding of the host species specificity of *Pneumocystis*. In a study of ∼20 different primate species or subspecies (including humans) based on sequence analysis of multiple *Pneumocystis* genes, a unique *Pneumocystis* sequence for all genes was obtained from each primate species or subspecies (108, 109), despite high genetic similarities among these host species. For example, *Pneumocystis* organisms from the Chinese rhesus macaque (*M. mulatta*) and the crab-

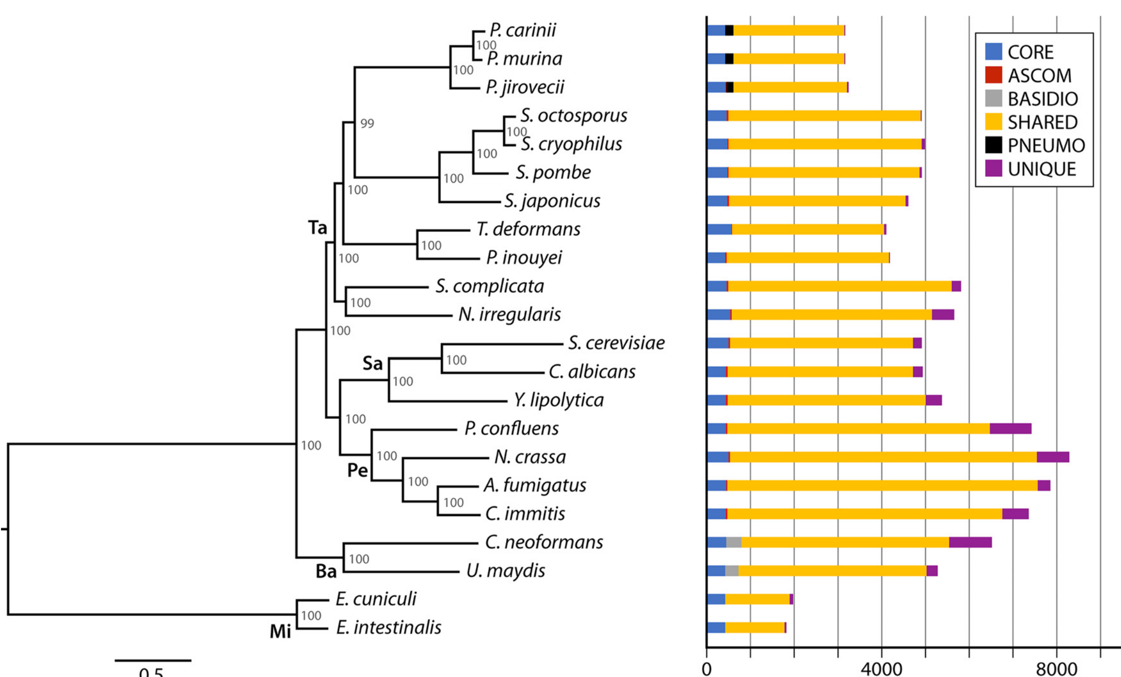

FIG 1 Phylogenetic relationships and ortholog conservation for *Pneumocystis* and related fungi. The maximum likelihood tree was inferred from 248 single-copy core orthologs. Ortholog conservation patterns highlighted include core orthologs found in all genomes (CORE), ascomycete-specific orthologs found in all ascomycetes but not in Microsporidia (ASCOM), basidiomycete-specific orthologs found in both *Cryptococcus neoformans* and *Ustilago maydis* but not in all other fungi (BASIDIO), *Pneumocystis*-specific orthologs (PNEUMO), orthologs shared in any two or more genomes (SHARED), and orthologs unique to only one genome (UNIQUE). Numbers on the branches of the tree indicate bootstrap support values. The phyla and subphyla are indicated on the main branches as follows: Ta, Taphrinomycotina; Sa, Saccharomycotina; Pe, Pezizomycotina; Ba, Basidiomycota; and Mi, Microsporidia.

eating macaque (*Macaca fascicularis*) differ by 3.5% in mtLSU sequence (108), though the genomes of these two macaque host species differ by only 0.34% (110), which further highlights the exceptionally high-level host species specificity. Parallel analysis of these *Pneumocystis* organisms and their primate host species found that the phylogenetic relationships between *Pneumocystis* organisms were well correlated with the phylogenetic relationships between their respective host species (108, 109). These findings suggest that the host species specificity of *Pneumocystis* might result from a long history of coevolution with or adaptation to its hosts. The hypothesis of coevolution is further supported by phylogenetic analysis of *Pneumocystis* species from broader mammalian taxa, including primates, rodents, carnivores, bats, lagomorphs, marsupials, and ungulates (111). Given the strict host species specificity and assuming coevolution with its host, each *Pneumocystis* species can serve as a signature of its host species, and studies of *Pneumocystis* phylogeny may complement studies of the phylogeny of the host.

The species divergence times for three *Pneumocystis* species were estimated based on a small number of genes (112). According to these estimations, *P. murina* and *P. carinii* diverged from each other between 51 and 71 million years ago, which appears to be earlier than the divergence time between rats and mice (~12 to 24 million years ago) (113); *P. carinii* and *P. jirovecii* diverged from each other between 90 and 100 million years ago, similar to the divergence time between humans and rodents (~80 million years ago) (114). These estimates should be interpreted with caution due to their reliance on nucleotide variations calculated from only a few genes, which may not contain enough information for estimating speciation timing. The availability of whole-genome sequences for multiple *Pneumocystis* species and strains, together with new, advanced bioinformatics tools, will potentially improve these estimates.

Morphology and Hypothetical Life Cycle

Although *Pneumocystis* was identified more than 100 years ago, its life cycle remains

poorly understood, largely due to the inability of researchers to culture the organism continuously *in vitro*. As extracellular parasites, *Pneumocystis* organisms have been found almost exclusively in the alveolar space in the lungs of mammals. At either the light or electron microscopic level, the morphologies of *Pneumocystis* spp. from different mammalian species are generally not distinguishable, although some subtle electron microscope-based differences have been reported (115). It has been hypothesized that the *Pneumocystis* life cycle consists of asexual and sexual phases, with two primary morphological forms: the trophic form (or trophozoite) and the cyst (ascus) form (116–118). While there are intermediate stages between these two forms, they are less defined. In fact, neither "cyst" nor "trophozoite" has been used to describe any stage of other fungi; both have been used for *Pneumocystis* because it was originally classified as a protozoan, for which trophozoite (Greek for "animal that feeds") is the active, replicating stage in the host, usually associated with pathogenesis, while cyst often refers to the dormant stage, with a thick protective cell wall enabling the parasite to survive in the outside environment (119). With the recognition that *Pneumocystis* is a fungus, the trophic form is thought to be equivalent to vegetative yeast and the cyst form to the asci of ascomycete fungi.

The trophic form is highly pleomorphic, varying in size from ~2 to 10 μm (for the long dimension), with a thin, flexible cell wall (~20 to 30 nm). In infected lungs, trophic forms are often clustered together or tightly attached to type I pneumocytes, and they usually predominate over cyst forms by a ratio of ~10 to 20:1. The majority of the trophic forms are haploid, but a minor population appears to be diploid (120, 121). Each trophic form contains a single nucleus, which is surrounded by cytoplasmic organelles, including mitochondria, rough and smooth endoplasmic reticula (ER), Golgi vesicles, and cytoplasmic vacuoles (122, 123). On the surface of the trophic form, there are many protrusions, termed tubular extensions or filopodia (122–126), which often protrude toward the host cell or penetrate into invaginations of the host cell (127, 128). The function of these structures remains unknown, but they have been hypothesized to play a role in nutrient uptake by interdigitating with the host membrane (125, 126, 129). The trophic forms are believed to replicate asexually by binary fission (130–132). In the sexual phase, two trophic forms can potentially mate and develop into cysts.

The cyst form has a spherical shape (~5 to 8 μm in diameter) with a thick, smooth cell wall (~100 to 160-nm thick) that is rich in β-glucans. Each mature cyst typically contains eight intracystic bodies (spores), which may represent precursors to trophic forms. Each intracystic body contains a nucleus, mitochondria, and abundant endoplasmic reticula (128). Compared to those of the trophic form, the cyst form has rare tubular extensions, which are typically attached just to the surface of the cell wall but do not extend into host cell invaginations. Studies of *Pneumocystis*-infected mice treated with β-glucan synthetase inhibitors have demonstrated that the cyst is the infective form responsible for transmission to new hosts (132). After inhalation, cysts are presumably deposited to the alveoli and release eight spores that subsequently develop into trophic forms and begin the life cycle again.

The occurrence of a sexual phase is supported by the observation of synaptonemal structures within *Pneumocystis* cells (118, 133, 134), the identification of a conserved meiotic pathway (135), and the presence and transcription of many sex-related genes in *Pneumocystis* genomes (69, 97, 136–138). Recent studies of the genomic structure at the mating-type (*mat*) loci suggested that sexual reproduction in *Pneumocystis* is achieved by a self-fertilizing mechanism known as primary homothallism (139), in which both mating-type idiomorphs are present within a single genome (140). Nevertheless, the identity and organization of the *mat* genes in *Pneumocystis* remain uncertain due to their significant divergence from those of a closely related sibling species, *S. pombe*, one of the best-studied fungal models for sexual reproduction (141). In addition, some key components involved in the mating process, including the mating factors (*map2* and *mfm1/2/3*), have not been identified in any *Pneumocystis* species. Mating and sexual reproduction presumably play a crucial role in the survival of *Pneumocystis*; elucidation of the related genetic pathways should improve the under-

TABLE 1 Genome statistics for *Pneumocystis* spp. and other fungi

Species	Size (Mb)	No. of genes	No. of exons per gene	No. of tRNAs	No. of rRNAs	GC (%)
P. murina	7.5	3,623	6.08	47	5	26.9
P. carinii	7.7	3,646	5.97	45	5	33.2
P. jirovecii	8.4	3,761	5.78	46	5	28.4
T. deformans	13.4	4,651	1.64	81	4	49.5
S. pombe	12.6	5,155	1.99	171	43	36.1
S. cerevisiae	12.1	5,863	1.06	275	25	38.3
C. albicans	14.3	6,189	1.06	126	4	33.4
A. fumigatus	29.4	9,782	2.92	179	34	49.8
C. immitis	28.9	9,757	3.31	121	41	46.0
E. cuniculi	2.5	1,996	1.00	46	9	47.3
E. intestinalis	2.2	1,833	1.01	46	13	41.5

standing of PCP pathogenesis and may identify new strategies to prevent or better manage disease in immunocompromised patients.

Genome Features

The mysterious lifestyle of *Pneumocystis* raised important questions about its genome. This led to an international proposal for a *Pneumocystis* genome sequencing project, which was announced in 1997 (142), only 1 year after the release of the first eukaryotic genome sequence, that of *S. cerevisiae* (143). However, this project progressed very slowly, lagging substantially behind those for many other pathogens, principally as a result of difficulties in obtaining high-quality DNA samples due to the lack of an efficient *in vitro* culture system. It was not until 2006 that the first *Pneumocystis* genome assembly was reported, though it was only a partial, highly fragmentary assembly for *P. carinii* (144). In 2012, the first *P. jirovecii* assembly, which utilized powerful next-generation sequencing (NGS) technologies, was published (97). Further application of multiple NGS technologies resulted in very-high-quality (at or near the chromosomal level) genome assemblies for *P. murina*, *P. carinii*, and *P. jirovecii* (69), allowing for a more reliable and thorough comparative genomic analysis (Table 1). Analyses of these genomes have yielded important insights into the biology of *Pneumocystis* (69, 97, 136, 145–147). This section briefly outlines various genome features, including genome structure, gene content, metabolic capacity, coding strategies for cell wall components, introns, and alternative splicing.

Genome structure. The genomes of the two species infecting rodents, *P. carinii* and *P. murina*, have very similar sizes and chromosomal organizations, with few chromosomal rearrangements, involving ~60- to 260-kb segments in five chromosomes of each species (69). In contrast, compared to that of rodent *Pneumocystis*, the *P. jirovecii* genome is highly rearranged both inter- and intrachromosomally, with each of the 17 largest scaffolds (potentially representing chromosomes) mapped to two to five different chromosomes of *P. murina*. A similar organization was seen in mitochondrial genome (mitogenome) studies. While all three species have nearly the same set of genes, the mitogenomes of *P. murina* and *P. carinii* are linear, with the same gene order; in contrast, *P. jirovecii* bears a circular mitogenome with a different gene order (99). The substantial variation in both the nuclear and mitochondrial genomes between human and rodent *Pneumocystis* spp. highlights the possibility that there may be clinically relevant differences between animal models of PCP and human disease.

