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# *Pneumocystis carinii*: What Is It, Exactly?

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## INTRODUCTION

The question posed in the title of this review is 86 years old, yet we still do not know what *Pneumocystis carinii* is, exactly. For the first 80 years after its discovery, clues to the identity of this microbe were very difficult to come by, principally because it fails to grow in a sustained manner in culture. Consequently, prior to 1988, it was not clear if *P. carinii* was a protozoan or a fungus. The last several years have resolved this question. *P. carinii* is definitely a fungus. Because the evidence supporting the placement of *P. carinii* in the kingdom Fungi has been reviewed previously (89), this review will provide only a brief synopsis of this topic and will focus instead on three additional questions: (i) What kind of fungus is *P. carinii*? (ii) What is the spectrum of variation among organisms known as *P. carinii*? (iii) Why is understanding this variation important?

## BRIEF HISTORY OF *P. CARINII*

Chagas described *P. carinii* in 1909 but thought it was a trypanosome (11). Three years later, Delanoe and Delanoe reported that the organism in question was distinct from trypanosomes and chose the genus name *Pneumocystis*, which was descriptive of the small but highly refractive and densely staining spherical cyst form of the microbe (19). Over the years, similar cysts were found associated with pneumonia in a large variety of mammalian species (reviewed in reference 14). Because all of these organisms looked the same, caused the same disease, and did not grow in culture, they were all referred to as *P. carinii*. *P. carinii* was studied little until the 1970s, when it became a frequent cause of pneumonia in immunosuppressed patients such as recipients of transplanted organs. In the search for anti-*P. carinii* drugs, it was established that *P. carinii* pneumonia did not respond to the broad-spectrum antifungal drug amphotericin B but that it could be successfully treated with drugs that were active against protozoa. This finding contributed to the idea that *P. carinii* must be a protozoan. A protozoan identity also seemed more consistent with the failure of *P. carinii* to grow continuously in culture. Although not universally accepted (103), this taxonomic assignment remained preeminent well into the era of AIDS, whose advent engendered new interest in *P. carinii*, which was causing the most common AIDS-defining illness in patients in North America.

## *P. CARINII* IS A FUNGUS

In 1988, the sequence of the *P. carinii* gene encoding the RNA in the small ribosomal subunit (16S-like rRNA gene) was determined. Surprisingly, the *P. carinii* 16S-like rRNA sequence was much more similar to fungal sequences than to any of the dozen available protozoan sequences (23, 24, 93, 94, 113). These data were greeted with great interest, but many investigators expressed reservations about concluding that *P. carinii* is a fungus on the basis of the sequence of one gene from such a limited number of organisms. These concerns were essentially eliminated by data published as early as 1992 (10, 22, 26, 55, 59, 80, 99, 120). There is now overwhelming phylogenetic evidence supporting the classification of *P. carinii* as a fungus.

An evolutionary tree that compared rat-derived *P. carinii* 16S-like rRNA sequence with 38 other fungal rRNA sequences placed *P. carinii* on its own branch, between the ascomycetes and the basidiomycetes but clearly within the fungal kingdom (99). A second independent analysis of 16S-like rRNA sequences came to the same conclusion (10). Analysis of other genes has supported the inference derived from rRNA data. Several protein-encoding genes have been isolated from rat-derived *P. carinii*, the most interesting for this discussion being the gene encoding elongation factor 3, a protein that has been found only in fungi so far (120). The *P. carinii* EF3 protein is 57% identical in sequence to elongation factor 3 from *Saccharomyces cerevisiae* and shares other structural features with elongation factor 3 proteins from *S. cerevisiae* and *Candida albicans*. Other cloned *P. carinii* genes that have been useful in the study of evolutionary relationships of *P. carinii* include those encoding dihydrofolate reductase (DHFR) (22), thymidylate synthase (TS) (26), β-tubulin (20, 21), α-tubulin (122), the TATA-binding protein (70, 95), and a P-type cation-trans-

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locating ATPase (70, 71); several genes carried in mitochondrial DNA (80); the actin gene (28); and arom, which is the gene encoding the multifunctional enzyme that makes aromatic amino acids (7). In every case, *P. carinii* genes and the proteins they encode are most similar to their counterparts from fungi. Phylogenetic trees have been derived from data on DHFR and TS (25, 45), β-tubulin (4, 54), and actin (28), and all of these trees place *P. carinii* among the fungi. Thus, the cumulative molecular genetic data firmly establish *P. carinii* as a member of the fungal lineage of eukaryotes.

Although it was not until recently that gene sequences furnished the definitive characters needed to establish the correct phylogenetic kingdom for *P. carinii*, it should be noted that it had long been known that some morphological features of these organisms resembled those of ascomycetous fungi (103). The structural and ultrastructural features of *P. carinii* have been reviewed recently by Ruffolo (82). Notable fungus-like features include the resemblance of the cyst form of *P. carinii* to fungal spore cases known as asci, the presence of lamellar cristae in the mitochondria, and the absence of highly visible organelles.

The reclassification of *P. carinii* has already had practical applications. Soon after the rRNA data were presented, investigators began testing new classes of antifungal agents for activity against *P. carinii*. It was quickly established that drugs that inhibit β-glucan synthetase, an enzyme involved in the synthesis of a component of the cell wall, are active against *P. carinii* in animal models (81, 84).

