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Pneumocystis carinii Karyotypes

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Pulsed-field gel electrophoresis techniques were used to examine the chromosomes of *Pneumocystis carinii* isolated from laboratory rats and two human subjects. *P. carinii* organisms isolated from each of four rat colonies and from two patients each produced a distinct band pattern, but in all cases the bands ranged in size from 300 to 700 kilobase pairs. *P. carinii* from three rat colonies produced patterns containing 15 prominent bands. Of these 15 bands, 2 stained more intensely than would be expected of bands of their size, suggesting that the *P. carinii* haploid genome contains 17 to 19 chromosomes. Summing the molecular sizes of the bands and accounting for staining intensities suggested that the haploid genome of rat-derived *P. carinii* contains on the order of 10^7 base pairs. Human-derived *P. carinii* produced patterns containing 10 to 12 bands which appeared to be similar to the 15-band patterns seen in rat-derived *P. carinii* with respect to the size range of the bands. *P. carinii* from the fourth rat colony produced a more complex band pattern containing approximately 22 bands, most of which appeared to comigrate with the bands present in one of the 15-band *P. carinii* patterns, suggesting that these animals were simultaneously infected by two different varieties of *P. carinii*. Hybridization experiments using oligonucleotide probes specific for the *P. carinii* 18S rRNA gene supported this possibility. The band pattern of *P. carinii* derived from a given rat colony was generally stable over time. *P. carinii* band patterns were not strictly rat strain specific and appeared to be transferrable between animals housed in the same room.

Pneumocystis carinii causes a pneumonia that is a principal cause of death among patients with acquired immunodeficiency syndrome (21, 28). Progress in characterizing this pathogen has been hampered by its fastidious growth requirements, which remain undefined. In vivo, the lung alveoli of immunocompromised mammals can accumulate very large numbers of *P. carinii* organisms, which are often found to be closely associated with type I pneumocytes (23, 47).

P. carinii populations derived from infected lungs are morphologically complex, containing many different forms of the organism which have been proposed to be stages in the life cycle (1, 7, 25, 41). The principal forms of the organism are the trophic form, which is a small uninucleated cell, and the cyst, which is a larger, spherical, multinucleated structure resembling sporangenous structures of certain protozoa and fungi.

Until recently, *P. carinii* had been widely considered to be a protozoan, primarily on the basis of morphological observations and drug susceptibilities. However, morphological and biochemical similarities between *P. carinii* and fungi have long been appreciated (32, 40). Recently, researchers in our laboratory and two others have examined the sequences of rRNA genes of *P. carinii* (9, 37, 45). These molecular genetic data suggest that *P. carinii* is more closely related to fungi than to protozoa.

The organism appears to infect a range of vertebrates. Over 75% of humans are seropositive for *P. carinii* by age 4, and many mammalian species host subclinical infections (16, 31). It is not clear whether *P. carinii* organisms found in these diverse hosts are the same organism or whether genetic diversity exists among *P. carinii* organisms found in a given host species. Antigenic variability among *P. carinii* isolates from different host species suggests that genetic variants of the pathogen exist (13, 14, 22, 42). Animal passage experiments and serological studies have suggested the presence of distinct strains (11, 18, 43). Furthermore, recent clinical studies have indicated that *P. carinii* antigens can change in people with recurrent *P. carinii* pneumonia (29). To address the question of genetic variability among *P. carinii* isolates, we used pulsed-field gel electrophoresis (PFGE) to examine the genomes of *P. carinii* isolates from immunosuppressed rats and from two humans. The data indicated that *P. carinii* populations isolated from a given source are generally composed of organisms that contain the same complement of chromosomes as judged by the size and number of bands resolved by PFGE. However, *P. carinii* populations isolated from four different colonies of infected rats each displayed a distinct pattern of bands, as did the two samples of *P. carinii* from humans. These findings indicate that substantial karyotypic variability exists among populations of the pathogen. Such karyotypic variability will be a powerful tool with which to explore the epidemiology of *P. carinii* infections.

MATERIALS AND METHODS

Rat colonies. Male rats were obtained from three sources. Outbred Sprague-Dawley rats (viral antibody positive) were exclusively from the colony kept in room 205H at Harlan Sprague-Dawley, Madison, Wis. Upon arrival, Sprague-Dawley rats were placed in colonies A and B, which were located in different buildings. Colony A had been established approximately 1 year previously, and colony B had been maintained for 7 years. Lewis rats were placed into colonies C and D (established 8 years previously), which were located

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in adjacent rooms in a different building from those of both colonies A and B. Two rats were housed per wire cage equipped with an automatic watering system. Absorbent paper was placed underneath and changed on a biweekly basis. Cages were held on double-sided stainless steel racks with a 50-cage capacity. When a new shipment of rats was received, the incoming animals were placed in separate cages and usually on separate racks; in some instances, new rats were placed on the same rack as infected animals but on the opposite side. Incoming rats were never placed in the same cage with immunosuppressed resident rats.

Provocation of *P. carinii* pneumonitis. Rats were rendered immunoincompetent by injection of methylprednisolone acetate (DepoMedrol; The Upjohn Co., Kalamazoo, Mich.) at 4 mg/week for 8 to 16 weeks, as previously described (6). To exacerbate *P. carinii* pneumonitis, malnutrition was induced by administration of a low (8%)-protein diet (ICN Biochemicals, Inc., Cleveland, Ohio). Water was supplemented with ampicillin (1 mg/ml) to suppress secondary bacterial infections.

Isolation of rat-derived *P. carinii* organisms. Immunosuppressed rats were monitored for weight loss and other signs associated with *P. carinii* pneumonitis, which usually developed after 8 to 16 weeks of the regimen described above. Infected rats were sacrificed, the lungs were removed, and impression smears were examined to determine the severity of infection. Grades of infection were assigned according to a semiquantitative grading system based on a previously described histological scoring technique (20). Lungs were homogenized in a blender (Stomacher; Tekmar Inc., Cincinnati, Ohio). The preparations were filtered through gauze to remove large particulates and then treated with a hypotonic lysis solution (0.85% ammonium chloride or 0.04 M KCl–0.025 M sodium citrate) to reduce contamination with host cells. Organism preparations were again filtered at least four to six times through 10-μm-pore-size filters (Mitex; Millipore Corp., Bedford, Mass.). Material that passed through the filter was examined microscopically to determine the number and purity of the organisms obtained. The preparations were then treated with DNase at 8.5 μg/ml for 30 min at 37°C in the presence of 10 mM $MgCl_2$ to digest host cell nuclei. The DNase was inactivated by adding EDTA to a final concentration of 0.250 M. Organisms were pelleted by brief centrifugation, and the pellet was suspended in 0.125 M EDTA (pH 7.4). The *P. carinii* organisms remained intact throughout these procedures (37). Surveillance cultures of each isolate were performed by streaking 10 μl of homogenate onto Sabouraud dextrose and Mueller-Hinton agars in plates, followed by incubation for up to 2 weeks at 25 and 35°C, respectively.