Genome contraction. Reductive evolution, which results in a loss of genes and a reduction in genome size, is a pervasive process that has long been considered a hallmark of parasitism and presumably occurs in part because the host can complement necessary biological functions; in fact, there is a growing body of evidence recognizing its impact as a major evolutionary force affecting a broad range of organisms (reviewed in reference 148). *Pneumocystis* species follow this paradigm, since they are the only lineage within the Taphrinomycotina to have evolved for animal parasitism, and they also harbor the smallest genome size. The *Pneumocystis* genomes

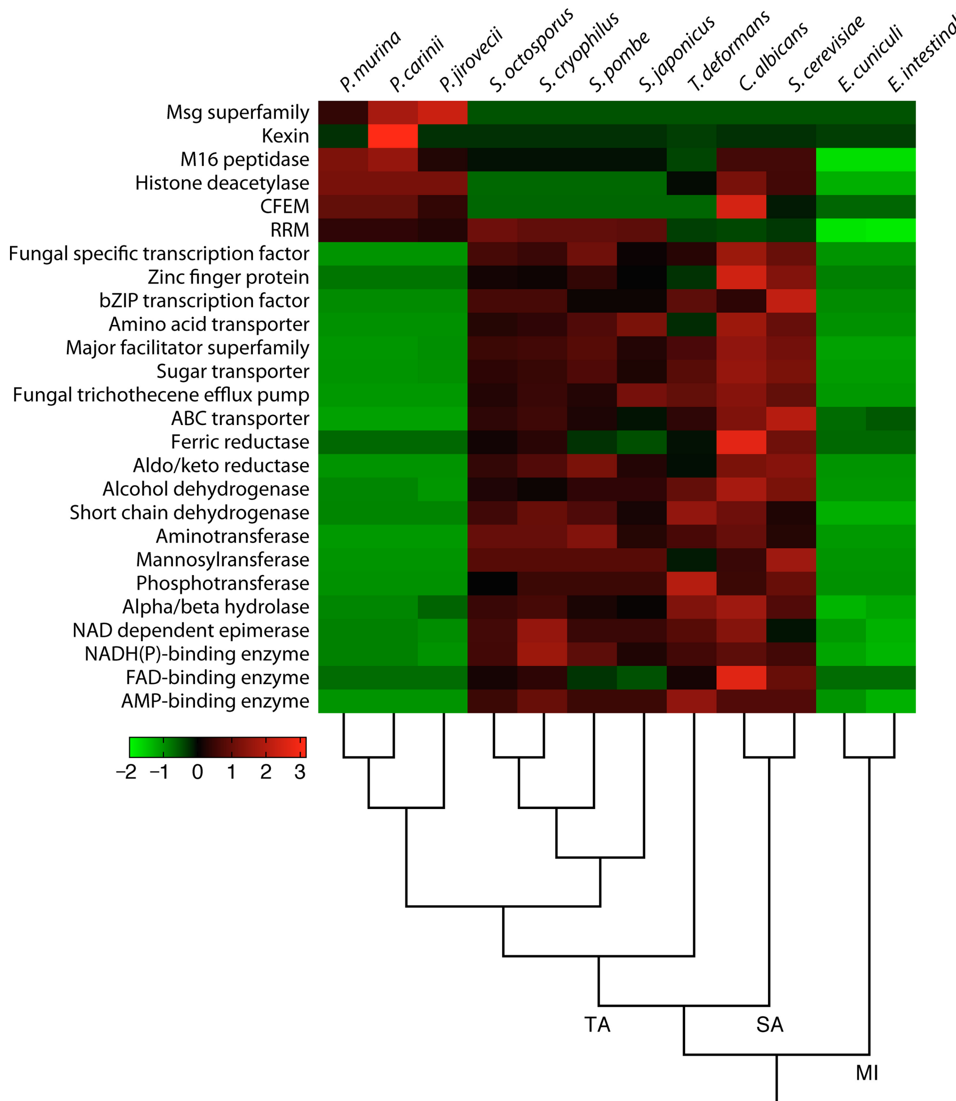

FIG 2 Top enriched and depleted protein families in *Pneumocystis*. Significantly enriched and depleted Pfam domains (Fisher's exact test; $q < 0.05$) were included in the heat map if the domains appeared at least twice in the following comparisons: *Pneumocystis* versus *Schizosaccharomyces*, *Pneumocystis* versus *Schizosaccharomyces* and *Taphrina deformans*, *Pneumocystis* versus *S. cerevisiae* and *C. albicans*, *Pneumocystis* versus *Encephalitozoon cuniculi* and *E. intestinalis*, and *Pneumocystis* versus all others shown. CFEM, common in fungal extracellular membrane domain; RRM, RNA recognition motif. The heat map is color coded based on Z scores from −2 to 3, as indicated by the key. Fungal species are ordered based on their phylogenetic relationships, as indicated at the bottom. The subphyla are indicated on the main branches, as follows: TA, Taphrinomycotina; SA, Saccharomycotina; and MI, Microsporidia. (Modified from Fig. 2 in reference 69.)

sequenced, to date, have all demonstrated a contracted genome compared to those of other closely related fungi (Table 1).

Genome reduction takes place via elimination of genes, reduction of gene length, reduction of intergenic spaces, contraction of gene families, and simplification of gene structures (e.g., loss of introns). Evidence of nearly all these processes can be found in the *Pneumocystis* genomes. Massive gene losses have been identified through comparative genomics (see "Lost metabolic functions," below). Intergenic spaces, which cover ~33% of the *Pneumocystis* genome, have reduced lengths compared to those in other ascomycetes (69). Gene families other than the subtelomeric *msg* superfamily display a net contraction (Fig. 2), and only a few instances of expansions have been reported (69, 145). The only discordant feature compared to those of other parasites is the high intron density (see "Introns and alternative splicing," below).

TABLE 2 Loss of biological pathways in *Pneumocystis*

Category	Type of loss	Biological pathway or component affected
Stress responses	Loss of many transcription factors	pH sensing
		Osmotic stress response
		Oxidative stress response
		Cell wall stress response
Protein synthesis	Loss of *de novo* synthesis	All 20 amino acids
Carbohydrate metabolism	Loss of pathway	Gluconeogenesis
		Glyoxylate cycle
		Fermentation
Lipid metabolism	Loss of synthesis	Ergosterol
		Cholesterol
		myo-Inositol
		Choline
		Ether lipids
		Complex sphingolipids
		Phosphatidylinositol
		Phosphatidylcholine
		Glycerol
	Loss of function	Fatty acid β-oxidation
Cofactor metabolism	Loss of synthesis	Coenzyme A
		Thiamine
		Biotin
		Siderophores
		Reductive iron uptake
	Loss of enzyme	Carbonic anhydrase

Understanding gene loss may give insights into the basis for *Pneumocystis* host specificity. However, the patterns of gene loss in multiple *Pneumocystis* species are roughly similar (69), which suggests that gene deletions occurred in a common ancestor, became fixed (i.e., not lethal), and were transmitted vertically, although formal validation of this scenario awaits genome sequencing for additional species. If this holds true, analysis of gene loss in currently sequenced species alone cannot fully explain the host specificity.

Lost metabolic functions. Many opportunistic pathogens, including bacteria (e.g., *Pseudomonas aeruginosa* [149]) and fungi (e.g., *Aspergillus fumigatus* and *Coccidioides immitis* [Table 1]), have large genomes encoding complex and often redundant metabolic pathways to allow the pathogens to live within diverse environments, including both inside and outside the host. While initial analysis of an early partial genome of *P. carinii* suggested the presence of most standard metabolic pathways (136), recent studies of the nearly complete genome of *P. carinii* as well as those of *P. murina* and *P. jirovecii* demonstrated a loss of many metabolic pathways (Table 2), with retention presumably of those critical to survival in the host environment (69, 97, 145).

(i) Amino acid metabolism. Amino acid metabolism is shifted to wholly scavenge from the host instead of performing *de novo* biosynthesis. All three *Pneumocystis* species lack ~80% of the genes for amino acid biosynthesis that are present in yeast (97, 150), and they are also defective in inorganic nitrogen and sulfur assimilation (145). As a result, *Pneumocystis* cannot synthesize any of the 20 standard amino acids. Moreover, there is only one potential plasma membrane-localized amino acid transporter (Ptr2) in each *Pneumocystis* species, in sharp contrast to the case in yeast species, which have more than 20 such transporters. Nevertheless, nearly 50% of the 26 amino acid transporters associated with mitochondria and vacuoles in yeast are conserved in *Pneumocystis*. These findings suggest that *Pneumocystis* scavenges amino acids from its host. This hypothesis is supported by the retention or relative expansion of genes encoding proteases and proteasome proteins in *Pneumocystis* and the presence of

abundant amino acids and peptides in alveolar fluid of the hosts (151, 152). Similarly, polyamine biosynthesis is completely lost in *Pneumocystis*, while one polyamine transporter is retained, supporting a mechanism for direct uptake of polyamines from the host.

(ii) Nucleotide metabolism. Nucleotide metabolism is focused solely on *de novo* biosynthesis of fundamental cellular components. The *Pneumocystis* genomes have retained the complete *de novo* biosynthesis pathways for purine and pyrimidine nucleotides but have lost nucleotide salvage and degradation pathways (69, 145), consistent with the reported absence of a thymidine salvage pathway (153). This is unusual for a highly compact genome, since the *de novo* biosynthetic pathways involve substantially more chemical reactions and thus require more energy than those for salvage pathways. In fact, although *de novo* nucleotide synthesis is a fundamental biological process that is highly conserved in fungi and other organisms, most organisms, including all known human fungal pathogens, also maintain functional salvage and degradation pathways (154). The exception to this are the intracellular fungal pathogen Microsporidia (155, 156) and parasitic protozoa (157, 158), which lack *de novo* nucleotide synthesis pathways but preserve the nucleotide salvage and degradation pathways. The retention of the energy-expensive *de novo* synthesis pathways in *Pneumocystis* suggests an absolute necessity to keep these pathways, presumably as a result of a lack of nucleosides and nucleobases in the host alveolar environment. These findings suggest that inhibitors of *de novo* nucleotide synthesis may be effective drugs for treating PCP.

(iii) Carbohydrate metabolism. Carbohydrate metabolism is streamlined to produce energy for cell wall synthesis, with limited output of metabolites. *Pneumocystis* genomes carry all genes required for glucose uptake and catabolism through glycolysis and the tricarboxylic acid cycle, as well as all key genes required for oxidative phosphorylation. In addition, all the enzymes required to catalyze mannose and fructose to glucose and for the metabolism of glycogen and trehalose are preserved, though critical enzymes required for converting sucrose or galactose to glucose are not present. The synthesis of glycogen is supported by the detection of abundant glycogen granules in the *Pneumocystis* cytoplasm in many electron microscopic studies (115, 159, 160). These findings indicate that *Pneumocystis* relies largely on glucose utilization via oxidative pathways for energy production.

The most striking losses in carbohydrate metabolism include the central regulatory enzyme (Fbp1) for gluconeogenesis, two key enzymes (Mls1 and Icl1) for glyoxylation, and all enzymes for pyruvate fermentation. Simultaneous loss of these pathways not only suggests an inability of *Pneumocystis* to use nonsugar carbon sources (including fatty acids and simple carbon compounds, such as ethanol and acetate) for energy production, further supporting a high reliance on glucose, but also indicates a limited capacity for biosynthesis of complex structural polysaccharides in the cell wall, as discussed below. In addition, the loss of the glyoxylate pathway may explain in part the apparent lack of virulence of *Pneumocystis* given that this pathway is believed to be required for virulence of some pathogenic fungi (161–163) as well as bacteria (164).

(iv) Lipid metabolism. Lipid metabolism is optimized to exploit host resources, resulting in distinctive lipid profiles compared to those of other fungi. *Pneumocystis* has unique sterol biosynthesis pathways contributing to distinct sterol compositions in the plasma membrane (69, 165, 166). Based on genome analysis, both human and rodent *Pneumocystis* spp. are able to synthesize lanosterol, zymosterol, episterol, and fecosterol but cannot further metabolize them to ergosterol due to the lack of one or two key late-stage enzymes (Erg3 and Erg5) (69), consistent with the results of membrane chemical composition analysis (64, 67, 167, 168) and with resistance to antifungal agents targeting ergosterol (64). In addition, all potential pathways for cholesterol biosynthesis found in mammals and a few fungal species appear to be disabled due to the absence of the key enzyme Dhcr24 in both human and rodent *Pneumocystis* spp. and of another two enzymes (Erg3 and Dhcr7) in rodent *Pneumocystis* only. Nevertheless, cholesterol has been identified as the most abundant sterol in both human and

rodent *Pneumocystis* spp. (64, 165, 169–171). There has been evidence suggesting that *Pneumocystis* can scavenge cholesterol from its host (67, 69, 169), but the genes involved in cholesterol acquisition are not identified in the genome data. Alternative possibilities include the possibility that the Dhcr24 homolog in *Pneumocystis* could not be identified based on sequence homology due to high sequence divergence or that the enzyme activities of Dhcr24 could be replaced by those of other enzymes. The utilization of cholesterol in the *Pneumocystis* membrane is very unusual within the fungal kingdom, especially since organisms within the most closely related subdivision, Taphrinomycotina (166), utilize ergosterol (fission yeasts) (172) and brassicasterol (*Taphrina* and *Protomyces*) (173, 174). The reason for utilization of cholesterol rather than ergosterol is not clear but may reflect optimized cellular and physiological functions as a result of adaptation to the host environment. First, it is less energetically expensive to synthesize cholesterol (175) or to scavenge it from the host than to synthesize ergosterol *de novo*. Second, since membranes containing cholesterol are less rigid than membranes containing ergosterol, utilization of cholesterol may create a more flexible cell wall and thus may have promoted development of trophic forms. Third, as the major sterol in mammalian cell membranes, cholesterol is an efficient mechanical stabilizer (176). Utilization of cholesterol may allow *Pneumocystis* to better interact (interdigitate) with the cholesterol-containing host cell membrane and to stabilize its cell structure. Lastly, studies of plant fungal pathogens have suggested that ergosterol is a pathogen-associated molecular pattern (PAMP) (177, 178), and loss of ergosterol in *Pneumocystis* may represent a mechanism of immune evasion.

In addition to the loss of *de novo* biosynthesis of ergosterol and/or cholesterol, each *Pneumocystis* species lacks enzymes for *de novo* biosynthesis of many other lipids, including glycerol, ether lipids, phosphatidylcholine, phosphatidylinositol, and complex sphingolipids, as well as other components (such as *myo*-inositol and choline) related to synthesis of complex lipids (69). Except for glycerol, for which two potential transporters are encoded in the genome (responsible for direct uptake and export), there are no direct transporters predicted for any of these lipids. Nevertheless, alternative mechanisms may supply these lipids. For example, each *Pneumocystis* species encodes the Dnf1-Lem3 flippase complex, involved in uptake of external lysophosphatidylcholine that can be converted to phosphatidylcholine and, subsequently, to choline. There is also a potential transporter (Git1) involved in uptake of external glycerophosphoinositol that may be hydrolyzed into inositol (179), though the enzyme responsible for this hydrolysis has not been identified definitively in *Pneumocystis* or other fungi. However, other studies have reported the identification of direct inositol transporters (147, 180). This discrepancy awaits further investigation using yeast complementation assays and other approaches.