## *P. CARINII* IS AN ATYPICAL FUNGUS

Compared with other fungal pathogens of humans and fungal model organisms, *P. carinii* exhibits a number of anomalous properties. Its refusal to grow continuously in vitro and its resistance to classical antifungal agents have already been noted. The reason for its failure to grow well in culture is still a mystery, but the basis for its resistance to amphotericin B is now known to be the absence of the fungal sterol, ergosterol, in the cell membrane. Instead of ergosterol, *P. carinii* contains abundant cholesterol (43). *P. carinii* also contains small amounts of sterols previously known to be made by rust and smut fungi (43). These sterols are presumably made by *P. carinii*, since they are not detectable in uninfected hosts.

Another anomalous feature of *P. carinii* is the fragility of the cell wall in the trophic form of the organism. *P. carinii* trophic forms are relatively small and nonrefractive compared with *P. carinii* cysts but are the predominant morphological form of the organism found in the lungs, where they generally outnumber cysts 10:1. Trophic forms are thought to be similar to vegetative yeasts and are presumed to be capable of generating cysts (or spore cases), perhaps by fusion of two haploid cells followed by meiotic divisions within the cyst wall. Data supporting this scenario are scant, but electron micrographs that show a structure that looks like a synaptonemal complex in a cyst have been published (66). In addition, haploidy for trophic forms is suggested by the rough agreement between the DNA content per nucleus, as measured by fluorescence microscopy, and the genome size of 7 megabases, which was calculated from the sizes of chromosomes resolved by gel electrophoresis (electrophoretic karyotyping) (41, 63, 117). Haploidy is also suggested by the observation that each of the 15 or so chromosomes of a given isolate of *P. carinii* migrates as a single band, indicating either that there is a single copy of each chromosome or that both homologs of a chromosome pair are always the same length. Haploidy seems more likely because comparison of electrophoretic karyotypes of different isolates of rat *P. carinii* have shown that many of the chromosomes in this organism can vary in length (16). Hence, if *P. carinii* were diploid, both homologs must change in concert. A mechanism for keeping homologs the same length is not obvious.

Instead of a rigid, tough cell wall, trophic forms of *P. carinii* have a flexible, fragile external layer containing β-glucan (65, 115) and glycoprotein (15, 34, 56, 64, 76, 118, 119). The β-glucan appears to be necessary for viability since inhibitors of β-glucan synthetase can cure *P. carinii* pneumonia in animals (81, 84). Other studies have reported the presence of material reactive with antibodies against chitin (32, 110). Whatever its composition, the surface layer of trophic forms is insufficient to protect the cells from lysis under conditions that do not affect the integrity of conventional yeasts (41). In addition, electron micrographs have shown that the surface of trophic forms can be very convoluted (see reference 82 for a review), and it appears that tubular projections from a trophic form can interdigitate with similar structures from the host cell membrane.

A third anomalous feature of *P. carinii* compared with other fungi is that *P. carinii* contains no more than two copies of the gene encoding 16S-like rRNA whereas most fungi contain hundreds of copies of this gene (37).

Given the phenotypic and genetic peculiarities of *P. carinii* vis-à-vis other fungi, perhaps it is not surprising that comparison of gene sequences has not identified a close relative of *P. carinii*. As mentioned above, phylogenetic trees based on 16S-like rRNA gene sequences placed *P. carinii* on a unique branch between the ascomycetes and basidiomycetes (10, 99). Phylogenetic trees constructed on the basis of sequences of several other genes (the DHFR, TS, β-tubulin, and actin genes) also place *P. carinii* near the ascomycetes (4, 25, 28, 45, 54). Eriksson has proposed placing *P. carinii* in a new taxonomic family, *Pneumocystidaceae*, and a new order, *Pneumocystidales*, in the class *Ascomycota* (27). While the available data do not conclusively show that *P. carinii* is a member of the ascomycetes, there is little reason to believe that it belongs in any of the other classes of the kingdom Fungi. Early work on 5S gene sequences led to the suggestion that *P. carinii* might be a zygomycete (113), but none of the other DNA sequence comparisons have indicated a relationship between *P. carinii* and zygomycetes. The possibility that *P. carinii* is a basidiomycete cannot be excluded, but no compelling evidence for this is available.

## THERE ARE MANY VARIETIES OF *PNEUMOCYSTIS* ORGANISMS

There are many different kinds of *Pneumocystis* organisms, some of which are sufficiently different to suggest that they are separate species.

### Variation among *Pneumocystis* Organisms Derived from Different Host Species

*P. carinii* isolates from different host species differ both phenotypically and genetically. Phenotypic characteristics that vary among *P. carinii* organisms include (i) host species specificity, (ii) antigens, (iii) electrophoretic mobility of enzymes, and (iv) morphology.