Isolation of *P. carinii* derived from human sources. *P. carinii* isolates from humans were obtained from four bronchoalveolar lavage fluid samples (026, 020, 009, and 133) and from two postmortem lung tissue specimens (samples 118 and 142). All specimens were from human immunodeficiency virus-infected patients and were obtained through agreements with Robert Baughman, Pulmonary Division, University of Cincinnati College of Medicine (bronchoalveolar lavage fluids), and the Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine (postmortem samples). Bronchoalveolar lavage fluids were treated with hypotonic lysis solution and DNase to reduce host cell contamination (as described above) and then immediately embedded in low-gelling agarose (see below). Postmortem specimens were processed as described above for isolation of *P. carinii* from rat lungs, except that all procedures were performed in a BSL-2 laboratory and biocontainment hood according to Centers for Disease Control directives for handling of human immunodeficiency virus-infected specimens (F. A. Murphy, W. J. Martone, J. W. Curran, and J. D. Millar, Morbid. Mortal. Weekly Rep. **39[RR-1]**:1–11, 1990). Human lung homogenates contained more host cell debris than did rat lungs and were filtered extensively (at least 8 to 10 times).

PFGE. *P. carinii* suspensions were mixed with a solution of low-gelling agarose (Sigma Chemical Co., St. Louis, Mo.) in 0.125 M EDTA to achieve a parasite density of 5×10^8 nuclei per ml in 0.6% agarose as described previously (3). Titration of *P. carinii* densities from 5×10^5 to 1×10^9 showed that this number of organisms was optimal for ethidium bromide visualization. Gel-embedded cells were treated with 0.25 mg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml and 1% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.) at 55°C for 18 h. Digested agarose plugs were stored in 0.5 M EDTA (pH 9.0) at 4°C.

Contour-clamped homogeneous electric field electrophoresis (CHEF; 5) and field inversion gel electrophoresis (FIGE; 3) gels containing 1% agarose were prepared in 45 mM Tris hydrochloride–45 mM boric acid–0.125 M EDTA (0.5× TBE) and electrophoresed at 14 to 16°C in the same buffer. CHEF was performed in a Bio-Rad CHEF DRII apparatus. FIGE was performed in an apparatus constructed as described by Carle et al. (3). CHEF gels were generally run at 90 V for 96 h with 50- to 100-s gradual switching, while FIGE gels were run at 105 V for 120 h with 50-s forward and 25-s backward switching. Occasionally, gels were run with different parameters, and these are noted in the figure legends.

Isolation of rat-derived *P. carinii* DNA. For each preparation of DNA, 2×10^{10} filtered, DNase-treated *P. carinii* organisms obtained from infected rat lung homogenates were lysed in 0.25 M EDTA containing 0.25 mg of proteinase K per ml and 1% Sarkosyl at 55°C. The lysate was then incubated at 55°C for 4 h. Digested lysates were extracted twice with phenol plus chloroform and twice with chloroform alone, and the DNA was precipitated with ethanol (24). Approximately 20 μg of DNA was obtained. The purity of DNA preparations was assessed as described in Results.

Production of a rat-derived *P. carinii* genomic library. Three micrograms of *P. carinii* DNA obtained from four Lewis rats (two from colony C and two from colony D) was partially cleaved with *Sau*3A (24). The ends of the DNA were partially filled by treatment with Klenow polymerase in the presence of dGTP and dATP. The DNA was fractionated by electrophoresis through a 0.7% agarose gel. DNA that migrated between size markers of 23 and 16 kilobase pairs (kb) was excised, extracted from the agarose, and ligated to 1 μg of the lambda Fix vector (Stratagene, La Jolla, Calif.). The ligated DNA was packaged in vitro by using Gigapack (Stratagene), and approximately 60,000 plaques were produced by infection of *Escherichia coli* LE392. Approximately 30% of these plaques were produced by chimeric bacteriophage as judged by plaque formation on *E. coli* P2392 and by hybridization to a radiolabeled probe prepared by nick translation of a sample of the agarose gel-purified DNA used in the ligation to lambda Fix.

Hybridization probes. A synthetic 25-base oligonucleotide probe designed to be specific for 18S rRNA sequences unique to *P. carinii* had the sequence GGTAATCCAGGA GGGAAGGATCAGT. This sequence was complementary to a sequence running from nucleotide residue 680 to residue

TABLE 1. Characteristics of *P. carinii* isolates studied

Sample	Colony	Date (mo/day/yr)[a]	No. of rats	Yield of *P. carinii* nuclei	Culture[b]	No. of bacteria/ *P. carinii* nuclei
SD(1)	A	11/29/88	7	4×10^9	G^+ cocci	2.4×10^{-6}
SD(3)	B	12/1/88	8	13×10^9	G^- rods	6.3×10^{-8}
SD(4)	B	5/17/89	7	140×10^9	G^+ cocci	2.2×10^{-8}
SD(6-4)	B	6/6/89	1	6×10^9	ND	
SD(8)	A	6/21/89	1	2×10^9	ND	
L(1)	C	11/29/88	2	3×10^9	ND	
L(3)	C	1/3/89	4	2×10^8	G^+ cocci	1.6×10^{-2}
L(4)	D	1/10/89	6	8×10^8	ND	7×10^{-7}
L(6)	D	5/18/89	4	28×10^9	ND	1×10^{-8}
L(7)	C	6/5/89	4	3×10^9	G^- rods	1.3×10^{-3}
L(8-1)	D	6/5/89	1	6×10^9	G^- rods	1.6×10^{-4}
L(8-2)	D	6/5/89	3	3×10^9	G^+ cocci	9.1×10^{-4}
L(8-3)	D	6/5/89	1	2×10^8	G^- rods	5.6×10^{-7}
L(8-5)	D	6/5/89	1	2×10^9	ND	
L(8-7)	D	6/5/89	1	9×10^8	G^+ cocci	1.2×10^{-6}
L(9-1)	C	6/5/89	1	1×10^7	G^- rods	1.1×10^{-1}
L(9-2)	C	6/5/89	1	9×10^7	G^- rods	1.1×10^{-2}
L(9-5)	C	6/5/89	3	9×10^8	G^+ cocci	3.8×10^{-3}
L(10-1)	C	6/7/89	1	2×10^8	G^+ cocci	5.9×10^{-7}
L(10-2)	C	6/7/89	1	2×10^8	G^- rods	5.6×10^{-3}
L(10-3)	C	6/7/89	1	3×10^8	G^- rods	2.9×10^{-3}
L(10-4)	C	6/7/89	1	5.2×10^8	G^+ cocci	1.9×10^{-7}
L(11-1)	C	6/21/89	1	6×10^7	G^- rods	2.0×10^{-2}
L(11-2)	C	6/21/89	1	2.9×10^7	ND	
L(11-C)	A	6/21/89	1	1.3×10^8	ND	
L(11-D)	A	6/21/89	2	5.0×10^7	G^+ rods	7.2×10^{-5}