Fatty acid metabolism in *Pneumocystis* is also unique among fungi. The absence of *fas1* and *fas2* genes in *Pneumocystis* suggests a loss of the cytosolic fatty acid synthesis (FAS) pathway, which is highly conserved in other fungi and eukaryotes. In addition, *Pneumocystis* lacks the majority of genes required for fatty acid β-oxidation that are conserved in other fungi, implying that fatty acids cannot be used by *Pneumocystis* for energy generation, further highlighting its dependence on glucose for energy production. Despite the loss of the cytosolic FAS pathway, rodent *Pneumocystis*, but not *P. jirovecii*, conserved the complete mitochondrial FAS pathway, which is composed of eight monofunctional enzymes resembling the bacterial FAS system, in contrast to the cytosolic eukaryotic multifunctional FAS complex. This selective conservation in rodent *Pneumocystis* spp. suggests its vital role in these organisms' survival, given that mitochondrial FAS is believed to be essential for cellular respiration, RNA processing, and mitochondrial biogenesis in eukaryotes (181–183). *P. jirovecii* lacks one of the eight genes (Mct1) involved in mitochondrial FAS, raising the possibility that this pathway is disabled in this organism. However, given the retention of the seven other genes of this pathway in this organism, as well as all the genes required for the downstream processes in the ER, including fatty acid chain elongation, desaturation, and hydroxylation, in all sequenced *Pneumocystis* species, it seems likely that *P. jirovecii* has also

retained mitochondrial FAS activities. It is possible that the *mct1* gene in *P. jirovecii* could not be identified due to sequence divergence or incomplete assembly of the genome, or the Mct1 activity in *P. jirovecii* may be replaced by activities of other enzymes.

(v) Cofactor metabolism. Cofactor metabolism is greatly consolidated. *Pneumocystis* genomes lack almost all genes involved in pantothenate biosynthesis and transport. However, they encode the enzymes required for synthesis of coenzyme A (CoA) from pantothenate and also encode a carrier protein (Leu5) to facilitate transport of CoA into mitochondria, suggesting a possible scavenging mechanism for pantothenate or its metabolites by another process, such as endocytosis, as discussed below. While *Pneumocystis* genomes are also missing key genes needed for *de novo* synthesis of thiamine (vitamin B_1), biotin (vitamin H), siderophores, and ubiquinone, they do encode transporters for each of them. *P. carinii* and *P. murina* have retained complete pathways for both biosynthesis and salvage of the coenzyme NAD. However, *P. jirovecii* is missing most of the enzymes required for NAD synthesis *de novo* but has preserved a salvage pathway to produce NAD by using nicotinic acid mononucleotide transported from an exogenous source via Tna1. All three *Pneumocystis* species lack almost all genes required for assimilation of reductive iron and biosynthesis of siderophores (184), but they do encode five proteins containing fungus-specific CFEM (common in fungal extracellular membrane) domains (185), raising the possibility that, like *Candida albicans* (186, 187), *Pneumocystis* can use a subset of these to scavenge iron from host hemoglobin and heme.

As discussed above, there is a lack of both *de novo* biosynthesis and direct transporters for some nutrients, implying other mechanisms for nutrient uptake. Genome analysis suggests that endocytosis may serve this purpose, as evidenced by the preservation of nearly all proteins associated with clathrin-dependent endocytosis and the presence of vesicles and other structures (including tubule-, filopodium- and basket-like structures) in numerous electron microscopic studies of different *Pneumocystis* species (115, 126, 188, 189).

In summary, the streamlining of *Pneumocystis* genomes has resulted in a significant reduction and consolidation of metabolic pathways compared to those of other closely related fungal species, presumably reflecting adaptation to the mammalian hosts, on which these organisms are highly dependent for nutrients and a stable environment. These reduced pathways may explain the slow growth of *Pneumocystis* organisms (190, 191) and their failure to grow continuously *in vitro*.

Cell wall reduction. Two main types of macromolecules are typically found in the cell walls of fungi: polysaccharides of different types (mainly chitin, chitosan, glucans, and mannans) and proteins with various modifications. These components are cross-linked to maintain the cell shape and structural integrity, protecting the cell from its surroundings and allowing the cell to interact with other cells and the environment (192–194). The importance of the cell wall in fungi is reflected by the fact that, in yeast, one-fifth of the genome is devoted to cell wall biosynthesis (193, 195). Given that the pathways needed for cell wall biosynthesis are largely limited to fungi, enzymes required for cell wall metabolism are potential targets for antifungal chemotherapies and fungicides. Due largely to an inability to culture *Pneumocystis*, its cell wall composition and structure have not been completely defined.

Early biochemical studies demonstrated an abundance of glycoproteins in the cell wall for all forms of the organism (196–202), while β-glucans were found only in cysts (71–73, 203, 204). Based on ultrastructural studies, the cell wall of the trophic form measures 20 to 30 nm and consists of two layers: an electron-dense outer layer and an inner layer containing the plasma membrane (205, 206). The cyst cell wall is ~100 nm thick and consists of a distinct, tightly packed, electron-dense outer layer and an inner plasma membrane separated by an electron-lucent middle layer (Fig. 3). Thiery's reagent stained only the electron-lucent middle layer (207), suggesting a limited abundance and distribution of polysaccharides. This is in contrast to the cell wall of *C. albicans*, which is ~200 to 300 nm thick, with five to nine layers (representing different

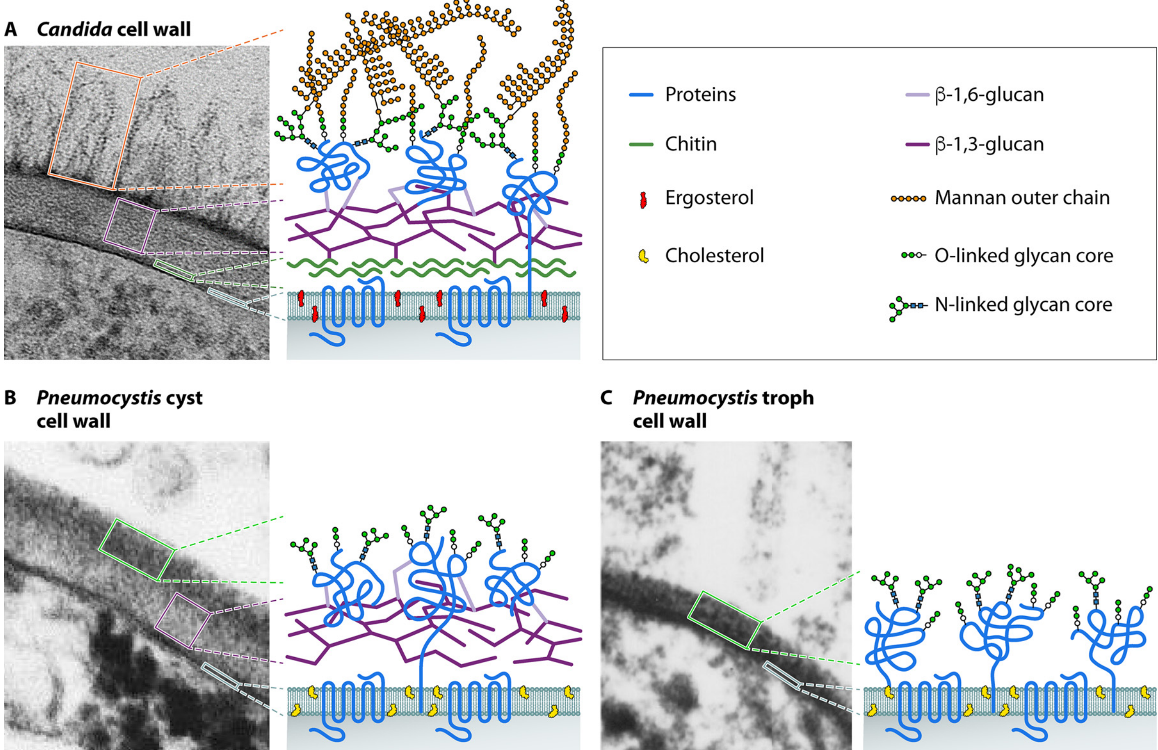

FIG 3 Cell wall structure of *Pneumocystis* compared to that of *C. albicans*. (A) *C. albicans* cell wall. The inner layer contains chitin and β-glucans, whereas the outer layer contains hypermannosylated N- and O-linked glycans (mannans) that are covalently linked with proteins to form glycoproteins. The plasma membrane contains ergosterol. (Electron micrograph courtesy of Louise Walker and Neil Gow, University of Aberdeen, United Kingdom; reprinted with permission.) (B) Cell wall of *Pneumocystis* cysts (asci). The inner layer contains β-glucans and no chitin, whereas the outer layer is highly enriched in proteins that are glycosylated via N- and O-linked glycans, but without the mannan outer chains. The plasma membrane contains cholesterol instead of ergosterol. (C) Cell wall of *Pneumocystis* trophic forms. The cell wall is the same as that of *Pneumocystis* cysts, except for the absence of β-glucans.

dominant polysaccharides) distinguishable by Thiery's staining (208). The recent sequencing of *Pneumocystis* genomes has allowed an improved understanding of the molecular landscape of the *Pneumocystis* cell wall (69, 97, 145), as illustrated in Fig. 3 and summarized below.

(i) Complete loss of chitin biosynthesis and degradation pathways. None of the *Pneumocystis* genomes sequenced (69) encodes chitin synthase, an enzyme critical for the synthesis of chitin, in contrast to all other sequenced fungal species, which contain up to 10 chitin synthase genes (209). *Pneumocystis* also lacks chitinases, which are required for chitin degradation during cell wall remodeling, encoded by up to 30 or more genes in other fungi (210–212). The absence of both chitin synthase and chitinase strongly suggests that the *Pneumocystis* cell wall lacks chitin, which was confirmed experimentally by mass spectrometric analysis of the cell wall content and by the absence of staining with a recombinant chitin binding domain specific for chitin (Fig. 4). While each *Pneumocystis* species encodes homologs of a few accessory proteins, such as Chs5 (213), not directly associated with chitin synthesis or degradation, these proteins do not have domains associated with either chitin synthase or chitinase activity. Note that the Chs5 protein of *S. cerevisiae* is part of a complex associated with exporting membrane proteins, including chitin synthase (214). A lack of chitin has not been reported previously for any other fungi.

(ii) Presence of β-glucans but not α-glucans. Both human and rodent *Pneumocystis* spp. have complete pathways for the biosynthesis and degradation of β-1,3-glucan and β-1,6-glucan (69), consistent with the detection of both types of glucans in the cell wall of cysts in previous studies (71, 73, 215–217) (Fig. 4). As in other fungi, β-1,3-glucan is found in the inner layer of the cyst cell wall (73) (Fig. 3). In the trophic form, both types

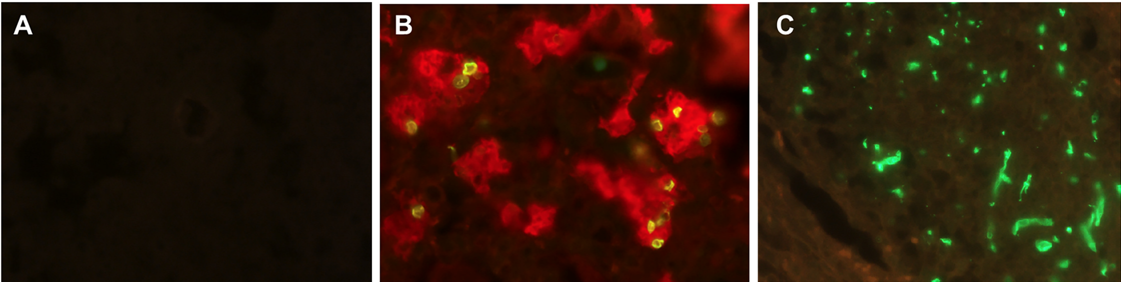

FIG 4 Loss of chitin in *Pneumocystis* cell wall. *P. murina*-infected lung tissue (A) and *C. albicans*-infected kidney tissue (positive control) (C) were stained with a recombinant chitin binding domain (green). Chitin staining is absent in *P. murina* (A) but readily detected in *C. albicans* (C). (B) *Pneumocystis* organisms are demonstrated by dual staining with anti-Msg (red), which labels both trophic forms and cysts, and dectin-Fc (green), which labels β-1,3-glucan in cysts. Original magnification, ×400.

of glucans are absent (71, 73, 215–217). *Pneumocystis* spp. are missing all genes required for biosynthesis and degradation of α-glucan (69), which is present in many other fungi and is able to prevent innate immune recognition by the β-glucan receptor (218). The retention of β-glucans in the absence of α-glucan, chitin, and mannans (see below) suggests that β-glucans are absolutely necessary for the survival of *Pneumocystis* cysts, supporting β-glucans as potential targets for treatment of PCP. Indeed, several studies have shown that inhibitors of β-1,3-glucan synthase are highly effective at reducing cyst numbers in animal models of PCP (132, 204, 216, 217, 219–221). In other fungi, β-1,3-glucan is found as a branched polymer with β-1,6 side interchains and is covalently linked to other wall components, such as chitin, mannans, and glycoproteins (193, 195, 222). How β-1,3- and β-1,6-glucans interact with each other and with other cyst cell wall components in *Pneumocystis* is currently unknown, though a recent study found that β-glucans are masked by surface proteins (216). Since β-glucans are well-known activators of innate immunity during infection by *Pneumocystis* (223–229) and other pathogens (230–232), their absence in the trophic form and their masking in cysts may represent a mechanism to escape host innate immunity. Given that immunocompromised hosts were likely rarely encountered during the evolution of *Pneumocystis*, this mechanism presumably evolved during infection of immunocompetent hosts, in whom the organism burden is low. In immunosuppressed hosts with high organism loads, release of β-glucans, presumably as a result of organism death, is a critical factor contributing to deleterious host inflammatory responses (216).