It is generally believed that *P. carinii* organisms are host specific, because several well-controlled experiments have shown that introduction of organisms isolated from one host species into another host species resulted in no discernible growth (1, 35, 86). Reports of successful transfer between host species must be regarded as inconclusive because it was not proved that the organisms present in the inoculated animals

were the same as the organisms used in the inoculum (85). While there is no experimental evidence for transmission of *P. carinii* between different species of laboratory animals, it has been suggested that humans can carry *P. carinii* normally found in rats (53, 62). This suggestion is based on the results of PCR experiments by one laboratory in which samples from some *P. carinii* pneumonia patients produced a PCR product with a sequence characteristic of rat *P. carinii*. The first report of this phenomenon involved experiments in which the PCR was used to amplify a segment of the mitochondrial gene encoding rRNA (mtrRNA gene). The 300-bp PCR product from 1 of 12 patients examined (sample pt12) began with a sequence that matched a sequence from rat *P. carinii*, switched to a sequence from human *P. carinii*, then switched back to rat, and then switched back to human (53). The same laboratory later reported that sample pt12 yielded two different DNA molecules after PCR amplification of the two internal transcribed spacers (ITS) of the nuclear gene encoding ribosomal RNA (62). One PCR product matched the sequence of human *P. carinii*, but the other matched the sequence of rat *P. carinii*. This could have been due to the presence of both rat and human *P. carinii*. However, since neither the rat nor the human *P. carinii* mtrRNA sequence was detected in sample pt12, it would seem that this sample did not contain either of these organisms. It has been suggested that sample pt12 contained an organism that was produced by mating of rat and human *P. carinii* and recombination between mitochondrial genomes (53). If this is the case, the presence of three recombinant junctions within the 300-bp region analyzed is remarkable. A third paper from this group reported that four of seven Italian patients yielded *P. carinii* samples that produced ITS PCR products from both rat and human *P. carinii*. A fifth patient in this group of seven produced a single PCR product whose sequence contained tracts identical to rat *P. carinii* and tracts identical to human *P. carinii* (61).

The basis of these observations is unclear. One possibility is that humans can be infected by rat *P. carinii* and that the patchwork sequences are the result of sexual recombination between human and rat *P. carinii*. However, at this point, an alternative explanation of the data has not been excluded. It is possible that some patient samples were contaminated with rat *P. carinii* or DNA from rat *P. carinii*. While the work in question employed the standard measures to minimize the chance of contamination, this possibility is an ever-present concern in PCR experiments. Questions about the source of the rat *P. carinii* PCR products in human-derived samples are also evoked by other aspects of the PCR data. It is difficult to understand why humans should be susceptible to rat *P. carinii* and not to *P. carinii* from other host species. While it is possible that humans are susceptible to infection by rat *P. carinii* but not by *P. carinii* found in other animals, it is at least as plausible to suppose that the prevalence of DNA from rat *P. carinii* in human samples reflects the prevalence of rat *P. carinii* and PCR products from rat *P. carinii* in the laboratory. Contamination can also explain the patchwork sequences seen in samples from two patients because the PCR is able to generate complex recombinant molecules from a mixture of related template DNAs (74).

While the above considerations are not easily dismissed, they do not prove that humans were not infected by rat *P. carinii*, and clarification of the source of the rat *P. carinii* DNA in some human samples will require additional data. The possibility of contamination by PCR products can be excluded by showing that additional loci from rat *P. carinii* are also present. This has been shown for two loci (mtrRNA and ITS) in sample pt12, but additional data would be reassuring. In addition, it is vital to show that the same recombinant molecule is produced each time a sample like pt12 is amplified, because this is extremely unlikely to occur if the patchwork sequence were generated by the PCR. Finally, all doubt would be eliminated by visualizing rat *P. carinii* organisms in human samples. This should be possible, because rat *P. carinii* DNA constituted a substantial fraction of the PCR products obtained from the human-derived samples in question (61). This implies that a substantial fraction of the organisms in these samples were rat *P. carinii*. Thus, it should be possible to detect these organisms by in situ hybridization. Alternatively, antibodies that discriminate between *P. carinii* from rats and humans could be used to determine if organisms containing rat *P. carinii* epitopes are present (33, 48, 57).

The second way in which *P. carinii* organisms isolated from different host species differ is with respect to antigens. Antigenic variation among *P. carinii* from different host species has been established by two lines of evidence. First, many antibodies elicited against *P. carinii* from one host species do not cross-react with preparations of *P. carinii* from other host species (9, 36, 38, 48, 49, 57, 112). Second, the antibodies that do cross-react with antigens from *P. carinii* from more than one host species typically recognize different molecules in different *P. carinii* organisms, as assessed by immunoblotting of denatured proteins separated by gel electrophoresis (36, 38, 49, 56).

A third form of phenotypic variation among *P. carinii* isolates from different host species has been brought to light by electrophoretic separation of enzymes in nondenaturing gels followed by visualization of the enzyme activity in situ. *P. carinii* isolates from rats, rabbits, and mice produced different band patterns when subjected to this assay (69).

The fourth kind of phenotypic variation that has been reported is a subtle morphological difference between mouse and rabbit *P. carinii* (18).

As might have been expected from the phenotypic differences described above, *P. carinii* isolates from different hosts are genetically distinct. Such genetic differences were first observed by analysis of chromosomes by gel electrophoresis, which showed that the electrophoretic karyotypes of *P. carinii* from rats and humans were similar but distinct (41, 121). Karyotypes from both human-derived and rat-derived *P. carinii* contained about 15 bands, ranging in size from 200 to 1,000 kb, but the band patterns of the two kinds of *P. carinii* were different. Later studies showed that *P. carinii* organisms from ferrets and mice also each have their own electrophoretic karyotypes and that the chromosomes from ferret and mouse *P. carinii* did not hybridize to any of three genes (DHFR, TS, and β-tubulin genes) from rat *P. carinii* (114). A similar result was obtained with a gene encoding an isoform of the major surface glycoprotein (MSG). MSG genes are repeated about 100 times per *P. carinii* genome and are distributed on all 15 chromosomes resolved by gel electrophoresis (40, 41, 50, 91, 92, 97, 104). An MSG gene from rat *P. carinii* hybridized to all of the bands in an electrophoretic karyotype from rat *P. carinii* but did not hybridize to an electrophoretic karyotype from human *P. carinii* (90, 91).