[a] Date when *P. carinii* was harvested.
[b] G^+, Gram positive; G^-, gram negative; ND, none detected.

705 in the *P. carinii* 18S rRNA gene (9). This region is highly variable among 18S rRNA genes. The 25-mer was 50% identical to the corresponding sequence in the 18S rRNA gene of *Neurospora crassa* and 25% identical to the corresponding *Saccharomyces cerevisiae* sequence, the two 18S sequences known to be most similar to that of *P. carinii*. Identity of the 25-mer with other 18S rRNA gene sequences ranged between 25 and 35% (*Oxytricha nova*, *Acanthamoeba castellani*, *Dictyostelium discoideum*, corn, frog, and rat sequences were among those analyzed) (17). A second synthetic oligonucleotide 26 bases long and complementary to nucleotide residues 636 to 662 of the *P. carinii* 18S rRNA gene was also used. This 26-mer corresponded to one of the three probes reported by Edman et al. (9) to hybridize to human *P. carinii* in situ. The 26-mer performed identically to the 25-mer described above in our hybridization experiments (data not shown).

Oligonucleotides were labeled by treatment with T4 polynucleotide kinase in the presence of [γ-^{32}P]dATP. Total rat cell DNA was isolated from cultured Rat-3 cells (38) and labeled by nick translation as described by Maniatis et al. (24). Radiolabeled cDNA corresponding to a segment of the *P. carinii* 18S rRNA was prepared by reverse transcription of total *P. carinii* RNA in the presence of universal 18S rRNA primer A as described previously (37). Other probes were prepared by nick translation (24).

Southern blotting. CHEF and FIGE gels were prepared for transfer by treatment with 0.25 M HCl for 20 min, followed by denaturation with 0.5 M NaOH and neutralization as described by Schwartz and Cantor (33). DNA was transferred by capillary flow onto nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) as recommended by the vendor. Prehybridization was done as described in reference 26 for oligonucleotide probes and as described in reference 36 for other probes. Hybridization and washing conditions for oligonucleotide probes were as follows: hybridization in 6× SSPE (0.9 M NaCl, 0.09 M NaH_2PO_4, 0.02 M disodium EDTA [pH 7.4])–10 μg of denatured salmon sperm DNA per ml–0.1% sodium dodecyl sulfate (SDS) at 42°C for 16 to 30 h; wash in 6× SSPE–5× Denhardt reagent–0.4% SDS–50% formamide at 42°C for 16 to 30 h. Washes were performed at 65°C in 0.2× SSPE–0.5% SDS. Radioactive signals were detected by radioautography as described in reference 24.

RESULTS

Analysis of *P. carinii* preparations. *P. carinii* organisms were isolated from infected lungs by using previously established methods (6, 7, 37, 42). The *P. carinii* preparations used in this study were characterized by microscopic examination and by inoculation of culture plates that would allow growth of other microorganisms.

Microscopic inspection and tinctorial staining were used to quantitate *P. carinii* organisms (Table 1) and to assess the presence of potential contaminants, such as other eucaryotic microbes, rat cells, and rat cell nuclei. None of the samples used in this study contained other eucaryotic microbes. No rat cells were detected, but rat cell nuclei were sometimes observed. Although in low numbers compared with the number of *P. carinii* nuclei (less than 1%), host nuclei were a significant problem because each mammalian nucleus contains on the order of 200 times more DNA than a *P. carinii* nucleus. This problem was minimized by the use of DNase, which was effective in specifically degrading rat DNA.

Inoculation of agar plates with samples of *P. carinii* preparations provided the second means with which to detect the presence of other microorganisms. Again, no eucaryotic microbes were detected, but bacterial contami-

nation was common. However, the presence of bacteria in *P. carinii* preparations did not influence the patterns obtained by PFGE. The ratio of bacterial cells to *P. carinii* nuclei present in *P. carinii* preparations varied from as high as 1.1×10^{-1} to as low as 10^{-8}, yet there was no correlation between the number of bacteria and the PFGE band pattern (Table 1).

Analysis of *P. carinii* nucleic acids. Microscopic and microbiological characterization of *P. carinii* preparations showed that few, if any, other eucaryotic microbes were present. In addition, we have previously shown that *P. carinii* prepared as described here produced essentially pure *P. carinii* rRNAs, as assessed by the sizes of the *P. carinii* rRNA molecules and sequence analysis of 18S rRNA species (37). Therefore, it seems unlikely that PFGE bands produced from such preparations could originate from anything other than *P. carinii*. Nevertheless, it seemed prudent to confirm that the lysis procedure used in preparing samples for PFGE did indeed release *P. carinii* DNA.

DNA was prepared from a *P. carinii* preparation [SD(3) in Table 1] by using the lysis procedure used to prepare gel-embedded samples for PFGE. To assess the presence of rat sequences, 1 μg of DNA was electrophoresed through 1% agarose. The gel was treated with HCl to mobilize high-molecular-weight DNA, which was then transferred from the gel to a nylon filter, and the filter was subjected to hybridization with radioactive DNA prepared from cultured rat cells. The only material that hybridized to the rat probe was less than 5 kb long (data not shown).