(iii) Partial loss of protein glycosylation. *Pneumocystis* genomes encode all enzymes residing in the endoplasmic reticulum that are necessary for biosynthesis of the core structure of N- and O-linked glycans (consisting of up to nine mannose residues), but they do not encode any enzymes to add mannose outer chains, including α-1,6-, α-1,2-, and α-1,3-mannosyltransferases as well as mannan polymerase complex I and complex II, all of which are located in the Golgi apparatus (69). These findings suggest that in contrast to those of other fungi, cell wall proteins of *Pneumocystis* have low levels of mannosylation (Fig. 5). This hypothesis was confirmed by direct examination of glycosylation in purified *P. carinii* Msg proteins (69). By liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of peptide-*N*-glycosidase F (PNGase F)-released N-linked glycans, the predominant N-linked glycan identified was M5N2 (hexose5 HexNAc2). Although trace levels of M6N2 to M9N2 were found, nothing larger than M9N2 was definitively identified. Additionally, glycopeptide mapping of Msg tryptic digests identified 15 Msg isoforms, with 31 N-linked glycans in, all with M5N2 as the major constituent. In only one Msg isoform, M6N2 was also identified as a minor constituent.

(iv) Expansion of the complex surface protein superfamily. Fungal wall proteins often form large families with a common multidomain structure (233, 234). They are usually heavily glycosylated and form a dense protein coat to mask the inner polysac-

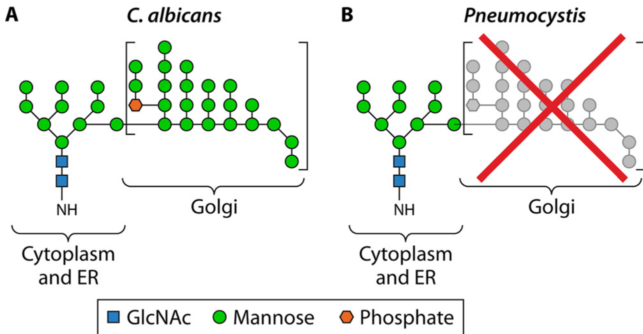

FIG 5 Lack of N-linked hypermannose (mannan) outer chains in *Pneumocystis*. Like *C. albicans*, *Pneumocystis* spp. are able to synthesize the N-linked glycan core structure (containing up to nine mannose residues, as indicated on the left) in the cytoplasm and the endoplasmic reticulum (ER). However, due to the loss of multiple enzymes, *Pneumocystis* spp. are unable to synthesize the α-1,6-linked mannose backbone as well as the α-1,2- and α-1,3-linked mannose outer chains seen in *C. albicans* (square brackets), which are synthesized in the Golgi apparatus. (Diagrams of the N-linked mannan structure in *C. albicans* and *Pneumocystis* were adapted from reference 69.)

charide layer (193, 234). They can function in mediating developmental states, optimizing mobility and adhesion ability, protecting the organism from harmful environmental stresses, and adapting to various niches. Characterization of *Pneumocystis* cell wall proteins is very challenging due in large part to the lack of a reproducible culture method, which prevents isolation of *Pneumocystis* proteins in large quantities and with high purity. Nevertheless, numerous studies have found that the most abundant cell wall protein in both the cyst and trophic forms of all *Pneumocystis* species studied, to date, is the major surface glycoprotein (Msg), also known as gp95, gp115, gp120, and gpA (196, 197, 201, 235–239).

Early studies of *Pneumocystis* in humans, rats, and mice showed that the Msg protein is encoded by a gene family with an estimated ~30 to 100 copies per genome (236–238, 240, 241). *msg* genes (up to ~3 kb each) are closely related to but clearly distinct from each other and are clustered in the subtelomeric regions of multiple chromosomes (69, 242). These features make it difficult to accurately determine the total number or complete sequences of *msg* family members. Thanks to advances in DNA sequencing technology, especially the PacBio long-read sequencing platform (243, 244), nearly complete sets of *msg* genes from three *Pneumocystis* species (*P. jirovecii*, *P. carinii*, and *P. murina*) were determined as part of the *Pneumocystis* genome project (69). This genome study identified multiple additional genes that are related to *msg*, based on the presence of one or more conserved domains, which are collectively termed the *msg* superfamily. Each *Pneumocystis* genome encodes 60 to 180 members of the Msg superfamily, which is the largest family of surface proteins found in fungi, to date (245). In each species, *msg* superfamily genes account for 3 to 6% of an otherwise highly compact genome, suggesting a vital role in the organism's survival in its host.

The availability of a nearly complete Msg repertoire in three *Pneumocystis* species has allowed, for the first time, a detailed analysis of the Msg domain structure, phylogeny, and classification. Like the cell wall proteins in other fungi (233, 234), Msg proteins are also composed of multiple conserved domains, named N1, M1 to M6, C1, and C2 (69). These domains are unique to *Pneumocystis* and are not shared with any other fungal species. The Msg superfamily can be grouped, based on phylogeny and domain organization, into five families, termed Msg-A to Msg-E. The sequences of these proteins show not only conservation among different families across different *Pneumocystis* species but also species-specific expansions or contractions.

The Msg-A family is the largest family and includes three subfamilies: Msg-A1, -A2, and -A3. The majority of members of this family contain all nine Msg signature domains, while a small number contain only three to eight domains. The Msg-A1 subfamily

primarily includes all classical Msgs, which are conserved in all known *Pneumocystis* species and whose gene expression is controlled by a unique, single-copy subtelomeric expression site known as the upstream conserved sequence (UCS) (246–249). The UCS is expressed in frame with one of the multiple *msg* gene variants; the region between the UCS and its downstream *msg* gene is termed the conserved recombination joint element (CRJE), which is highly conserved among all classical *msg* genes and potentially serves as an anchor for recombination (250). Different *msg* variants are presumably expressed by recombination downstream of the UCS. The Msg-A2 subfamily primarily represents *msg*-related (*msr*) genes present in both *P. carinii* (251, 252) and *P. murina* but absent in *P. jirovecii* (69). Each *msr* gene contains a highly conserved exon at the 5′ end; its expression is not dependent on the UCS. The Msg-A3 subfamily includes genes with substantial sequence identity to the Msg-A1 and Msg-A2 subfamilies, but without the CRJE of the classical *msg* genes or the highly conserved exon 1 of the *msr* genes. This subfamily has 33 members in *P. jirovecii* but only 1 and 2 members in *P. murina* and *P. carinii*, respectively.

The Msg-B family is present only in *P. jirovecii*; most genes encode only three Msg signature domains. The Msg-C family is encoded by a tandem array of six genes in *P. murina*, with each copy containing three Msg signature domains. Only one and two short copies are present in *P. carinii* and *P. jirovecii*, respectively. The Msg-D family is related to the previously reported A12 antigen gene in *P. murina* (253, 254); this family is encoded by a single gene in *P. murina* and *P. carinii* but is expanded to 20 copies in *P. jirovecii*, with the majority encoding six Msg signature domains. The Msg-E family is related to two previously reported p55 genes (255–260); there are five to seven Msg-E genes in each *Pneumocystis* species, and each of them encodes only one Msg signature domain.

Currently, the functions of the vast majority of Msgs remain poorly understood or uncharacterized. The best-studied proteins are the classical Msgs of the Msg-A1 subfamily. It has long been hypothesized that this subfamily plays a critical role in pathogen-host interactions, including adhesion to host cells and extracellular matrix proteins (261–264), evasion of host immune attacks via antigenic variation (237, 265–268), and masking of immune activation by β-glucans (216). Evidence in support of their role in antigenic variation includes the preservation of all required components of the DNA recombination machinery (69), the clustering of *msg* genes almost exclusively in subtelomeric regions to facilitate recombination (69, 242), the presence of strong serological responses to Msg proteins in patients with PCP (30, 269–271), the selective expression of Msg variants among different organisms in the same infected lung (267), and the occurrence of discordant antibody and cellular responses to Msg variants in animal models (268). The last observation suggests that antigenic variation may target T-cell responses, not antibody responses. In addition, transcriptome sequencing (RNA-Seq) data indicate that all *msg* genes in *P. murina* and *P. carinii* are transcribed in a population of organisms; the UCS gene in both species is the most highly expressed protein-encoding gene, consistent with a very high level of expression of the *msg*-A1 gene subfamily as a whole (69). Due to the lack of a culture system and an inability to genetically manipulate *Pneumocystis*, it is not currently feasible to use more direct methods to address the underlying mechanism of *msg* gene recombination and antigenic variation, such as by studying organisms expressing only one *msg* gene. Although they are recognized as glycoproteins, classical Msg proteins are not highly mannosylated (69), unlike glycoproteins in other fungi (272, 273).

The functions of all nonclassical *msg* genes remain unknown, although studies of animal models have suggested that p55-related proteins in the Msg-E family (258, 259, 274, 275) and A12-related proteins in the Msg-D family (253, 254) are antigenic and can potentially generate protective immune responses in hosts. Given that the *Pneumocystis* genome is highly compact and that the recombination system associated with classical *msg* genes is presumably sufficient for antigenic variation and immune evasion, the diverse nonclassical Msgs may provide other advantages for *Pneumocystis* to

survive in the host, such as mediation of life-stage development (276), optimization of cell mobility and adhesion ability, and adaptation to specific host niches.

Despite the Msg proteins being classified as cell wall proteins, their subcellular locations and interactions with other cell wall components remain largely uncharacterized. Cell surface proteins, including the classical Msgs, mask β-glucans, but the chemical linkages between them are still unknown. One study suggested that the p55 antigen in *P. carinii* may be at least partially masked by β-glucans (257). While *Pneumocystis* genomes encode all key enzymes required for glycosylphosphatidylinositol (GPI) anchor synthesis (69), there is a lack of direct proof of GPI-anchored proteins on the cell wall, though potentially functional GPI signal sequences have been reported from indirect studies of the ferret *Pneumocystis* glycoprotein (277) and the *P. carinii* kexin genes (278).

(v) Other cell wall components. Melanin is present in some pathogenic fungi, with an important role in protecting against harmful environmental exposures that can include ionizing radiation, UV light, or oxidizing agents (279). While there are reports of detection of melanin in the *Pneumocystis* cell wall (280, 281), none of the key enzymes involved in melanin biosynthesis, including polyketide synthase, laccase, tyrosinase, and phenoloxidase (279, 282), is encoded in the *Pneumocystis* genome. It is possible that the melanin biosynthesis pathway was lost in *Pneumocystis* as a result of adaptation to an environment largely devoid of exposure to UV light and ionizing radiation.

In summary, compared to those of other fungi, the *Pneumocystis* cell wall has a significantly reduced composition, thickness, and rigidity. This reduction presumably reflects its adaptation to the mammalian host environment. Given that chitin, mannan, and β-glucans are all known PAMPs that trigger the host innate immunity through host pattern recognition receptors (HPRRs) that include DC-SIGN, dectin-1, and dectin-2 (272, 283), their simultaneous loss or masking may represent a highly efficient mechanism adapted by *Pneumocystis* organisms, especially the trophic form, to evade host innate immunity. The absence of these PAMPs is consistent with the absence in the *Pneumocystis* genome of LysM effector genes, which are widely conserved among fungi and function to suppress the PAMP-triggered host innate immune response (284, 285). The potential for antigenic variation conferred by the Msg family may provide additional protection from host adaptive immunity. Given the losses noted above, retention of β-glucans in cysts is noteworthy, suggesting that they are essential to the organism, possibly by providing an aerodynamically efficient cell wall enabling airborne transmission to other hosts (132) and/or contributing to formation of protective biofilms with Msg proteins (286, 287). The absence of both β-glucan and chitin in the cell wall suggests that trophic forms are fragile (with a "wall-less" state) and presumably unable to withstand harsher environmental conditions outside the host lung environment. This may reflect the adaptation of *Pneumocystis* to the stable environment in the host lungs. The absence of β-glucan and chitin in trophic forms may also confer some advantages on *Pneumocystis*, including a cell wall with greater malleability, which may permit a closer connection with host cells to obtain nutrients.

Introns and alternative splicing. Introns can provide mRNA stability, act as regulators of gene expression (288), and promote proteome diversity via alternative splicing. Introns can also mediate RNA interference and microRNA biogenesis (289, 290). Despite the reduced genome and low gene content, the intron density is exceptionally high in *Pneumocystis* (averaging about 5 introns per gene) compared to those in many other fungi in which widespread loss of introns is apparent, as in *S. pombe* (Fig. 6) and Microsporidia (291, 292). *Pneumocystis* introns are small (48 bp, on average) and have a strong adenine and thymine (A+T) bias. They harbor canonical spliceosomal splicing motifs (293) and are present throughout the genomes. Intron-rich genes (>4 introns) represent 42 to 46% of the gene content.

Transcription and splicing of intron-rich genes require a considerable expenditure of energy and cellular resources, as evidenced by the relative expansion in the *Pneumocystis* genome of RNA recognition motif (RRM)-containing genes (Fig. 2) as well as genes related to mRNA surveillance and the spliceosome (69). Such deployment in an

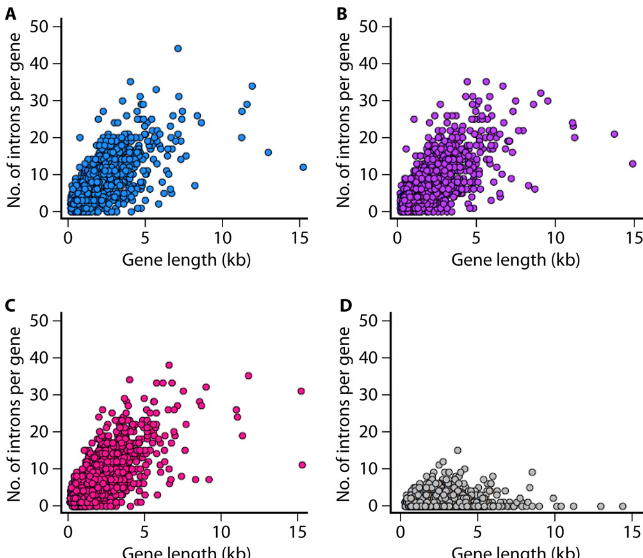

FIG 6 High intron densities in *Pneumocystis* genomes. The graphs show numbers of introns per gene as a function of gene length (measured in kilobases) for three *Pneumocystis* species as well as for the fission yeast *Schizosaccharomyces pombe*. Each dot represents a single gene. Intron densities per gene for 1,624 orthologous genes are systematically higher for *Pneumocystis* spp. than those for *S. pombe*. Intron positions and sizes were extracted from annotated GenBank files for *P. jirovecii* (accession no. GCA_001477535.1) in panel A, *P. carinii* (accession no. GCA_001477545.1) in panel B, *P. murina* (accession no. GCF_000349005.1) in panel C, and *S. pombe* (accession no. GCF_000002945.1) in panel D.

otherwise highly compact genome suggests an essential role of introns in the organism's survival. It is possible that alternative splicing allows *Pneumocystis* to regulate gene expression and to increase transcript diversity from its reduced genome. In consonance with this hypothesis, earlier studies showed that *Pneumocystis* spp. are apparently able to use alternative splicing to respond to changes in their environment, e.g., different isoforms of the IMP dehydrogenase are produced by *P. carinii* in response to different short-term culture conditions (294).