In keeping with the results of DNA hybridization experiments, gene sequencing has shown that *P. carinii* isolates from different hosts differ at numerous loci (6, 7, 17, 18, 20, 21, 23, 26, 31, 40, 45, 50, 71, 77, 79, 90, 91, 95, 97, 104, 108, 109, 116, 122). Table 1 lists the genes that have been sequenced from more than one kind of *P. carinii*. The broadest database of *P. carinii* gene sequence is for a 300-bp segment of the mtrRNA, which has been studied in organisms from nine host species (rat, mouse, shrew, rabbit, ferret, pig, horse, monkey, and human). The sequence variation at this locus ranges between 4

TABLE 1. Gene sequences available from at least two different special forms of *P. carinii*

| Gene[a] | Type of *P. carinii*: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Rat[b] | Rat[c] | Mouse | Human | Ferret | Horse | Pig | Rabbit | Shrew | Monkey |
| mtrRNA | 87[d] | 45 | 79 | 87 | 79 | 79 | 45 | 79 | 75 | 30 |
| nucrRNA | 23 | 17 | | 58 | | | | | | |
| ITS | 73 | 73 | | 73 | | | | | | |
| TS | 26 | 45 | 68 | 68 | 45 | | | 68 | | |
| β-Tubulin | 21 | | | 21 | | | | | | |
| α-Tubulin | 122 | | | 90 | | | | | | |
| ATPase | 71 | 71 | | | | | | | | |
| TBP | 95 | 95 | | | | | | | | |
| gpA/MSG | 50 | | 116 | 31 | 40 | | | | | |
| arom | 7 | | 5 | 5 | 5 | | | 5 | | |

[a] mtrRNA, 23S rRNA from mitochondrial genome; nucrRNA, 16S-like ribosomal RNA from nuclear genome; ITS, internal transcribed spacer from nuclear rRNA gene locus; TS, thymidylate synthase; ATPase, proton pump; TBP, TATA-binding protein; gpA/MSG, glycoprotein genes; arom, gene for a single polypeptide that catalyzes five consecutive steps in prechorismate aromatic amino acid biosynthesis.
[b] *P. carinii* f. sp. *carinii*.
[c] *P. carinii* f. sp. *ratti*.
[d] Numbers indicate references. Spaces indicate sequences that have not yet been determined.

and 27%. The most extensively studied pair of *P. carinii* organisms are those from humans and rats, in which eight different loci have been compared at the sequence level. All of these loci differ between the two organisms, with variation approaching 50% at the ITS region of the nuclear gene encoding rRNAs. The possible significance of these differences in terms of species identification will be discussed after consideration of genetic variation of *P. carinii* found in the same host species.

### Variation among *Pneumocystis* Organisms Found in the Same Host Species

Genetic differences among *P. carinii* organisms isolated from different rats were first observed as differences in the migration of chromosomes resolved by gel electrophoresis. These electrophoretic karyotype differences were of two types. In the more common type of variation, the number of bands remained the same but some of the bands migrated differently in one sample than in another (16, 17, 41). This is now understood to reflect variation within what was originally termed the prototype rat *P. carinii*, which was the organism most commonly found in laboratory rats (16, 17). Prototype *P. carinii* has also been termed Pc1 (59) but has now been renamed *P. carinii* special form *carinii* (*P. carinii* f. sp. *carinii*) (see below). The karyotypic variation within *P. carinii* f. sp. *carinii* is probably due to the existence of multiple strains of this organism. In the second type of karyotype variation, some samples had many more bands than others (41). This turned out to be due to the presence of *P. carinii* f. sp. *carinii* and an additional kind of *P. carinii*, which was originally called variant (17) or Pc2 (59) but is now known as *P. carinii* f. sp. *ratti*.

The genetic differences between *P. carinii* f. sp. *carinii* and *P. carinii* f. sp. *ratti* are substantial. Gene sequencing showed that the 16S-like rRNA genes of *P. carinii* f. sp. *carinii* and *P. carinii* f. sp. *ratti* were 6.6% divergent in the 121-bp region analyzed (17). Comparison of complete 16S-like sequences showed them to be nearly 2% divergent (73). Variation was also found in genes encoding the 26S rRNA (59), other parts of the locus encoding rRNAs (73), and genes encoding TATA-binding factor (95), an ATPase (71), thymidylate synthase (45), and mitochondrial rRNA (45). Hybridization experiments also indicated that *P. carinii* f. sp. *carinii* and *P. carinii* f. sp. *ratti* are more divergent than are strains of *P. carinii* f. sp. *carinii*. This was shown by using an MSG gene from *P. carinii* f. sp. *carinii* as a hybridization probe. This gene probe hybridized to every chromosome from four strains of *P. carinii* f. sp. *carinii* but did not hybridize to *P. carinii* f. sp. *ratti* chromosomes under conditions that would preclude the detection of hybrids that were more than 15% mismatched (16, 17, 41). Cross-hybridization was observed under conditions that permitted annealing of DNA strands less than 85% identical in sequence (91). Preliminary data have suggested that ferrets also harbor two types of *P. carinii* that may be as divergent as *P. carinii* f. sp. *ratti* and *P. carinii* f. sp. *carinii* (6).