Three micrograms of the *P. carinii* DNA preparation was partially digested with *Sau*3A and electrophoresed through 1% agarose, and fragments that migrated between 15 and 23 kb were isolated. These fragments were ligated to lambda Fix (Stratagene). Approximately 20,000 recombinant phage plaques were obtained, as judged by plating on the spi(+) host, *E. coli* P2392, and by hybridization to the gel-purified DNA fraction used in the ligation. The library was screened for the presence of rat sequences by hybridization of 2,000 plaques to radioactive rat DNA; 1 plaque hybridized.

The library was next screened with radioactive cDNA made by reverse transcription of the 18S rRNA from *P. carinii*. Approximately 2% of the plaques hybridized to the probe, a percentage consistent with expectations based on the assumption that rRNA genes are as numerous in the *P. carinii* genome as they are in *S. cerevisiae* (50 copies per haploid genome [30]). Restriction analysis of four chimeric phage showed that they all contained related inserts and that each insert restriction map was consistent with what would be expected on the basis of a previously reported restriction map of the rRNA locus of *P. carinii* (10; data not shown). The phage all hybridized to an oligonucleotide complementary to a unique region of the *P. carinii* 18S rRNA gene (data not shown). The likelihood that this oligonucleotide can hybridize to DNA not from *P. carinii* is very low because the probe corresponds to a sequence that varies considerably, even among close relatives. Analysis of the 18S rRNA sequence data base showed that the oligonucleotide shared less than 50% analogy to any other rRNA sequence (17; see Materials and Methods for details).

PFGE analysis. Information regarding the rat-derived *P. carinii* isolates analyzed by PFGE can be found in Table 1. Seven *P. carinii* preparations were obtained from Sprague-Dawley colonies A and B. Nineteen *P. carinii* preparations were obtained from Lewis rats, which were divided between colonies C and D. Initial preparations of *P. carinii* originated from pooled homogenates of infected animals, but in all cases, animals grouped for *P. carinii* isolation were from the same colony. Later preparations of *P. carinii* were usually from individual animals.

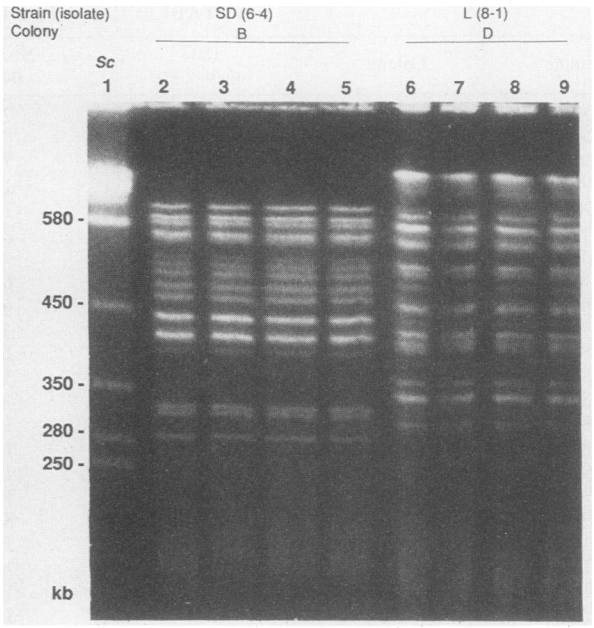

FIG. 1. *P. carinii* chromosomes resolved by CHEF. Samples prepared from colonies B and D were subjected to CHEF at 90 V for 96 h with an initial switching interval of 50 s. The switching interval was gradually increased to 100 s. Lane 1 contained *S. cerevisiae* (*Sc*). Lanes 2 to 5 contained replicate plugs containing *P. carinii* preparation SD(6-4) (Table 1). Lanes 6 to 9 contained replicate samples of preparation L(8-1).

Figure 1 shows the results of CHEF analysis of two samples of *P. carinii* DNA, one from colony B [SD(6-4)] and one from colony D [L(8-1)]. Both *P. carinii* samples produced a series of bands ranging from approximately 300 to 700 kb, but the band patterns for the two samples were different. [The DNA that migrated in the megabase range in sample L(8-1) was later shown to be rat DNA (see below).]

To verify that the CHEF bands were from *P. carinii*, gels were blotted and hybridized to either an rRNA gene probe to locate the band carrying the rRNA locus (Fig. 2B) or an oligonucleotide probe specific for the *P. carinii* 18S rRNA gene (Fig. 2A). Both probes hybridized to a band of approximately 500 kb in each of the *P. carinii* samples. The rRNA gene probe hybridized strongly to the yeast genome (Fig. 2B, lane 4), but the oligonucleotide probe hybridized to *P. carinii* bands and not to the yeast genome. In sample L(4), the rRNA oligonucleotide probe hybridized to two bands, one at approximately 500 kb and the other at approximately 535 kb (Fig. 2A, lanes 5 and 11). The significance of the presence of two bands carrying rRNA genes is discussed further below.

Figure 3 shows FIGE analysis of sample SD(3). FIGE produced the same general pattern as CHEF but resolved an additional band that migrated at approximately 570 kb. As in Fig. 2, both rRNA probes hybridized to a single band in the SD(3) pattern.

Figure 3 also shows the results of hybridization of FIGE bands to a chimeric lambda phage, Rp3-1, that was isolated from the *P. carinii* library. The plaque from which Rp3-1 was isolated hybridized particularly well to total *P. carinii* DNA. The Rp3-1 probe hybridized to all of the bands (Fig. 3). The radioactive signal intensities deviated from the ethidium