The self-splicing group I introns in *Pneumocystis* have been well studied because of their potential as drug targets (295). They have been identified in numerous genes, such as rRNA genes (296). These introns catalyze self-excision from RNA, a process that can be inhibited by drugs, such as pentamidine and its analogues (297), which correlates with growth inhibition in *C. albicans* (298). Significant intron variations in rRNA genes within and among multiple *Pneumocystis* species have been described (299). The intron of the *msg* expression site of *P. jirovecii* is also variable, which can help in identifying strain variation (300).

Approximately 30 to 40% of introns are efficiently removed from transcripts (69), indicating a high intron retention rate, which is consistent with the dominant tendency in the fungal kingdom. The nonsense-mediated mRNA decay machinery is conserved in *Pneumocystis*, which suggests that mRNAs containing nonspliced introns, which encode aberrant proteins, are tagged for destruction and recycled. Although the functions of some genes of *Pneumocystis* can be evaluated via complementation in other fungi, direct testing of *Pneumocystis* introns in fungal genetic models, such as *S. pombe*, has failed because of an inability of the yeast spliceosome to splice them (301).

The origin and mode of acquisition of introns are unknown. The high intron density suggests that there is an advantage to conserving them, or alternatively, these elements may represent a transient stage after a massive intron proliferation. This is intriguing because intron gain is rare in many eukaryotes (302, 303); retracing the intron evolutionary history of many eukaryotic lineages shows little support for intron creation (304).

Introns evolve without the biologic constraints placed on exons and, as a conse-

quence, have higher evolutionary change rates. This accelerated evolution can erase sequence homology clues necessary to determine their origin as well as the mechanisms that created them. Intron densities are roughly similar in all three *Pneumocystis* species (69), and their locations are often highly conserved, suggesting a single origin.

EPIDEMIOLOGY OF *PNEUMOCYSTIS*

Methods for Molecular Typing

A variety of molecular typing methods have been utilized to study strain variation and the epidemiology of *Pneumocystis* infection in humans. Early reviews on this topic are available (305–307). The present review serves as an update on this expanding field, with an emphasis on newer typing methods leading to new insights into the epidemiology of PCP.

Single-locus Sanger DNA sequencing. Traditional Sanger DNA sequencing remains the most commonly used approach for single-locus typing of *P. jirovecii*. This method has the advantage of being able to detect all known or potentially new sequence variants in the target regions. While the emergence of newer typing methods has decreased its popularity, Sanger sequencing is still an attractive choice in many circumstances, especially with the advent of fast and low-cost commercial sequencing services. The main disadvantages of Sanger sequencing include its low throughput and the inability to differentiate mixed sequences within an amplicon, which are frequently encountered for some loci, as discussed below. Circumventing such limitations usually requires subcloning before sequencing.

Almost all genetic markers used for *P. jirovecii* genotyping were initially validated by Sanger sequencing, including the internal transcribed spacer 1 and 2 (ITS1 and ITS2, respectively) regions and the intron of the 26S subunit (26S rRNA) of the nuclear rRNA operon (308, 309), mitochondrial small- and large-subunit rRNA genes (mtSSU and mtLSU, respectively) (310, 311), and genes encoding cytochrome *b* (*cob*) (312, 313), thymidylate synthase (*ts*) (314), beta-tubulin (*β-tub*) (315), superoxide dismutase (*sod*) (106), the multifunctional product of *arom* (310, 316), dihydropteroate synthase (*dhps*) (56, 317), dihydrofolate reductase (*dhfr*) (54, 318), kexin (*kex1*) (319), and thioredoxin reductase 1 (*trr1*) (320).

Among these loci, ITS1 and ITS2 are the most polymorphic loci and have been used widely, often simultaneously, for typing *P. jirovecii* (305, 306, 309). So far, at least 60 and 62 unique genotypes at ITS1 and ITS2, respectively, have been reported in GenBank, based on worldwide studies. One drawback with these two loci is the presence of poly(T) and poly(A) tracts, which often have variable lengths within the same strains and which prevent Sanger sequencing from accurately determining the sequences downstream of these tracts (308, 309, 321). It remains unclear whether the variation in these tracts is caused by slipped-strand mispairing during *in vivo* DNA replication or represents artifacts generated during PCR and sequencing. Since this results in reporting the presence of two or more sequence populations within the same patient samples (309, 322–324), such polymorphisms are typically not used to identify different strains.

While mtLSU and mtSSU have also been used frequently for *P. jirovecii* strain typing, both appear to be less discriminatory than ITS1 and ITS2 (306, 325). Approximately 5 and 25 unique genotypes at mtLSU and mtSSU, respectively, have been reported in GenBank, based on worldwide studies. Recently, sequencing of the complete mitochondrial genomes of multiple *P. jirovecii* isolates identified a 1-kb noncoding region rich in polymorphic sites, including both tandem repeats and single nucleotide polymorphisms (SNPs) (99); targeted sequencing of this region identified at least 20 unique *P. jirovecii* genotypes in 23 clinical samples, suggesting its potential utility for typing of human isolates.

MLST. Multilocus sequence typing (MLST) involves PCR amplification followed by DNA sequencing of multiple genes (326). The sequence of each gene in an isolate is digitally assigned a distinct allele, and the combination of alleles at all genes in each isolate defines the allelic profile or sequence type (327, 328).

Almost all known genetic markers for *P. jirovecii* have been evaluated for their potential to develop an MLST system (14, 329–336). While MLST has higher discriminatory power than that of single-locus typing methods, no consensus MLST scheme is currently available. Various schemes involving different genetic loci have been reported (307, 333–338), making it difficult to compare data from different laboratories (339). In fact, the application of MLST to *P. jirovecii* has lagged behind that for many other pathogens (340), due in part to the previously limited availability of sequenced genetic loci for this pathogen. Recent reports of whole-genome sequences of *P. jirovecii* (69, 97), together with increasing application of NGS, should facilitate the development of MLST methods for this pathogen.

Since it is costly and labor-intensive to amplify and sequence individual loci from individual patients, an attempt has been made to overcome this drawback by employing either DNA pooling strategies (341, 342) or simultaneous amplification of multiple loci followed by single-base extension analysis (343, 344). These approaches have potential for high-throughput application but have not been evaluated by different laboratories.

RFLP. Restriction fragment length polymorphism analysis (RFLP), the most popular method for studying genetic variation during the 1980s and early 1990s (345), is still used frequently for typing of many organisms. This method usually involves PCR amplification of the genetic targets followed by restriction enzyme digestion, resulting in restriction fragments which are then separated by size by use of gel electrophoresis; hybridization can usually increase the sensitivity of DNA band detection but is not always needed. Similarities or differences in the band patterns reflect sequence similarities or differences. The advantages of this method include no requirement for expensive instruments, a potentially short processing time (without hybridization), and a higher sensitivity (with hybridization). The major disadvantages are its reliance on the availability of restriction sites within the targeted DNA and the interrogation of fewer polymorphic sites than those used for sequencing. Other potential disadvantages include challenges in interpretation of band patterns with minor differences and in data exchangeability between different laboratories. RFLP has been used most extensively to detect mutations in the *dhps* gene of *P. jirovecii* (346–354). Recently, RFLP was adapted to identify polymorphisms of the *P. jirovecii msg* repertoire (355–357); we refer to this method as *msg*-RFLP henceforth.

The *msg*-RFLP system targets an ~1,300-bp fragment of the *msg* gene family in *P. jirovecii* referred to as the classical *msg* genes or the *msg*-A1 subfamily, comprising ~80 genes per genome based on whole-genome sequencing (69). This target is amplified by PCR, using primers targeting highly conserved regions, followed by restriction digestion and then conventional agarose gel electrophoresis (Fig. 7) (355, 356). The main strength of *msg*-RFLP is that rather than examining a single or very limited number of nucleotide polymorphisms, as is the case with many available typing methods, it interrogates the entire *msg* repertoire of the *P. jirovecii* genome, thus permitting an exceptionally powerful discriminability. Indeed, in the initial report, no two isolates from different AIDS patients with PCP showed identical RFLP patterns (356). In contrast, sequential samples from the same patient (obtained within intervals of ≤3 months) displayed identical patterns, indicating a high stability within the same patient. Despite its high discriminability and stability, *msg*-RFLP has a major disadvantage in that it requires a minimum of ~1,000 *msg* gene copies per RFLP PCR, which can be quantified by real-time quantitative PCR (356). Samples with low *msg* copy numbers may produce weak signals or inconsistent results. This system has been used successfully to investigate outbreaks of PCP, as discussed below.

SSCP. Originally developed to detect polymorphisms of human DNA, single-strand conformation polymorphism analysis (SSCP) relies on the ability of individual nucleotide polymorphisms to change the mobility of single-stranded DNA under nondenaturing electrophoretic conditions (358). The method consists of PCR amplification followed by gel electrophoresis under nondenaturing conditions. SSCP is a relatively simple and effective method for identifying nucleotide variations within and between

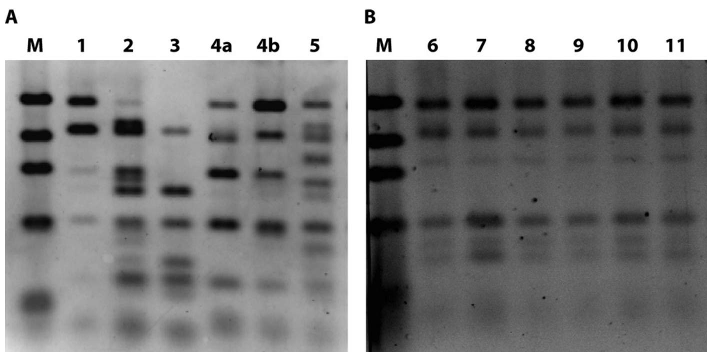

FIG 7 *msg*-RFLP analysis of *P. jirovecii*. Msg is encoded by a multicopy *msg* gene family (*msg*-A1 subfamily) in *P. jirovecii*, with an estimated 80 to 90 variable copies per genome. DNAs were extracted from respiratory samples from patients with PCP. The downstream region of the *msg* gene repertoire was amplified by PCR, using primers targeting conserved regions, followed by restriction digestion with the enzyme DraI and then electrophoresis in conventional agarose gels stained with SYBR green. Labels at the top represent the DNA marker (lane M) and individual patient samples (numbered lanes). (A) Samples from different HIV-infected patients with PCP, except for samples 4a and 4b, which were sequential samples from the same patient. (B) Samples from different renal transplant patients with PCP from an outbreak in Germany (345). Note that the RFLP patterns among samples from unrelated HIV patients are different from each other, whereas the RFLP patterns among the renal transplant patients are identical to each other.

amplicons (~100 to 500 bp), with the potential for rapid screening of large numbers of samples per day. The main disadvantage of this method is that its detection efficiency varies depending on various parameters, such as the size and base composition of the sequence, the electrophoresis temperature, and/or the gel composition. Thus, it is possible that some nucleotide changes may not be identified. The genetic targets typically utilized in SSCP typing of *P. jirovecii* include ITS1, the 26S rRNA gene, mtSSU, mtLSU, *β-tub*, and *dhps* (55, 321, 335, 338, 359–364). The main strength of SSCP for typing of *P. jirovecii* is its ability to distinguish multiple sequences in patients with coinfections, with a detection threshold of as little as 10%, which is usually not achievable by direct Sanger sequencing (361). This is important for studying the epidemiology of PCP given the high prevalence of coinfections (up to 92%) based on studies at various genetic loci (300, 307, 309, 361, 365–368).

VNTR analysis. Unlike typing methods that rely on identifying nucleotide substitutions or indels in nonrepetitive loci, variable-number tandem-repeat (VNTR) analysis relies on quantifying the repeat copy numbers of short tandem repeats, also known as microsatellites. VNTR loci generally mutate at 10 to 100,000 times higher frequencies than those of nonrepetitive sequences in the genome (369), as a result of slipped-strand mispairing during DNA replication (370).

The first VNTR locus used for typing of *P. jirovecii* is located in the intron of the UCS of the *msg* gene, which contains a 10-bp VNTR motif (247, 300). The number of repeats can be determined by PCR amplification of the VNTR-containing region followed by high-resolution denaturing gel electrophoresis (300) (Fig. 8) or fluorescence capillary electrophoresis (371). A major benefit of this method is the high sensitivity for detection of a minor population in mixed populations, which can be seen in up to 92% of patients with PCP (see Table S1 in the supplemental material).