In addition to the possible species level divergence seen between *P. carinii* f. sp. *carinii* and *P. carinii* f. sp. *ratti*, more modest gene sequence variation occurs among *P. carinii* isolates from a single host species. Most of the data pertaining to this more modest genetic variation are from patients and are discussed further below.

## ARE THERE MULTIPLE SPECIES OF *PNEUMOCYSTIS*?

While characterization of chromosome sizes and gene sequences has demonstrated widespread genetic variation within the genus *Pneumocystis*, the significance of these differences in terms of species identification is difficult to assess. A species is a group of phenotypically similar organisms, living in a certain way, which do not acquire genes from organisms outside the group. Each kind of *P. carinii* found in a particular mammalian host clearly exhibits the first two features required of a species, but whether genetic exchange occurs between the various kinds of *P. carinii* remains an open question. Direct experimentation on the sexual capabilities of *P. carinii* organisms is precluded by the lack of a culture system adequate for such investigations, so the arguments on this point rest on circumstantial evidence. With the exception of the reports described above regarding *P. carinii* f. sp. *carinii* PCR products being amplified from samples obtained from humans (53, 61), the available circumstantial evidence weighs against the possibility of genetic exchange between *P. carinii* organisms found in different host species. The first line of such evidence is host specificity itself, which would lead to the physical isolation of *P. carinii* organisms adept at growing in different host species. A second line of evidence suggestive of speciation within the *Pneumocystis* genus is that the degree of genetic divergence between some of these organisms exceeds that between other fungi known to be different species. The sequences of the 16S-like rRNA genes from rat and human *P. carinii* are 4% divergent in the 441

TABLE 2. Divergence of gene sequences among different *P. carinii* isolates[a]

| Gene[b] | % Divergence in: | | |
|---|---|---|---|
| | Class I[c] | Class II[d] | Class III[e] |
| TS | 0 | 4 | 20 |
| mtrRNA | 0.5 | 4 | 20 |
| β-Tubulin | 0.5 | 4 | 15 |
| arom | 0.8 | 7 | 18 |
| ITS | 2–4 | 20–30 | 40–50 |

[a] See references 5, 7, 17, 20, 21, 26, 30, 44–47, 52–55, 58–62, 68, 73, 77, 79, 87, 90, and 107.
[b] Genes are as defined in Table 1.
[c] Divergence between human isolates.
[d] Divergence between *P. carinii* f. sp. *carinii*, *P. carinii* f. sp. *ratti*, and *P. carinii* f. sp. *muris*.
[e] Divergence between human, ferret, and rodent *P. carinii*.

bases compared (90). This divergence value is four times higher than that between two species of *Saccharomyces* (*S. rosei* and *S. cerevisiae*), three times higher than that between *Candida albicans* and *C. tropicalis*, and nearly as great as that between *S. cerevisiae* and *C. albicans* (6.6%). The degree of divergence between the two *P. carinii* isolates is at least as great as that between 13 pairs of ascomycetes from different genera, such as *Podospora anserina* compared with *Neurospora crassa* and *Histoplasma capsulatum* compared with *Coccidioides immitis*. The rRNA data have been echoed by analysis of two other genes. Analysis of the TS gene from five kinds of *P. carinii* (human, ferret, mouse, and two types from rat) produced a phylogenetic tree in which the distance between human and rat *P. carinii* is three-quarters as great as the distance between *C. albicans* and *S. cerevisiae* (45). Similar results were obtained when sequences from the mtrRNA gene were used to construct the phylogenetic tree. In this case, the five sequences used in the TS study were joined by sequences from *P. carinii* isolates from the pig, rabbit, and horse (46). Interestingly, trees derived from TS and mtrRNA sequences grouped the three *P. carinii* isolates from rodents together.

Table 2 lists the cumulative data on genetic divergence among different *P. carinii* organisms. These data suggest three classes of divergence. The greatest divergence is seen among *P. carinii* organisms from different host species that are not themselves closely related (class III). Class III divergence reaches or exceeds levels seen among bona fide species of other genera. *P. carinii* isolates from closely related host species show class II divergence. Class II divergence is much less than class III but also reaches levels near those seen among species. Class I divergence is seen among isolates of human *P. carinii*.

Class III genetic divergence among various *P. carinii* organisms is difficult but not impossible to reconcile with the idea that the genus *Pneumocystis* contains a single species. More work is needed in this area. A glaring deficiency in the *P. carinii* gene sequence database is the lack of a systematic study of 16S-like rRNA gene sequences. Currently, 16S-like sequences are available from only three organisms, *P. carinii* f. sp. *hominis* (90), *P. carinii* f. sp. *carinii*, and *P. carinii* f. sp. *ratti* (17, 23, 73). Complete sequences for the 16S-like genes from all of the possible species of *Pneumocystis* would allow investigators to fully exploit the power of the largest database for fungal genes. A second valuable avenue of systematic research that has not been developed for *P. carinii* is measurement of DNA relatedness, which is the fractional identity of two complete genomes. This parameter has been widely applied in fungal taxonomy, and a large body of work has defined the relationship between DNA relatedness and speciation (51). DNA relatedness is usually measured by hybridization in solution. This is more difficult for *P. carinii* studies because *P. carinii* DNA preparations always contain DNA from host cells, but it should be possible to overcome these problems. DNA relatedness of *P. carinii* from humans, rats, and mice has been assessed by hybridization of labeled probe DNA from one *P. carinii* organism to the electrophoretically separated chromosomes of the other (90, 91). There was essentially no cross-hybridization between the genomes of the different *P. carinii* organisms under stringent conditions, suggesting that DNA relatedness is less than 85%. The same results were obtained when a probe was prepared from a copy of an MSG gene from *P. carinii* f. sp. *carinii*.