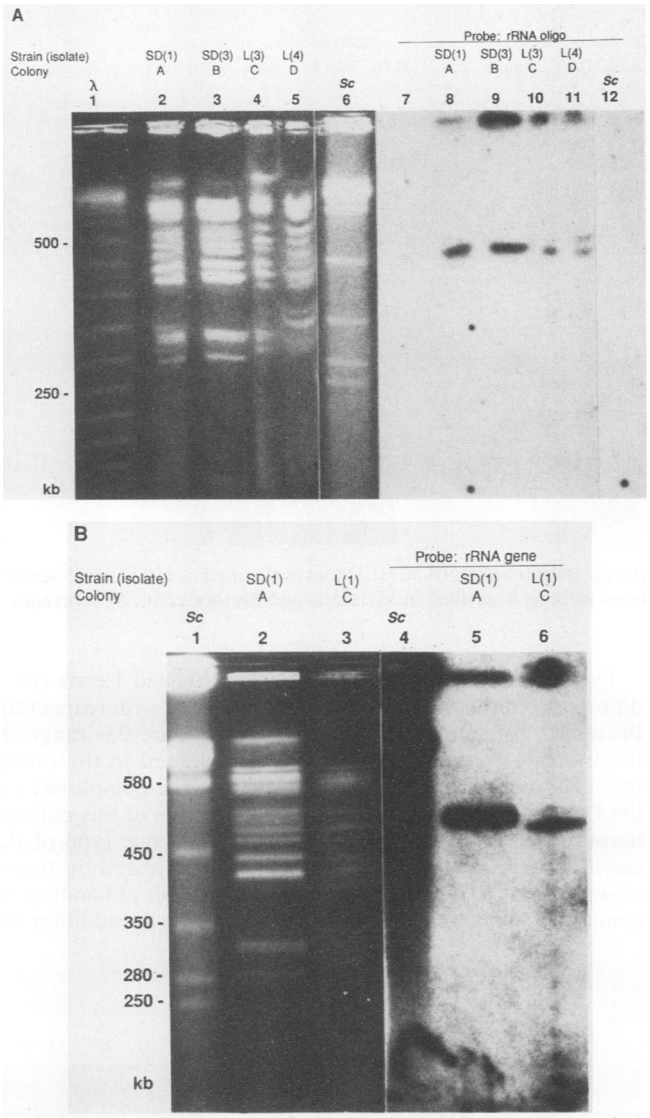

FIG. 2. Hybridization of *P. carinii* chromosomes to 18S rRNA gene probes. The gels in panels A and B were run under the conditions stated in the legend to Fig. 1. Probes were prepared and used as described in Materials and Methods. The lane labeled λ contained concatenated lambda genomes. Hybridization and wash conditions were as follows. For panel A, the blot was hybridized in 6× SSPE–10 μg of salmon sperm DNA per ml–0.1% SDS at 42°C and washed in 6× SSPE–0.5% SDS at 65°C. For panel B, the blot was hybridized in 50% formamide–6× SSPE–5× Denhardt solution–100 μg of salmon sperm DNA per ml–0.4% SDS and washed in 0.2× SSPE–0.5% SDS at 68°C. *Sc*, *S. cerevisiae*.

bromide fluorescence intensities, suggesting that this chimeric phage carried a sequence that is repeated in the *P. carinii* genome and is present on all chromosomes but that the number of repeats varies among chromosomes. That the hybridization signal was not due to the presence of lambda sequences in the probe was verified by the experiment shown in Fig. 2B, in which a lambda phage carrying a fragment from the *P. carinii* rRNA locus hybridized to a single band. *P. carinii* DNAs from all four rat colonies contained the repeated sequence present in the Rp3-1 cloned DNA fragment, but rat and yeast DNAs showed no homology to Rp3-1. Rp3-1 hybridized to bands that migrated between 300 and 700 kb and, in some cases, to smaller DNAs

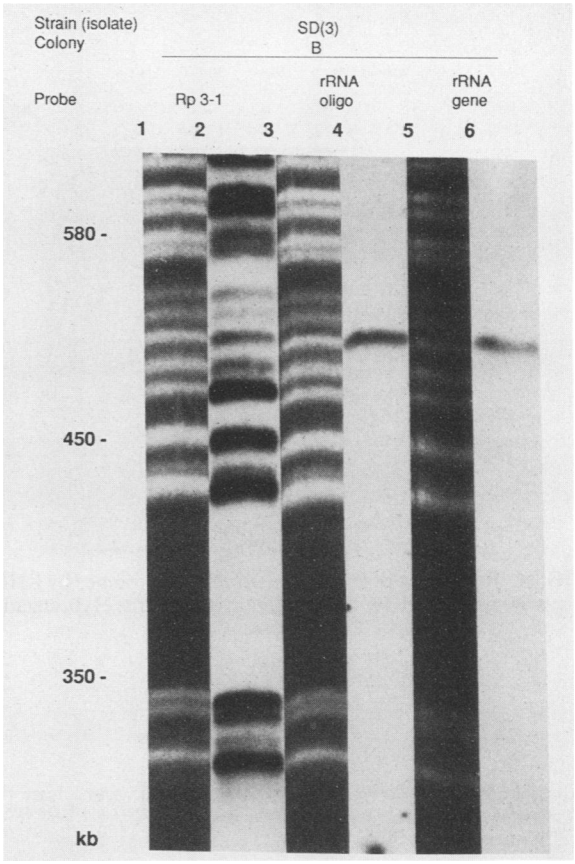

FIG. 3. *P. carinii* chromosomes resolved by FIGE hybridized to a repeated sequence. Multiple lanes of a 1% agarose gel were loaded with sample SD(3) and subjected to FIGE at 105 V for 120 h with switching forward for 50 s and backward for 25 s. The DNA was transferred to a nylon membrane which was cut into strips which were hybridized to the probes indicated under the conditions described in Materials and Methods.

that migrated as a smear at the bottom of the gel (Fig. 4). The Rp3-1 probe did not hybridize to the DNA that migrated in the megabase range. The data shown in Fig. 4C identified this megabase DNA as originating from a rat, which explains why this large DNA was present in only some of the *P. carinii* samples.

Comparative analysis of *P. carinii* from rats. The experiments described above showed that *P. carinii* derived from different colonies of rats produced distinct band patterns on CHEF gels. To better resolve these patterns, samples from each of the four colonies were analyzed by FIGE. Each colony produced a characteristic band pattern (Fig. 5). The patterns produced by Sprague-Dawley rats from colonies A and B were very similar, the only differences being that pattern B contained a prominent 560-kb band that was not present in the A pattern, and in pattern B the 560-kb band and the 545-kb band stained less intensely than the 545-kb band in pattern A. This can be seen more clearly in Fig. 3. [Note that the bands in lane 2 are misaligned because this lane was from a different gel. The bands in sample SD(1) comigrated with samples from colony B (see Fig. 2A and 9).] Colony C, which housed Lewis rats, produced a band pattern that was quite different from those of colonies A and B. However, the patterns from colonies A, B, and C all contained 15 prominent bands that ranged between 300 and 700 kb.