The discriminatory ability of the VNTR assay can be improved by sequencing the amplified DNA, since the 10-bp repeat units are not identical but have at least three different sequence types (types 1, 2, and 3) (Table S2). Isolates with the same number of repeat units can have different distribution patterns of repeat types. Application of this VNTR method to different PCP patient populations worldwide has found that the number of repeat units varies from 2 to 6, with 2, 3, and 4 repeats being the most common (300, 319, 371–374). The 6-repeat allele contains only one repeat pattern, while all other alleles reported, to date, harbor two or more different repeat patterns. In addition, SNPs are present in two positions upstream and five positions downstream

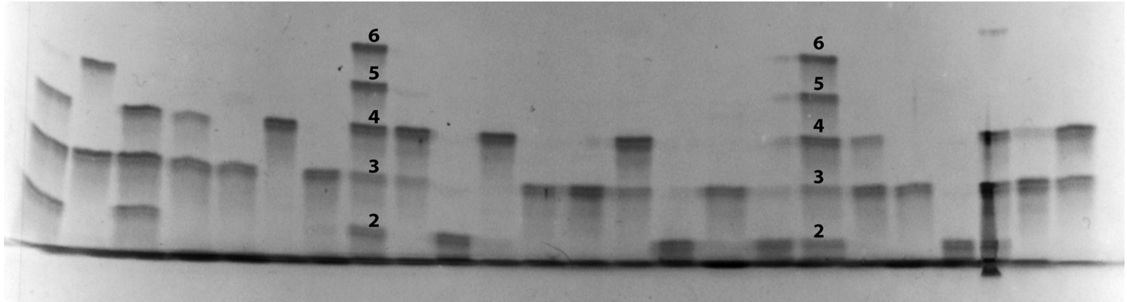

FIG 8 Quantification of the tandem repeat copy number in the *msg*-UCS of *P. jirovecii.* The tandem repeat region in the intron of the single-copy *msg*-UCS gene was amplified by PCR, separated in an acrylamide sizing gel, and stained by silver staining as described by Ma et al. (300). Numbers above each lane represent individual patients. Lanes M contain a DNA size marker, with the number of repeats indicated above each DNA band. Each band within a lane represents a unique *Pneumocystis* strain identified in that patient. For example, lane 1 was obtained from a patient infected with three strains, lane 2 from a patient infected with two strains, and lane 5 from a patient infected with a single strain.

of the repeat region (Table S2). Recently, an additional VNTR locus containing a 5-bp repeat unit was identified 228 bp downstream of the above-mentioned 10-bp VNTR locus, and it also contains three different sequence types (types a, b, and c) (Table S2) (373). Combining these two VNTR markers and their adjacent SNPs can improve the discriminatory power (319, 373). In fact, simultaneous use of VNTRs at different loci, termed multilocus VNTR analysis (MLVA), is a very popular approach in studying many organisms (375). This approach is facilitated by the availability of *P. jirovecii* genome sequences (69, 97), which has allowed the identification of a large number of tandem repeats. Two MLVA schemes, based on six or eight VNTR markers (with only one locus common to both), have been reported for samples from different patient populations (368, 376). Both schemes achieved a high discriminatory power, though additional studies with this approach are needed, including the selection of the best VNTR markers and standardization of PCR amplification protocols, repeat number quantification methods, and data reporting and transfer systems.

Contributions of NGS to molecular typing. NGS is invaluable for the discovery, validation, and evaluation of genetic markers for strain typing. The 2012 release of the first *P. jirovecii* genome assembly, for a Swiss strain (97), allowed the identification of multiple VNTR loci, leading to the development of the first two MLVA schemes, reported in 2014 and 2015 (368, 376). The 2015 release of the second *P. jirovecii* genome assembly, for an American strain, allowed, for the first time, a genome-wide comparison of these two geographically diverse strains; 24,902 SNPs were identified by such an analysis (~1 per 337 bases) (69).

While whole-genome sequencing and genome-wide SNP analysis are more informative than traditional typing methods (377, 378), their application to *P. jirovecii* is still challenging, not only because the cost of NGS is still fairly high, preventing its routine use, but also because it is difficult to obtain sufficient quantities of high-quality *P. jirovecii* DNA for NGS, due partly to the absence of a reliable culture method. Studies have reported enrichment to about 20% for *P. jirovecii* DNA by use of immunoaffinity purification followed by random whole-genome amplification (97) or oligonucleotide hybrid selection (69). However, these approaches are expensive, time-consuming, and labor-intensive; additionally, their reproducibility has not yet been established, especially for utilizing the small amounts of *Pneumocystis* typically available in clinical samples. There is a clear need to develop more cost-effective and innovative strategies for whole-genome sequencing of *P. jirovecii*.

Despite the difficulty in using NGS for *P. jirovecii* whole-genome sequencing, NGS was applied successfully in three studies for targeted sequencing of several commonly used genetic markers for *P. jirovecii*, including mtLSU, ITS2, *dhfr*, *cob*, *sod*, and *β-tub* (379–381). In these studies, three or four genetic loci from different clinical samples

were amplified by PCR, pooled after barcoding, and then subjected to NGS. This approach enabled highly efficient detection of all known and new SNPs in the targets. Together with the availability of the *P. jirovecii* whole-genome sequence, this approach could be used to evaluate additional loci for the development of novel and more efficient MLST as well as MLVA systems.

Transmission

As ubiquitous fungal pathogens distributed throughout the world, *Pneumocystis* organisms found in humans and animals are morphologically similar but genetically distinct. While many questions are still unanswered regarding their epidemiology and transmission, advances in the molecular biology of *Pneumocystis* spp. have opened a window and afforded a better view of the following three basic questions. Where is the reservoir, what is the infectious form, and how are these organisms transmitted? Understanding these questions is essential for the development of effective control and prevention measures.

Where is the reservoir of *Pneumocystis*? Initially after the recognition that *Pneumocystis* causes pneumonia in humans (382), PCP was believed to be a zoonosis, largely because of the morphological similarity and wide distribution of this pathogen across mammals (383). In addition, this belief was supported by a few studies showing cross-infection of nude or *scid* mice by intrarespiratory inoculation of *Pneumocystis* organisms from rats and humans (384–386). However, increasing reports of an inability to experimentally transmit *Pneumocystis* between various mammalian species (74, 387–391), as well as the presence of significant genetic differences among *Pneumocystis* organisms from different animal species (60, 392–395), led to the recognition that *Pneumocystis* organisms are host species specific. The organism specific for humans, *P. jirovecii*, has been found only in humans, not in any other mammalian species, including nonhuman primates (108, 111). *Pneumocystis* organisms from animals have never reliably been found in humans; rare reports of *P. carinii* or *P. wakefieldiae* DNA (identified by PCR) in patient samples (97, 308, 396–398) may represent experimental contamination (60). Taken together, the high host species specificity and the results of genome-wide sequence analyses exclude the possibility that *Pneumocystis* infection in humans represents zoonotic transmission.

After excluding animals as the reservoir, the next question is whether an environmental reservoir (outside the mammalian host) exists for *Pneumocystis*. This question has been addressed in ~20 studies by use of PCR to detect *Pneumocystis* DNA or RNA (Table S3). The vast majority of these studies were carried out on air samples. Wakefield reported the detection of both *P. jirovecii* and *P. carinii* DNAs in outdoor air in rural areas of England (399). In the same study and another six studies (400–405), indoor air was also collected from animal facilities housing *Pneumocystis*-infected rats or rabbits, and *Pneumocystis* DNA was identified by PCR in all these studies. Two of these studies compared the genotypes found in air samples and infected-animal lung samples and found identical genotypes (402, 405). One study reported the detection of *Pneumocystis* DNA in air samples from zoological parks for monkeys, with identical sequences seen between air samples and infected-monkey lung samples (108). Another 12 studies all reported the detection of *P. jirovecii* DNA in indoor air samples from various locations, including homes and hospital rooms occupied by PCP patients and hospital rooms and ward corridors without PCP patients. In seven of these studies, the genotypes of the air samples were well matched to those of patient samples from the same room (325, 406–411).

The consistent detection of *Pneumocystis* DNA in the air in many independent studies from diverse locations strongly suggests the presence of viable *Pneumocystis* organisms in the air, especially in close proximity to infected hosts. Direct proof of this hypothesis would require identification of intact, viable organisms, which is challenging and has never been reported due to the very low organism load in the air (399) and the inability to culture *Pneumocystis*. Nevertheless, Chin et al. (412) reported the detection of *Pneumocystis* organisms with a thick wall by immunofluorescence staining of mate-

rials retrieved from air blower prefilters and air filters attached to microisolator cages housing *P. carinii*-infected rats. After storage at −80°C or room temperature for 3 to 21 weeks, these prefilters were placed in cages with immunosuppressed *P. carinii*-free rats; 76% of these rats developed PCP, in contrast to only 4% of control rats kept under the same conditions except for exposure to autoclaved air prefilters (413), suggesting that the organisms remain viable and infective outside mammals. The viability of organisms is also supported by the detection of *P. jirovecii* RNA by reverse transcription-PCR for air samples from an HIV clinic (409) and from hospital rooms occupied by patients with PCP (408).

One study from the United States reported the detection of *P. jirovecii* DNA in pond water samples (414). It remains uncertain if the DNA in water represents deposition of organisms from air or infected humans, or possibly a PCR artifact. Further, a study using immunosuppressed rats suggested that water is not an environmental source of *Pneumocystis* infection (415). Note that waterborne fungal pathogens are very rare, with only two species reported so far, including the microsporidia *Encephalitozoon intestinalis* and *Encephalitozoon bieneusi*, which cause gastrointestinal tract infections through contaminated water (416, 417).

Another potential environmental source of *Pneumocystis* is the soil. Many human fungal pathogens, such as *Cryptococcus*, *Coccidioides*, *Blastomyces*, *Aspergillus*, *Histoplasma*, and *Sporothrix*, normally live in soil or soil-like environments but are able to infect humans when conditions are appropriate (418, 419). One study from Japan reported the detection of *P. jirovecii* DNA from a hospital floor swab, which showed a genotype matching the PCP outbreak strain (420) and which may represent deposition from air. There has been no report describing *Pneumocystis* in soil samples, although certain soil exposures were linked to a higher risk of PCP in humans in one study (421). Nevertheless, Hughes and colleagues found that immunosuppressed rats did not develop PCP after exposure to soil spiked with *P. carinii* cysts (422), which argues against soil as an environmental source of *Pneumocystis* infection. This is consistent with the results of NGS deep sequencing of fungal communities in >14,600 soil samples from across the world, in which ~100,000 fungal operational taxonomic units were identified, none of which matched *Pneumocystis* sequences (423, 424). While the failure to detect *Pneumocystis* sequences in these studies does not necessarily indicate its absolute absence in soil, these data suggest that, unlike the case for other fungal pathogens, soil is not a reservoir of *Pneumocystis*.

Based on recent genome analyses of human and rodent *Pneumocystis* spp., *Pneumocystis* has adapted to growth as an obligate pulmonary pathogen which depends on the lung for a stable supply of gas and nutrients as well as a stress-free environment (including stable pH, temperature, and osmatic pressure). This organism, particularly its trophic form, appears to be unable to survive in a harsh environment with low CO_2 concentrations and other potentially harmful factors. From this point of view, soil, water, and ambient air all do not favor the growth of *Pneumocystis* due to the presence of too much stress and a lack of accessible nutrients and sufficient CO_2 concentrations (i.e., ~0.25% in soil, ~0.001% in surface water, and ~0.04% in ambient air versus ~5% in the lung). Moreover, *Pneumocystis* lacks carbonic anhydrase, an enzyme critical for regulation of pH. *S. cerevisiae* strains that lack this enzyme are able to grow normally in 5% CO_2 but demonstrate severe growth restriction in ambient air; extrapolating to *Pneumocystis*, vegetative growth would be difficult outside the host. However, given the presence of a relatively rigid cell wall containing β-glucans, along with the consistent detection of DNA in the air, the cyst form is likely able to survive in the air, at least transiently or in a dormant state following exhalation from the infected host. The air serves as the medium for transmission rather than as a reservoir.

Thus, current evidence, including the high level of dependence on the host, the strict host species specificity, and the potential coevolution with its host, points strongly to the conclusion that *Pneumocystis* can propagate only within its specific mammalian host, which also serves as the natural reservoir of infection. This is strikingly different from the case for other fungal pathogens that normally reside in the envi-

ronment without a requirement for a specific mammalian host for propagation and development. The multilayered immune systems of the mammalian host play an important role in inhibiting and killing fungal invaders. However, unique among fungal pathogens, *Pneumocystis* has developed multilayered mechanisms to avoid both host innate and acquired immune defenses, allowing the organism to live and replicate within the host, as discussed above.

What is the infectious form of *Pneumocystis*? Based on the discussion above, the cyst form of *Pneumocystis* may be the only form that can survive transiently outside the mammalian host, suggesting its potential role as the infectious form responsible for transmission. While this possibility has been hypothesized since at least the 1980s (425, 426), it has only recently been supported by studies with immunosuppressed rat and mouse PCP models (132). Treatment of *Pneumocystis*-infected rats and mice with echinocandins, which inhibit β-1,3-glucan synthesis, resulted in elimination of cysts while leaving trophic forms largely unaffected. Animals receiving this treatment could not transmit *Pneumocystis* to cohoused immunosuppressed *Pneumocystis*-free animals, while untreated infected animals were able to transmit infection, supporting the hypothesis of the cyst being the infectious form. This finding was further strengthened by a study using nude rats and cell-sorted *P. carinii* cyst and trophic forms (427). Rats infected with cysts alone were able to transmit the infection to receiver rats after 12 h of cohousing, while rats infected with trophic forms alone were not.

Theoretically, the cyst can initiate infection efficiently, since each cyst contains eight intracystic bodies/spores (191, 428), which presumably are released and develop into trophic forms that attach to type I pneumocytes (429–431). Further, assuming it is exhaled as a particle from an infected lung, the size of the cyst ($\sim$5 to 8 μm) is in a range that can be deposited directly in the alveolar space following inhalation (432).