The genetic and functional differences between *Pneumocystis* organisms from different host species are substantial, but many investigators are reluctant to assign new species names to these organisms at present. This conservative approach to nomenclature changes is understandable, given the complexity of the problem at hand and our limited understanding of microbial speciation in general. Although different species are always genetically divergent, genetic divergence can also occur within a species. The relationship between genetic divergence and speciation in microbes is particularly difficult to establish when the organisms in question can undergo prolonged uniparental reproduction (such as binary fission), which gives rise to genetic divergence among clones (98). The maximum divergence that could occur among clones from within a species that grows primarily by uniparental reproduction is not known. Consequently, divergence in the absence of speciation cannot be excluded as a possibility for *P. carinii*. In addition, it is not possible to exclude the possibility that *P. carinii* organisms found in different host species will mate in some other milieu.

Although designating new *Pneumocystis* species is currently unpopular, many researchers in the field have agreed that the single name *P. carinii* is inadequate at this juncture, and a trinomial system which distinguishes the major types of *P. carinii* organisms (i.e., those displaying class III and class II divergence [Table 2]) has been proposed. Table 3 shows the proposed new names (3). A similar trinomial nomenclature has been proposed by others (27).

It should be noted that host specificity and antigen differences led Frenkel to previously propose the name *Pneumocystis jiroveci* for the human-associated organism (29). However, the proposed new name did not gain general acceptance. Later, Hughes and Gigliotti proposed a trinomial system similar to that now in use (42).

## EACH SPECIAL FORM OF *P. CARINII* THAT HAS BEEN EXAMINED IS HETEROGENEOUS

Heterogeneity among *P. carinii* organisms isolated from the same host species was first seen by electrophoretic karyotyping of *P. carinii* from rats. Four different "karyotype forms" of *P. carinii* f. sp. *carinii* were identified by a study of eight rat strains from four different commercial animal vendors (16). These karyotype forms were found to be stable over a 1-year period and to be associated with specific commercial animal vendors but not with a particular strain of rat. This suggests that different commercial animal facilities harbor different strains of *P. carinii* f. sp. *carinii*, which are sustained in the colony by transmission from adults to pups.

Electrophoretic karyotypes have been obtained for *P. carinii* f. sp. *hominis* (41), but attempts to apply this technique to *P. carinii* f. sp. *hominis* have been hampered by the small numbers of organisms that can usually be obtained from patients. Ge-

TABLE 3. Trinomial nomenclature for *P. carinii*

| Organism | Name |
|---|---|
| Rat prototype or Pc1 | *P. carinii* f. sp. *carinii* |
| Rat variant or Pc2 | *P. carinii* f. sp. *ratti* |
| Human or Pc3 | *P. carinii* f. sp. *hominis* |
| Ferret | *P. carinii* f. sp. *mustelae* |
| Mouse | *P. carinii* f. sp. *muris* |
| Horse | *P. carinii* f. sp. *equi* |
| Pig | *P. carinii* f. sp. *suis* |
| Rabbit | *P. carinii* f. sp. *oryctolagi* |

netic divergence among isolates of *P. carinii* f. sp. *hominis* has been most often demonstrated by sequencing gene fragments derived by the PCR. Analysis of 12 patient-derived samples at the mtrRNA locus showed that 7 differed from each other and from the other 5 (53). The differences principally involved two polymorphic sites within a 300-bp segment. Isolates from other patients from different areas of the world also showed variation within this segment but only at a single site (107). Isolates from patients in France also showed variation at this site (52). Similar results were reported for the locus encoding cytoplasmic rRNAs, in which the ITS sequences (ITS1 and ITS2) were especially variable among *P. carinii* f. sp. *hominis* samples derived from different patients (60). These data suggest that different humans can be infected by different strains of *P. carinii* f. sp. *hominis*. The stability of the putative strains of *P. carinii* f. sp. *hominis* is not known, but no differences were seen in samples taken from the same patient 3 weeks apart (47). In addition, the same strain has been observed in samples obtained from patients in Cincinnati over a 3-year period (44).

As assessed by gene sequencing, *P. carinii* f. sp. *hominis* isolates are more variable than are *P. carinii* f. sp. *carinii* isolates (73, 83). The reason for this apparent difference in gene sequence diversity is not known, but one possibility is that a colony of laboratory rats is a relatively closed system, which militates against the incursion of exogenous microbes. As new animals are born, they would most probably be first exposed to the *P. carinii* f. sp. *carinii* strain present in adult colony members, which could lessen the chance of other strains gaining access to the colony. The fact that the multiple karyotypic forms of *P. carinii* f. sp. *carinii* show little variation in gene sequences suggests that changes in chromosome length occur more frequently than do changes in gene sequences in *P. carinii* f. sp. *carinii* (83). Such chromosome length polymorphism is common in fungi (100).