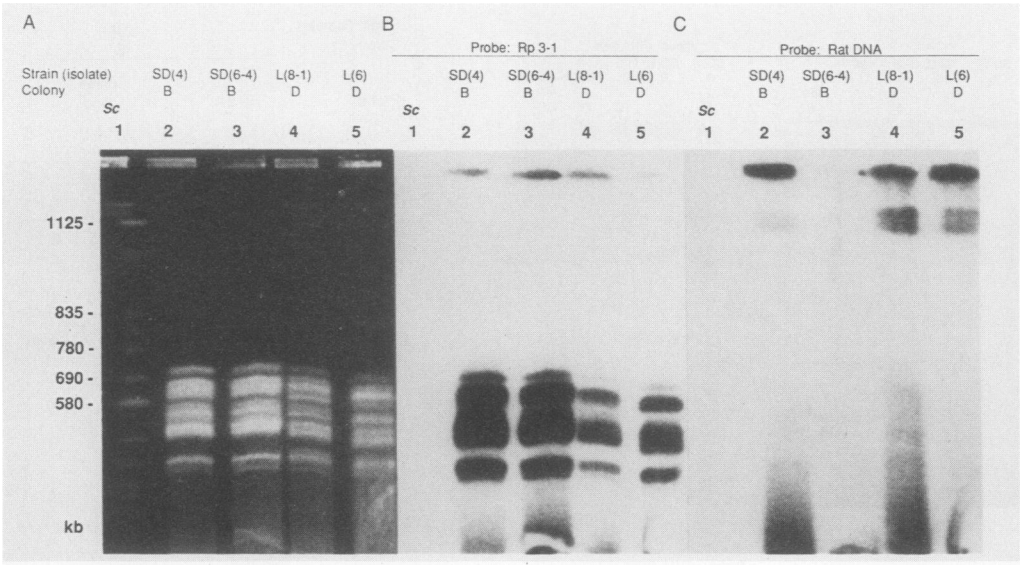

FIG. 4. Resolution of largest *P. carinii* chromosomes by CHEF and hybridization to rat DNA. CHEF was performed at 150 V for 45 h with 100-s switching on a 1.5% agarose gel in 0.5× TBE. Hybridization conditions were as described in Materials and Methods. *Sc*, *S. cerevisiae*.

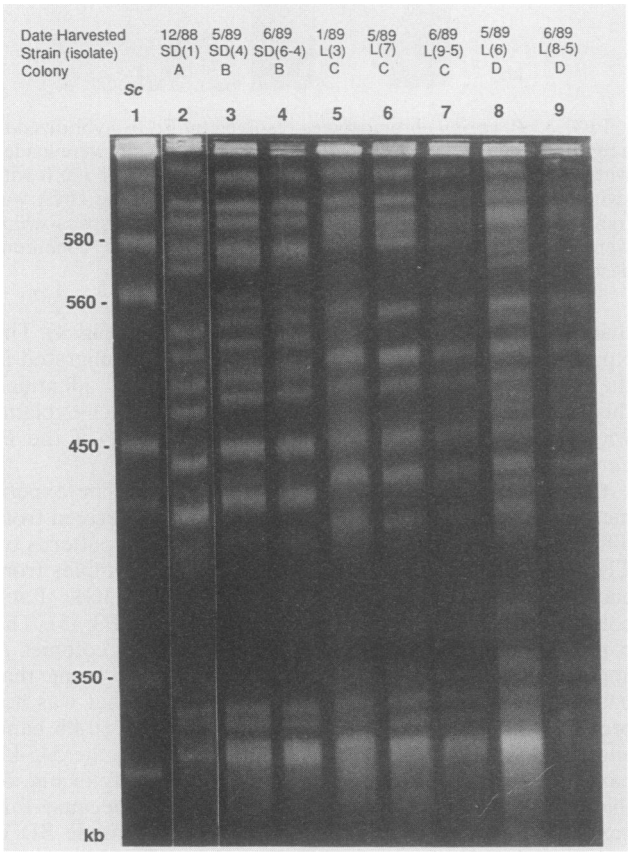

FIG. 5. Variation of band patterns among *P. carinii* samples from different rat colonies and stability of the pattern from a given colony over time. FIGE parameters were as described in the legend to Fig. 3. Note that lane 2 was from an earlier gel. The bands in sample SD(1) comigrated with colony B bands in experiments in which both were run on the same gel (Fig. 2A). *Sc*, *S. cerevisiae*.

The pattern of colony D, which also housed Lewis rats, differed from the other three colony patterns with respect to the number of bands, showing 22; however, the size range of these bands conformed to the range observed in the other three colonies (Fig. 6, lanes 1 through 6). The complexity of the D band pattern suggested that the animals of this colony harbored an infection caused by more than one type of *P. carinii* organism. A mixed infection was indicated by three observations. (i) The D pattern contained a set of bands that comigrated with the bands in the C pattern in addition to

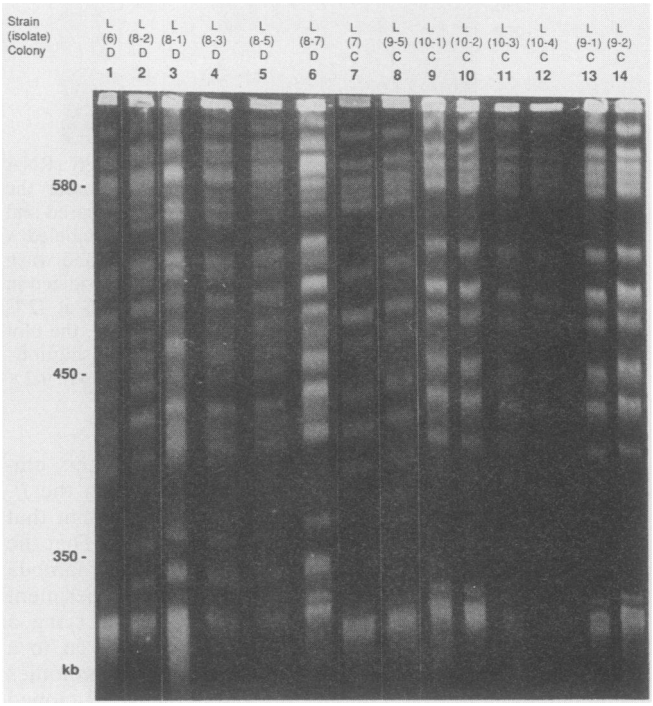

FIG. 6. FIGE analysis of multiple isolates from colonies C and D. The FIGE parameters used were as described in the legend to Fig. 3. The *P. carinii* isolates are described in Table 1.

several other bands. (ii) The rRNA oligonucleotide probe hybridized to two bands in the D pattern, one of which comigrated with the rRNA gene-containing band in the C pattern (Fig. 2A). (iii) All of the bands in the D pattern hybridized to the *P. carinii* repeat probe (Fig. 4B), which implies that all of these bands were from *P. carinii* and were not due to contaminants. The same pattern was produced whether pooled homogenates or isolates from individual rats were used, indicating that each animal in the D colony was infected by the same varieties of the organism in the same relative amounts.