How is *Pneumocystis* transmitted? The question of how *Pneumocystis* is transmitted has been investigated extensively, beginning soon after *Pneumocystis* was recognized as the causative agent of interstitial plasma cell pneumonia that occurred in malnourished infants (382). The lungs have been shown to be the primary infection site in patients and animals, consistent with genome analysis, which suggests that *Pneumocystis* has adapted efficiently to life in the lung environment of its mammalian host and thus is unlikely to inhabit extrapulmonary sites except in extraordinarily rare circumstances, as noted above. This concept is consistent with the absence of *Pneumocystis* DNA in NGS deep sequencing of fungal amplicons or metagenomic DNAs from human gut and skin samples (433–436). The absence of *Pneumocystis* in the skin and gut indicates that infection is not transmitted by skin-to-skin contact or the fecal-oral route.

There is overwhelming evidence that infection with *Pneumocystis* is acquired by inhalation. Airborne transmission has been proven under controlled conditions with various animal models, including rats, mice, rabbits, and macaques, and has also been suggested in numerous reports of humans, as reviewed by Morris et al. (437) and Nevez et al. (438). The question that remains unanswered is whether the mode of transmission is via host-to-host spread or involves a common environmental source. Given the absence of an identified environmental reservoir, in addition to the evidence supporting mammalian hosts infected with *Pneumocystis* as the natural reservoir of infection, with cysts as the transmissive agent (as discussed above), it seems likely that this pathogen is transmitted via airborne spread of cysts from infected hosts, rather than from a common environmental source, to susceptible hosts. Infected hosts presumably discharge infectious particles containing cysts through wheezing, coughing, or sneezing, and these are subsequently inhaled by susceptible hosts. The finding that the incidence and organism burdens of *P. jirovecii* in air samples decreased as the distance from PCP patients increased (439) suggests that close person-to-person contact (e.g., in the same room) can facilitate transmission.

Various animal studies have confirmed that both immunocompetent and immunodeficient hosts can become reservoirs of *Pneumocystis* and that airborne transmission can occur between immunocompetent hosts (440, 441), between immunodeficient hosts (132, 427, 442, 443), or between immunocompetent and immunodeficient hosts

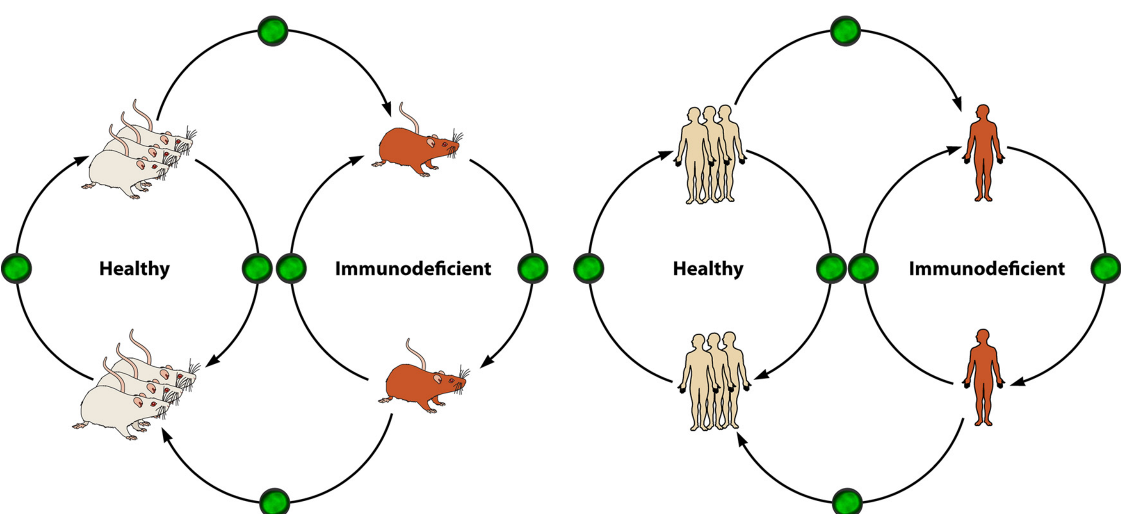

FIG 9 Hypothetical transmission mode of *Pneumocystis* in animals and humans. In this mode, cysts (indicated by green, as seen with immunofluorescence staining) serve as the infectious form and mammalian hosts (e.g., rodents on the left and humans on the right) as the reservoir for infection; transmission occurs via the airborne route between immunocompetent hosts, between immunodeficient hosts, or between immunocompetent and immunodeficient hosts. The healthy population (indicated by three individuals) is substantially larger than the immunodeficient population (indicated by one individual).

(190, 440, 444, 445), as illustrated in Fig. 9. A 12- to 24-h period of cohousing is sufficient to allow transmission in animal models (427, 440). For humans, airborne transmission has been suggested by the occurrence of clustered cases or outbreaks of PCP in different settings, including solid organ transplant units, hematology wards, pediatric oncology wards, and wards of other medical specialties (discussed below in more detail). Potential human reservoirs include people with active PCP and people with *Pneumocystis* colonization, which is defined as the presence of *Pneumocystis* organisms (usually identified by PCR-based techniques) without signs or symptoms of acute pneumonia (446, 447) and which was reviewed extensively by Morris and Norris (16).

Colonization occurs with a highly variable prevalence in both healthy and diseased individuals. The prevalence of *P. jirovecii* colonization among healthy adults in most studies varies from 0 to 20% (16). Higher prevalences of 32 to 100% have been reported for immunocompetent infants based on microscopic or PCR studies of autopsy or nasopharyngeal samples (1, 2, 448–450). Primary exposure to *Pneumocystis* likely occurs commonly at an early age, as evidenced by the increasing serum antibody titers against *Pneumocystis* found in the first few years of life (2, 451, 452). Healthy pregnant women have been found to have a colonization prevalence of 16% during the third trimester, based on PCR studies of nasopharyngeal swabs (450, 453). Health care workers (HCWs) in close contact with PCP patients with active PCP may have an increased risk of colonization (24%) compared to that for noncontact HCWs (11%) (454). This observation is consistent with the demonstration of higher *P. jirovecii* antibody titers in contact HCWs than in noncontact HCWs (455, 456). These studies suggest that HCWs may participate in transmission, though no definitive studies have addressed this directly (457).

Compared to immunocompetent individuals, immunocompromised patients generally have a higher colonization prevalence. Particularly for patients with HIV infection, colonization rates of 20 to 69% have been reported (16). Colonization is also commonly seen in patients without HIV infection but with other immunocompromised conditions, including cancers, immunosuppressive therapies, autoimmune disorders, and chronic lung diseases, especially chronic obstructive pulmonary disease (COPD). These colonized patients may be a source of transmission of *P. jirovecii* to HCWs and other susceptible individuals.

The clinical significance of colonization remains uncertain. One study suggested that

P. jirovecii colonization was associated with the pathogenesis of sudden infant death syndrome (SIDS) (458); however, this was not supported by later studies that utilized better controls (1, 459, 460). Given the high prevalence of *P. jirovecii* colonization in numerous studies of COPD patients (10 to 55%), as reviewed by Khodavaisy et al. (461), it has been hypothesized that colonization may contribute to the development of some lung diseases, particularly COPD (16, 462). In support of this hypothesis, studies with different patient populations have demonstrated that *P. jirovecii* colonization is associated with a higher risk of airway obstruction (463) and severe COPD (464) and an increased systemic inflammatory response in COPD (465, 466). In addition, studies of nonhuman primates with humanized simian-human immunodeficiency virus (SHIV) infection have suggested that *Pneumocystis* colonization may contribute to the development of COPD (467). However, whether this represents a causal association or indicates that damaged lungs provide a better environment for *Pneumocystis* colonization remains to be determined.

Understanding the reservoir of infection, mode of transmission, and susceptibility of the host has important implications for practicing and developing infection control strategies. According to the latest guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents, issued in 2017 by the U.S. Department of Health and Human Services and the Infectious Diseases Society of America (IDSA) (51), PCP infection control measures include treating patients with PCP together with prophylaxis of susceptible individuals (including HIV-infected adults and adolescents with CD4 counts of $<$200 cells/μl or CD4 percentages of $<$14%); respiratory isolation is not recommended as standard practice due to a lack of sufficient data. Nevertheless, since 2007, the Centers for Disease Control and Prevention (CDC) has recommended that a patient with PCP should not be placed in the same room with an immunodeficient patient (468). Note that the most effective approach for ending outbreaks of PCP has been widespread institution of prophylaxis among transplant recipients (14, 469, 564).

Strain Variation of *P. jirovecii*

Strain variation of *Pneumocystis* has been less well characterized than that for many other pathogens due to an inability to culture the organism. To date, no type strain (or reference strain) is available from any public microbial collection. Strain variation has been defined exclusively by molecular studies; no clear phenotypic variation has been observed within any *Pneumocystis* species. Strain variation of *Pneumocystis* was first observed in the early 1990s among *Pneumocystis* organisms from rats, based on electrophoretic karyotyping (470–472). Different karyotypes were identified among *Pneumocystis* organisms from different rat colonies. Some of these karyotypic differences were due to infections with *P. carinii* and *P. wakefieldiae*, either alone or in mixed infections (299, 473, 474). Electrophoretic karyotypes for *P. jirovecii* have also been determined (471), but application of this technique has been hampered severely by the limited amounts of organisms available from clinical samples. Therefore, determination of strain variation of *P. jirovecii* has relied largely on analysis of genetic loci after PCR amplification.

Prevalence of coinfection with multiple *P. jirovecii* strains in humans. Coinfections with two or more *P. jirovecii* genotypes in the same patients have been observed for almost all genetic loci studied. The prevalence of coinfection is highly variable (0 to 92%), depending on the genetic loci and the detection methods used (e.g., Sanger sequencing, gel electrophoresis, or NGS). It appears clear that the detection method has a more significant impact on the prevalence than the genetic locus utilized. The majority of the strain variation studies, especially those before 2000, used Sanger sequencing and revealed coinfection prevalences of up to 30% (see Table S1 in the supplemental material). In contrast, other methods, including NGS and high-resolution gel electrophoresis (for SSCP and VNTR analyses), have consistently shown high prevalences of coinfection (up to 92%).

The lower prevalence revealed by Sanger sequencing is attributed to the low

sensitivity of this method for detecting the minority sequence in samples containing mixed populations. Sanger sequencing is unable to detect a minority genotype if it is below 10 to 20% of the population (361, 475). These observations suggest that the coinfection prevalence has been underestimated in studies using Sanger sequencing. Indeed, a very recent study that analyzed 25 *P. jirovecii* samples by NGS identified a coinfection prevalence of 92% using mtLSU, 80% using ITS2, and 32% using *dhfr* as the PCR target (379). In this study, NGS was able to detect a minority genotype with a frequency as low as 0.5%; five patients (20%) showed coinfection with six distinct genotypes for mtLSU. Coinfection with six or seven genotypes was also reported in a study of ITS regions by Sanger sequencing (309), which represents the greatest heterogeneity of coinfection with *Pneumocystis* strains reported so far.

Relatively high prevalences of coinfection (23 to 70%) were also detected by VNTR analyses based on high-resolution gel (300) and capillary (367, 368, 376, 379) electrophoresis (Table S1). It is noteworthy that some VNTR loci, especially mono-, di-, and trinucleotide repeats, are highly unstable as a result of slipped-strand mispairing during *in vivo* DNA replication (370). Intrastrain instability has been observed in some di- or trinucleotide repeats of *P. jirovecii* (368). Variation detected at such loci may reflect their instability but not coinfection with different strains.

Despite the high prevalence of coinfection, there are no published studies on how coinfection occurs and what its potential consequences are. It is unknown if coinfection results from a single exposure to multiple strains simultaneously, resulting, e.g., from exposure to a host with preexisting infection with multiple strains, or from multiple exposures to different strains over time; mutation of an existing strain under selection pressures, including host immunity and drug pressure, seems unlikely to account for the substantial differences seen at multiple loci. It is also unknown how coinfections are distributed among immunocompetent and immunodeficient populations, how coinfecting strains interact with each other and with the host, and whether they affect host immune responses. Furthermore, it is unknown whether coinfection affects transmission and virulence (if it exists), alters disease dynamics, or affects treatment outcomes, as demonstrated in studies of more than 50 other human pathogens (476–478).

In addition to coinfection with multiple strains of *P. jirovecii*, there are considerable case reports of pulmonary coinfection of *P. jirovecii* with one or more other pathogens, including *Aspergillus* spp. (479–482), *Cryptococcus* spp. (483–485), *Candida* spp. (486), *Histoplasma capsulatum* (487), *Mycobacterium tuberculosis* (488–491), *Legionella pneumophila* (492–495), *Salmonella enterica* serovar Enteritidis (496), cytomegalovirus (497–506), influenza virus (507–509), herpesvirus 6 (510), *Strongyloides stercoralis* (511, 512), *Toxoplasma gondii* (513), and *Trichomonas vaginalis* (514). The majority of these cases occurred in patients with severe immunodeficiency. Most likely, these coinfections are more difficult to treat, potentially resulting in poorer outcomes. Further studies are need to elucidate how these coinfections behave in the lungs and what impacts they have on each other.

Strain variation provides insights into the pathogenesis of PCP. A long-debated question has been whether clinical PCP results from reactivation of latent infection or from acquisition of a new infection. This question has been reviewed in detail previously (16, 437, 515). Briefly, epidemiological evidence in favor of the former mechanism includes the high seroprevalence of anti-*Pneumocystis* antibodies in healthy children early in life (452, 516), the high prevalence of colonization of *Pneumocystis* detected by PCR in immunocompetent infants (1) and neonatal rats (517), and the high prevalence of PCP in children with immunodeficiency or malnutrition (4, 518, 519). Epidemiological evidence supporting the latter mechanism includes the presence of *Pneumocystis* DNA in the air (Table S3), animal-to-animal air transmission under controlled conditions, geographic clustering of PCP in HIV-infected patients (520, 521), and outbreaks of PCP in various organ transplant centers (14, 522, 523). However, it is hard to differentiate these two mechanisms based simply on these epidemiological data.