## PROGRAMMED GENE REARRANGEMENTS PRODUCE ANTIGENIC DIVERSITY WITHIN POPULATIONS OF *P. CARINII*

The *P. carinii* organism is covered by MSG, also known as gpA (39). MSG is a family of related proteins that are encoded by a gene family containing approximately 100 members, which are located on all 15 chromosomes (31, 40, 50, 91, 92, 97, 104, 116). MSG genes are organized in clusters of three or more genes arranged as head-to-tail tandem arrays (31, 97, 104). The expression of MSG genes appears to be regulated by programmed genetic rearrangements which place different MSG genes at a single site in the genome from which MSG mRNAs are produced (105). This "expression site" is at the end of a chromosome (96). The details of the mechanism by which different MSG genes become linked to the expression site are not clear, but the process must involve recombination between the expression site and the MSG genes at other loci. The expression site can be occupied by different MSG gene arrays in different organisms within a population of *P. carinii*, which predicts that different organisms within a population will express different isoforms of MSG. Data obtained with monoclonal antibodies specific for MSG are consistent with this prediction (57, 96). Data suggestive of population-wide variation in MSG expression have also been reported (2, 102). The ability of *P. carinii* to express different surface proteins may be used to escape attack by the host immune system, to facilitate colonization, or to do both.

Antigenic variation is a property of many and diverse pathogenic microbes, including many bacteria and several protozoa. Finding a system for antigen variation in *P. carinii* expands the breadth of this strategy to include the fungal kingdom. In eukaryotes, the best-studied surface variation system is the variable surface glycoprotein (VSG) system of the protozoan parasite *Trypanosoma brucei* (see reference 100 for a review), which also uses recombination to install different VSG genes at telomeric expression sites. It should be appreciated that similarity between the mechanism by which *P. carinii* achieves surface variation and the VSG system of the protozoan parasite *T. brucei* does not call the phylogenetic classification of *P. carinii* into question. *P. carinii* is undoubtedly a member of the fungi on the basis of genetic criteria (89). Instead, finding surface variation in *P. carinii* suggests that this capacity has evolved independently in two kingdoms of eukaryotes and that *P. carinii* has a parasitic lifestyle.

## IMPORTANCE OF UNDERSTANDING WHAT *P. CARINII* IS, EXACTLY

Recognizing and understanding the genetic divergence among *P. carinii* organisms is important for several reasons. First, the *P. carinii* organisms associated with humans are of primary interest but are difficult to study. Until a culture system is devised, researchers must continue to rely on animals as a source of organisms. Even if a culture system were devised, animal model systems would be necessary to study the normal and pathological interactions of the organism with its host and as a means to test anti-*P. carinii* drugs in vivo. These things can be done more effectively with knowledge of the genetic differences between human-associated *P. carinii* organisms and those found in animal models. Genetic differences should be taken into account in the development of drugs designed to interact with *P. carinii* proteins. The design of such drugs will rely on cloned *P. carinii* genes, both for predicting peptide sequences and for producing pure proteins. In such undertakings, it would be advantageous to work with genes and proteins from human-derived *P. carinii* isolates. Genetic divergence should also be considered in the development and application of DNA-based and immunodiagnostic reagents, which should be designed with knowledge of the target gene or antigen in the special form of *P. carinii* under study. Studies on the interactions between the host and pathogen, such as adherence and alteration of host cell function, also would best be done with *P. carinii* derived from the host under study. Finally, immunotherapies are more likely to succeed if target antigens are from human *P. carinii*.

It is also important to define genetic diversity among organisms associated with the same host. As an example, genetic variation might play a role in variation in serological responses to *P. carinii* antigens (31, 75, 88). Variation with respect to MSG epitopes can be expected to be a common feature within and between *P. carinii* populations because these organisms have a special mechanism to achieve this end. Additional variation can be expected to occur between strains of *P. carinii* f.

sp. *hominis*. Work with rats has established that the different special forms of *P. carinii* harbored by rats are genetically and antigenically distinct (17, 59, 73, 102). There may be more than one special form of *P. carinii* capable of infecting humans, either singly or in combinations. Ignorance of such a situation could complicate the clinical picture and confound efforts to understand the origin and course of *P. carinii* pneumonia in a given patient or patient population.

While genetic variation among *P. carinii* f. sp. *hominis* isolates potentially complicates patient analysis, it also provides a valuable tool for epidemiological studies. This approach is beginning to yield interesting results. Data have been reported that challenge the common idea that *P. carinii* infections in adults are necessarily caused by organisms acquired early in life, which are carried in a latent state unless immunity fails. A study of 10 AIDS patients who had two episodes of *P. carinii* pneumonia separated by at least 6 months showed that 5 of these patients had different *P. carinii* f. sp. *hominis* isolates in the first and second disease episodes, as assessed by the sequence of the mtrRNA gene (47). These data support the hypothesis that recurrence of *P. carinii* pneumonia can be due to reinfection rather than to reactivation of latent organisms. This hypothesis is also consistent with reports of outbreaks of *P. carinii* pneumonia and of increased incidence of *P. carinii* pneumonia in patients exposed to individuals with active *P. carinii* pneumonia (12, 111; reviewed in reference 14).

The idea that *P. carinii* organisms establish long-term latent infections also has been challenged by the negative results of recent attempts to detect *P. carinii* in tissues of immunocompetent individuals (67, 72, 78). In addition, experiments with animals have suggested that latent infection is short-lived in an immunocompetent host. In one study, infected SCID mice were restored to immunocompetency by injection of T cells active against *P. carinii*, after which the mice were cured of *P. carinii* pneumonia. More importantly, *P. carinii* did not return after the transplanted T cells were lost, showing that latency is not an inevitable outcome of *P. carinii* infection (13). Studies with immunosuppressed rats led to the same conclusion (101).