Analysis of *P. carinii* from humans. Samples were prepared from six individuals. Four samples were collected as bronchoalveolar lavage fluids (samples 026, 020, 009, and 133), and two were prepared from lung tissue (samples 118 and 142). The CHEF band pattern produced by sample 142 is shown in Fig. 7A. The intense band that migrated near the 250-kb marker was of unknown origin and was not present in the other human-derived *P. carinii* sample that produced a band pattern (Fig. 7B).

Sample 142 was also analyzed by FIGE, along with the other five samples prepared from human sources (Fig. 7B). Only samples 133 and 142 produced FIGE band patterns. The patterns produced by samples 133 and 142 were different from each other and from any of the patterns produced by rat-derived *P. carinii*, although the sizes of the bands present in both human- and rat-derived specimens were within the same 300- to 700-kb range. The number of human-*P. carinii* bands in these samples appeared to be 10 to 12. Visualization of the *P. carinii* ethidium bromide-stained bands obtained from human sources was more difficult than analysis of rat-derived specimens because of problems inherent to human specimens, including low organism number, decreased organism viability, and suboptimal sample preservation.

Attempts to hybridize the bands seen in the human-derived samples to total rat-derived *P. carinii* DNA, to a *P. carinii* 18S rRNA probe, or to an oligonucleotide specific for the *P. carinii* 18S rRNA gene were unsuccessful. The failure of the 18S rRNA probe to hybridize to the human *P. carinii* bands suggests that the lack of hybridization was due to technical factors, such as poor transfer of the bands to the nylon membrane or low organism numbers. The rRNA gene probe would have been expected to hybridize to any eucaryotic 18S rRNA gene, and this probe did, in fact, hybridize to yeast DNA (Fig. 2B). When we performed reconstruction experiments in which diminishing numbers of rat-derived *P. carinii* organisms were subjected to PFGE analysis, followed by blot hybridizations, bands as faint as those seen in the PFGE patterns produced from human-derived *P. carinii* were not detected by the rRNA gene probe and were barely in evidence following hybridization to a radioactive probe prepared from total rat-derived *P. carinii* DNA. Further study of this issue will require isolation of more human-derived organisms, which have not yet become available.

Size estimates of *P. carinii* chromosomes. Figure 8 is a schematic representation of band patterns of *P. carinii* organisms from each of the four rat colonies resolved by CHEF. Although FIGE has superior resolving power, molecular sizes of *P. carinii* bands were estimated by CHEF because sizing of FIGE-resolved bands is complicated by unpredictable migration of some chromosomes due to unknown factors (3). In our FIGE experiments, lambda markers appeared to migrate according to size above 400 kb, but the band pattern was aberrant in the small-molecular-size range. *S. cerevisiae* chromosomes also migrated aberrantly on FIGE. By contrast, lambda and *S. cerevisiae* markers migrated according to size on CHEF gels. The CHEF run shown in Fig. 1 separated DNA molecules between 50 and 600 kb in a manner that allowed the molecular sizes of all but the largest bands in the *P. carinii* samples to be deduced by interpolation. A second CHEF analysis was performed to better estimate the sizes of the largest three bands (Fig. 4).

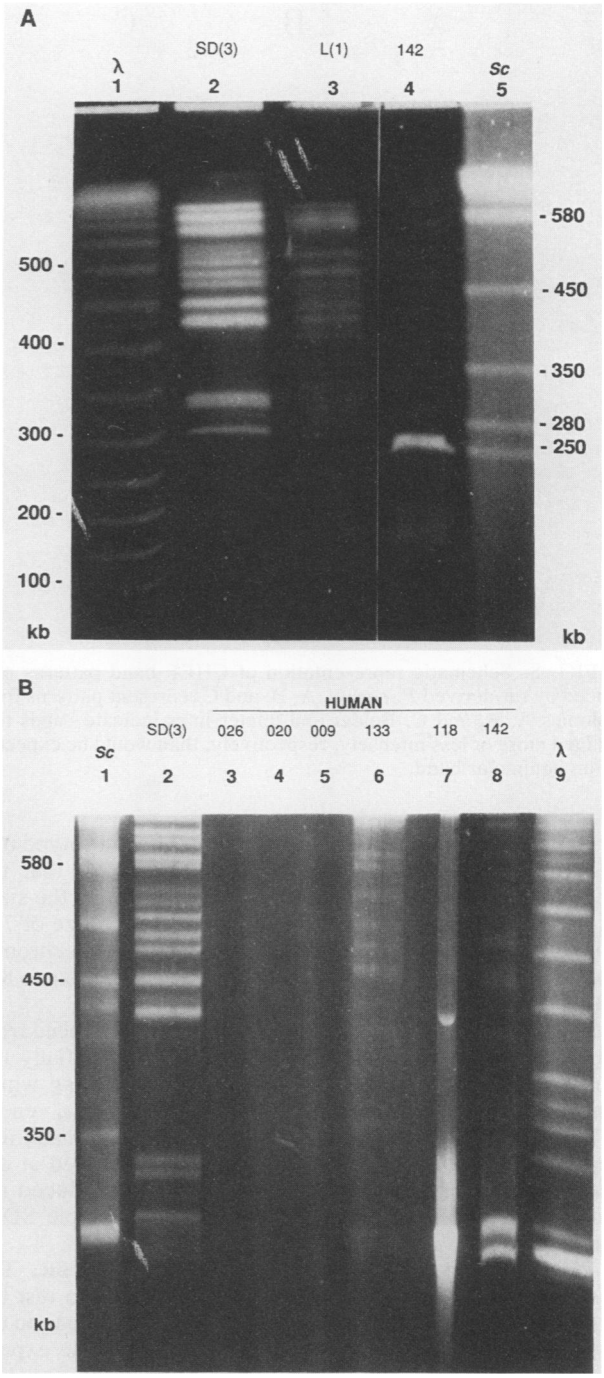

FIG. 7. Analysis of human-derived *P. carinii*. (A) CHEF through 1% agarose at 90 V for 96 h with an initial switching interval of 50 s gradually increasing to 100 s. Lane 4 contained a sample derived from human lung tissue. (B) The FIGE parameters used were as described in the legend to Fig. 3. Lanes 3 to 6 contained bronchoalveolar lavage fluid samples. Lanes 7 and 8 contained samples from human lung tissue. *Sc*, *S. cerevisiae*.