Strain variation studies performed by genotyping have significantly extended our understanding of these two mechanisms. There is increasingly convincing evidence

from genotyping studies supporting recent acquisition in at least some cases. This includes the presence of different genotypes between the first and second episodes in patients with recurrent infection (366, 524, 525); outbreaks of PCP caused by a single strain within one or more organ transplant centers, based on highly discriminative *msg*-RFLP or MLST, as discussed below (355, 357, 526, 527); and the occurrence of sulfa resistance-associated *dhps* mutations in patients without prior exposure to sulfa drugs (54, 57, 528, 529). Recent acquisition is further supported by the high prevalence of multistrain coinfections as discussed in the preceding section. These coinfections, particular those involving a mixture of three to seven strains, may represent continuous acquisition of new strains from different infected or colonized individuals over time.

Nonetheless, genotyping studies have also provided evidence in favor of reactivation of latent infection, including the presence of the same genotypes between the first and second episodes in patients with recurrent infection (366) and the high diversity of genotypes among different patients in many studies, especially the *msg*-RFLP studies (356, 530). The *msg*-RFLP system is highly discriminatory and has found remarkable variation among unrelated *P. jirovecii* isolates: no two isolates from >50 different patients shared an identical genotype profile. This implies that each patient is infected with a unique strain. This finding, together with the high level of adaptation to the lung environment and strict host specificity, suggests possible colonization and persistence in a latent state. The existence of a latent state for *Pneumocystis* is supported by its slow growth and ability to simultaneously evade both the host innate and acquired immunities as suggested by genome analysis (69). However, proving reactivation of a latent infection in humans is extremely difficult and would ideally require demonstrating genotypic identity between a strain acquired earlier in life (e.g., during primary infection as an infant) and one that is subsequently causing clinical pneumonia many years later.

Thus, while there is strong evidence to support recent acquisition in some cases, reactivation in other cases cannot currently be excluded. It is possible that both mechanisms can occur. A more detailed understanding of these mechanisms may facilitate the rational design of approaches for disease management and control. If reactivation of latent infection is the primary mechanism, there is no need for respiratory isolation of patients with PCP other than protection of susceptible individuals by prophylaxis. If *de novo* infection is the primary mechanism, preventing infected patients from transmitting *Pneumocystis* to others, particularly to immunocompromised patients, would have a more important role.

Strain variation and PCP outbreaks in organ transplant patients. As an infectious agent, *Pneumocystis* appears to be generally less pathogenic or virulent than many other human pathogens. However, outbreaks of PCP have been reported under various conditions across the world. The epidemic of interstitial pneumonia in European premature infants and malnourished children in the mid-20th century led to the initial recognition of *Pneumocystis* as a human pathogen (3, 4). Multiple outbreaks of PCP among apparently healthy individuals in the early 1980s (5, 531–533) not only heralded the onset of the HIV/AIDS epidemic but also led to the recognition of PCP as the leading cause of morbidity and mortality in HIV/AIDS. Although the incidence of PCP in HIV-infected patients dramatically declined with the introduction of cART and prophylaxis, its incidence among non-HIV-infected immunocompromised patients has been increasing. Over the last 2 decades, outbreaks of PCP have been reported frequently, as recently reviewed by de Boer et al. (14) and Yiannakis and Boswell (15). These two reviews summarize 30 nosocomial outbreaks of PCP between 1982 and 2013, involving 486 patients across 12 countries, predominantly in Europe. One of the most striking features of these outbreaks is that the majority (83%) of them occurred in patients undergoing solid organ transplantation, particularly renal transplantation. A minor portion of outbreaks occurred in pediatric oncology (6%), hematological malignancy (6%), and rheumatoid arthritis (3%) patients. In all these outbreaks, the affected patients received no or suboptimal prophylaxis for PCP.

These outbreaks have attracted intense global efforts to understand their causes,

underlying mechanisms, and potential intervention strategies. Early epidemiological investigations implicated certain immunosuppressive regimens as a risk factor for PCP development in outbreaks (534, 535), but this observation was not supported in other studies (536). In the majority of epidemiological studies, contact tracing analysis found colocalization of cases within clinic areas, suggesting the possibility of person-to-person or nosocomial spread of infection. Molecular typing studies, primarily for outbreaks in renal transplant patients occurring after 2000, provided strong evidence in support of this (15). All these typing studies, which primarily utilized MLST, identified cases with an identical genotype profile in more than one patient. More strikingly, a predominant strain, often even a single strain, of *P. jirovecii* was identified in 13 (81%) of the 16 outbreaks. Furthermore, three distant outbreaks, in Switzerland and Germany, were linked to a single *P. jirovecii* strain (355, 537). These findings strongly support the recent acquisition of infection through person-to-person transmission and also highlight that outbreaks of PCP can be understood better by an improved understanding of organism strain variation as well as patterns of transmission.

However, the origins of these outbreaks have not been defined. It remains unknown why such outbreaks prevail in renal transplant recipients and whether they are due to the introduction of specific *P. jirovecii* strains (e.g., with enhanced virulence) into the transplant environment or to specific conditions that may increase patients' susceptibility to infection (such as certain immunosuppressive regimens or rejection treatment protocols). The experience from these outbreaks illustrates the practical utility of strain variation in elucidating the epidemiology of PCP, underlines the necessity of utilizing prophylaxis in renal transplant recipients, and supports the recommendation to avoid placement of a patient with PCP in the same room as that of an immunocompromised patient per the current CDC guidelines (468).

Strain variation and drug resistance. The widespread use of TMP-SMX over the last 3 decades for both the treatment and prevention of PCP has raised concern about the development of drug resistance by *P. jirovecii*. The lack of a culture system has precluded the use of routine *in vitro* susceptibility testing to determine drug resistance in *Pneumocystis*. To overcome this problem, researchers have attempted to address this concern indirectly by determining genetic variations in the *P. jirovecii dhfr* and *dhps* genes, the targets of TMP and sulfa (including SMX), respectively, in clinical strains and then correlating strain variations with clinical characteristics of the PCP patients and with homologous mutations known to be associated with drug resistance in other organisms.

Genetic variation in the *P. jirovecii dhps* gene was first described in 1997 for six patient isolates; nonsynonymous nucleotide mutations involving amino acid changes were identified at six codons, suggesting a positive selective pressure, possibly as a result of sulfa exposure. Subsequently, two of these mutations (at amino acids 55 and 57) were identified in numerous studies of PCP patients throughout the world, with prevalences of 3 to 81% (reviewed in references 53, 538, and 539). The majority of these studies demonstrated that both mutations are associated with prior exposure to and failure of TMP-SMX and dapsone, primarily when administered as prophylaxis. Both mutations are at the active sites of the Dhps enzyme, based on homology to the crystal structure of Dhps in *Escherichia coli* (540). Mutations at or very near these positions are likely to alter the local structure and thus affect the binding of the substrate and sulfa. Mutations at these positions confer resistance to sulfa drugs in other organisms, including *E. coli* (541), *Streptococcus pneumoniae* (542), *Neisseria meningitidis* (543), and *Plasmodium falciparum* (544). All these observations strongly suggest that *P. jirovecii* has developed some level of resistance to sulfa drugs.

The clinical significance of *dhps* mutations in relation to sulfa resistance remains unclear. Several studies showed associations of *dhps* mutations with poor outcomes for HIV-infected patients with PCP, including increased mortality (545) and treatment failure (58, 546), but these associations were not supported in other studies (547, 548).

Genetic variations in the *P. jirovecii dhfr* gene have been less well studied. In about a dozen studies of clinical isolates worldwide (54, 528, 549–551), no *dhfr* mutations

were detected, other than rare synonymous nucleotide substitutions. In contrast, extensive mutations at more than 30 amino acid positions were reported in another half dozen studies, from Japan (318, 552), Portugal (320, 553), South Africa (554), Switzerland, and France (555). Each of these mutations occurred at a low frequency (≤1%); a small portion of isolates (2%) contained mutations at two or more positions simultaneously (555, 556). The greatest number of *dhfr* mutations was reported by Nahimana et al. (555), who identified 16 mutations in 11 (33%) of 33 clinical isolates. In the same study, patients with failure of prophylaxis, including that with a Dhfr inhibitor, were found to be more likely to harbor at least one *dhfr* mutation than those without such prophylaxis, though no single mutation or pattern of mutations was associated with failure. Notably, mutations were seen primarily in patients receiving pyrimethamine rather than trimethoprim as part of their prophylactic regimens; this drug is used infrequently as prophylaxis in other centers. By *in vitro* testing of recombinant *P. jirovecii* Dhfr enzymes containing known mutations, six Dhfr variants were found to be resistant to TMP, with K_i values that were 4- to 100-fold higher than those for the native enzyme (52), suggesting that these mutations may contribute to clinical resistance.

The consistently lower prevalence of mutations in *dhfr* than in *dhps* in the vast majority of reported studies suggests less selective pressure on *dhfr* than on *dhps* and further suggests that the *dhps* mutations are not random occurrences but rather the result of drug pressure. In agreement with the hypothesis of low pressure on *dhfr*, *in vitro* inhibition assays using recombinant enzymes (557) and yeast complementation (558) demonstrated that TMP as well as, to a lesser extent, pyrimethamine is a relatively poor inhibitor of wild-type *P. jirovecii* Dhfr. These findings support the concept that TMP contributes little to the effectiveness of TMP-SMX against *P. jirovecii*, which may in fact function as sulfa monotherapy (26, 54).

In addition to the emergence of sulfa resistance, there are a few studies suggesting that *P. jirovecii* may also be developing resistance to atovaquone, an alternative regimen for treating and preventing PCP (559). Atovaquone targets the mitochondrial gene cytochrome *b* (*cob*). Mutations in *cob* associated with atovaquone resistance have been well documented for malaria (560). Sequence analysis of the *P. jirovecii cob* genes from >70 clinical isolates from the United States and the United Kingdom identified seven different mutations, with only two present in more than one isolate (313, 561). These mutations were significantly more frequent in patients who received atovaquone, implying a positive selection pressure (561). When these seven mutations were introduced into the *S. cerevisiae cob* gene, five resulted in increases of the atovaquone 50% inhibitory concentration (IC_{50}), to >150 nM, compared to 25 nM for the wild type (562). In addition, structural modeling analysis suggests that these mutations directly interfere with atovaquone binding (562). These data support the hypothesis that *cob* mutations are involved in the development of atovaquone resistance in *P. jirovecii*. However, no reports, to date, have demonstrated an association of these mutations with treatment outcomes.

Based on currently available data, genotypic resistance testing remains an investigational tool to be utilized in a research setting, and clinical decisions should not be made based on such testing; prophylaxis should continue to be administered to susceptible individuals. Most patients with retrospectively identified *dhps* mutations responded appropriately to treatment with standard doses of TMP-SMX. *dhps* mutations thus appear to represent a low-level resistance that can be overcome by higher doses of TMP-SMX. Given that *Pneumocystis* has already shown that it can develop mutations in response to drug pressure, a greater concern is that subsequent additional mutations may result in high-level resistance, potentially leading to the loss of the most effective drug for managing *Pneumocystis* infection.

Note that mutations in all three drug targets described above (*dhps*, *dhfr*, and *cob*) were also detected in PCP patients who had no known prior exposure to the implicated drugs. The presence of mutations in these patients may suggest direct acquisition of mutant *P. jirovecii* strains via person-to-person transmission. Therefore, these muta-

tions, together with synonymous nucleotide changes in these genes, may be useful as markers in epidemiological studies (320, 336, 339, 563).

CONCLUDING REMARKS

Pneumocystis is truly a unique organism with unusual lifestyle and biological traits, which makes it a fascinating and at the same time difficult organism to study. Thanks to advances in science and technology and the continuing commitment of the *Pneumocystis* research community, a new window has been opened into the biology and epidemiology of *Pneumocystis*. Much of the mystery surrounding this somewhat elusive pathogen has begun to unravel, especially its refined taxonomic classification as a member of the Taphrinomycotina, its extremely reduced genome and concomitant inability to metabolize and grow independently of a mammalian host, its reliance on cysts as the infectious form and on mammalian hosts as the infection reservoir for airborne transmission, its widespread colonization in both immunocompetent and immunodeficient hosts, and its strain variation related to drug resistance, pathogenesis, and outbreaks of infection among transplant patients. These advances have greatly improved our understanding of *Pneumocystis* as a prominent pathogen. However, significant questions remain unanswered about many aspects of *Pneumocystis* biology and epidemiology. The most challenging issue is the lack of a continuous *in vitro* culture method, which has increasingly been recognized as the major bottleneck of *Pneumocystis* research. In order to resolve this issue, it may be necessary to leverage new and innovative strategies, which may include collaboration across different research teams. A reliable culture system is urgently needed to provide a better understanding of the *Pneumocystis* life cycle, host specificity, strain variation, and mechanisms of antigenic variation and development of drug resistance. This will allow the development of improved methods for identifying potential new therapeutic targets and screening of new drugs and will potentially allow the development of methods to genetically manipulate the organism. Nonetheless, the application of modern molecular biologic techniques has provided a strong foundation upon which to build the next major advances in understanding the biology and epidemiology of this family of atypical fungi.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CMR.00009-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare that we have no competing interests.

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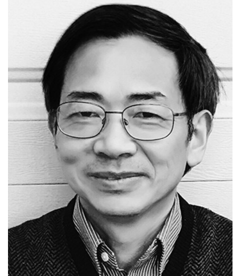

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Joseph A. Kovacs received his medical degree from Cornell University Medical College. Following his residency in internal medicine at The New York Hospital/Cornell, he completed a fellowship in infectious diseases and critical care at the NIH, Bethesda, MD. He remained at the NIH and is currently a Senior Investigator and Section Chief in the Critical Care Medicine Department, NIH Clinical Center, and an attending physician in the NIAID-CCMD HIV/AIDS program. He is also an Associate Clinical Professor of Medicine, The George Washington University School of Medicine, and an attending physician at the Medstar Washington Hospital Center. His research interests include understanding the basic biology of and host immune responses to *Pneumocystis* infection and developing improved methods for diagnosis, treatment, and prevention of *Pneumocystis* pneumonia. His clinical research focuses on better understanding the infectious and noninfectious complications of HIV infection and evaluating novel immune-based therapies for HIV infection.