If *P. carinii* pneumonia is not always due to reactivation of latent organisms, the frequent occurrence of this disease in susceptible patients implies that the pathogen is commonly encountered by humans. The possible sources of *P. carinii* in the environment include other infected humans and a hypothetical free-living form of *P. carinii*. A third possibility, i.e., that humans might be at risk of infection from animals, has been suggested by the observation that PCR amplification of some human-derived samples produced DNA with a sequence normally found in *P. carinii* from rats (53, 60). However, it has not been established that the source of this PCR product was rat *P. carinii* organisms.

No matter whether it comes from people, animals, or some other source, *P. carinii* would be expected to be present in the air because it has been shown that the infection is acquired by inhalation (see reference 14 for a review). Two reports of using PCR to detect *P. carinii* DNA in air samples have appeared. In one study, indoor air was sampled. *P. carinii* f. sp. *carinii* DNA was detected in air from a room containing infected rats, and *P. carinii* f. sp. *hominis* DNA was detected in air from rooms housing *P. carinii* pneumonia patients but not in air from offices in the same hospital (8). In the other study, air from a rural outdoor location was sampled (106). In the outdoor air samples, a number of different sequences were obtained, most of which were identical to *P. carinii* from rats or humans but some of which differed from the published *P. carinii* sequences by a small number of bases. Given what has been learned about diversity among *P. carinii* organisms from mammalian hosts, it would not be surprising to find at least as much diversity in air samples. An intriguing possibility is that the sequences detected in organisms isolated from outdoor air are from close relatives of known *Pneumocystis* species. Perhaps there are hundreds of *Pneumocystis* species, some of which are capable of invading certain mammalian hosts when the hosts are either immunoincompetent or immunonaive.

## SUMMARY AND FUTURE RESEARCH DIRECTIONS

*P. carinii* has long been known to be quite different from the organisms typically encountered by medical mycologists. Unlike other fungal pathogens of humans, its fastidious growth requirements have yet to be met in vitro and its lack of ergosterol renders it invulnerable to standard antifungal drugs. Recent research has revealed additional features of *P. carinii* that are atypical of fungi. Among these are a flimsy cell wall, very few rRNA genes, and a sophisticated mechanism for surface antigen variation. These phenotypic departures from what we have come to expect from most fungi concealed the heritage of *P. carinii* for a long time, but its genes have divulged the secret. *P. carinii* is definitely a member of the kingdom Fungi.

This strange fungus is found in many different host species. The various *P. carinii* organisms found in different hosts are morphologically very similar, but other phenotypic features differ dramatically. Molecular genetic analysis has established that these phenotypic differences have a genetic basis and that the genus *Pneumocystis* encompasses a group of organisms whose members differ as much from one another as do individual species of other fungal genera. It is important to recognize and understand this diversity in basic and clinical research and development endeavors.

While the complexity of the *Pneumocystis* group has been established, many parts of the question "What is *Pneumocystis*?" need to be investigated further. We need to clarify the relationship between *P. carinii* and other fungi. Progress in this area may lead to the identification of a close relative that can be cultured and will expand our understanding of what appears to be an unusual branch of the fungal family tree. Another area that needs further study involves the phylogenetic relationships of the members of this group. Systematic study of the same genes from each organism is needed to better understand the range of diversity among organisms recognized as *P. carinii* and to calibrate this range by comparison with the range of diversity seen among the members of other groups of fungi. Systematic genotypic information will also be necessary for research into the habitat of *P. carinii*. While it is clear that *P. carinii* can grow in mammals, whether mammals are their only habitat is an open question. This question is being addressed by looking for *P. carinii* DNA on filtrates from air. This approach is exceedingly sensitive but is limited by the absence of an organism to go with a particular PCR product. If we are forced to rely exclusively on PCR data, we must formulate genotypic criteria that are able to identify organisms detected by PCR of air filtrates. Formulation of adequate genotypic criteria will require a substantial body of systematic genotypic information about the various *P. carinii* organisms that can be obtained from animals. With this information in hand, it will be possible to determine if the organisms derived from mammals are representative of a much larger group or if mammals appear to be the only habitat of *P. carinii*.

Another issue that needs further study is the specificity of *P. carinii* for a given mammalian host. Although attempts to transfer *P. carinii* from one host to another have uniformly failed, data from patients have led some investigators to sug-

gest that the same organisms can infect both rats and humans. Further study is needed to determine if this is possible.

In addition to the need for further understanding of the relationships between members of the *Pneumocystis* genus (currently known as special forms of *P. carinii*), more work is needed on the genetic structure of populations of each specific form of *P. carinii*. It is already clear that different populations of *P. carinii* f. sp. *hominis* can contain different alleles of at least two genes. Such differences have allowed studies on latency and reactivation. In the future, genetic markers that vary among populations of a special form should be useful in a variety of investigations, including studies on the mechanism of transmission, latency, reactivation, virulence, and drug resistance. However, the battery of genetic markers available for such studies is limited, and much more work is needed in marker development. Molecular genetic comparisons of populations of the same special form of *P. carinii* may also provide insight into the reproductive life of this organism, since organisms that reproduce primarily via sexual means tend to have population structures that differ from those of microbes that seldom or never use sexual reproduction.

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