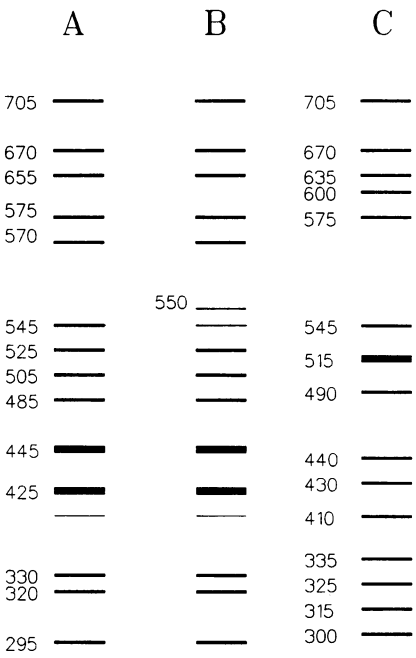

FIG. 8. Schematic representation of CHEF band patterns produced by rat-derived *P. carinii*. A, B, and C represent patterns from colonies A, B, and C. Bolder and fainter lines indicate bands that stained more or less intensely, respectively, than would be expected of an equimolar band.

If the simplest band pattern represents the karyotype of a *P. carinii* strain, patterns A, B, and C indicate that the genome contains 15 to 19 chromosomes. Summing the sizes of the 15 discrete bands gives a haploid genome size of 7×10^6 base pairs. Inclusion of putative comigrating chromosomes would increase the estimate of the genome size to 8×10^6 to 1×10^7 base pairs.

Stability of band patterns. The band pattern produced from *P. carinii* isolated from colony C remained essentially unchanged over a period of 6 months (Fig. 5), during which time new animals were received from the commercial vendor (Table 1), placed in the colony with infected animals, and rendered immunoincompetent. Organisms harvested at different times from other colonies also usually produced the same band pattern, the one exception being sample SD(8) (see below).

Band patterns were not strictly rat strain specific. The temporal constancy of band patterns allowed us to test the possibility that animals introduced into a colony acquired the organism from resident infected animals. The same experiment addressed the possibility that there might be Sprague-Dawley-specific *P. carinii* strains and Lewis-specific *P. carinii* strains. Lewis rats were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.), and upon arrival they were divided into two groups. Lewis rats from this vendor had been previously shown to produce *P. carinii* organisms with pattern C (Fig. 6, lanes 7 through 14). One group was placed with infected Lewis rats in colony C; the other group was housed with *P. carinii*-infected Sprague-Dawley rats in colony A. *P. carinii* band patterns from two individuals from each colony are shown in Fig. 9. As expected, the two Lewis rats placed in colony C yielded *P. carinii* organisms with pattern C (Fig. 9, lanes 6 and 7). The results obtained with Lewis rats placed in colony A were more complex but showed that Lewis rats can acquire *P. carinii* of the type characteristic of Sprague-Dawley rats residing in colony A. One animal produced *P. carinii* organisms with the characteristic A pattern (lane 4). The second Lewis rat from colony A produced a band pattern that suggested the presence of organisms typical of colony A, but the pattern contained additional bands, perhaps due to a mixed infection (lane 5). These results showed that Lewis rats can be infected by *P. carinii* formerly found only in Sprague-Dawley rats.

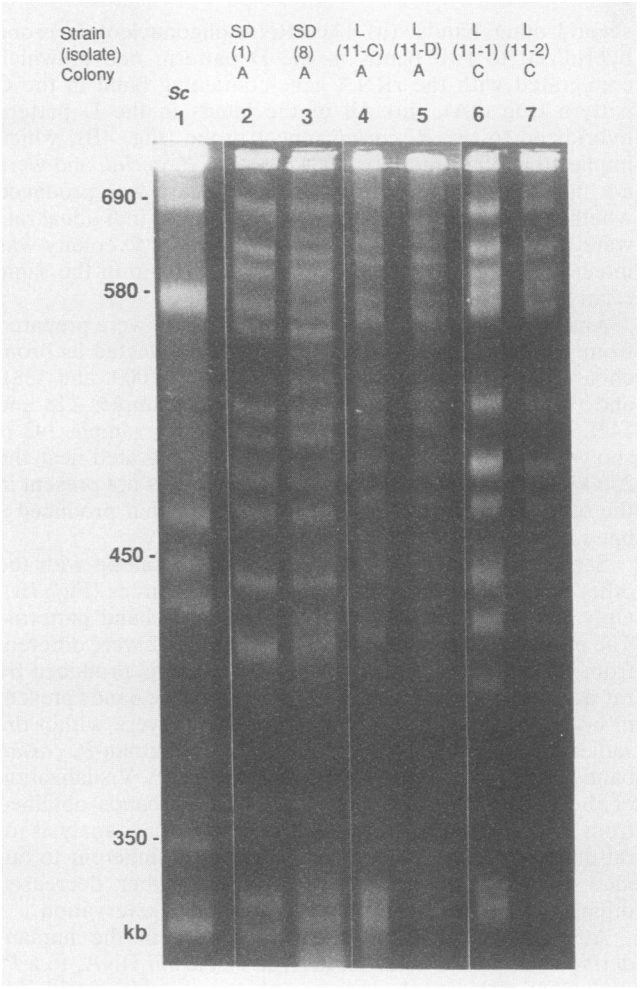

FIG. 9. Band patterns were not strictly rat strain specific. Organisms were prepared from Sprague-Dawley rats from colony A and from Lewis rats that had been housed in either colony C or colony A. The FIGE parameters used were as described in the legend to Fig. 3. *Sc*, *S. cerevisiae*.

Interestingly, a *P. carinii* isolate from a Sprague-Dawley rat from the A colony obtained at the same time as the two Lewis rat-derived *P. carinii* samples were obtained (Fig. 9, lane 3) also produced a band pattern different from that previously observed in *P. carinii* samples isolated from colony A (lane 2). This pattern appeared to contain all of the bands in the A pattern, as well as other bands, also suggesting a mixed infection. It is possible that this change in the band pattern was due to introduction of Lewis rats into the colony, but further experiments are necessary to identify the exact origin of the infecting organism.

***P. carinii* organisms with different PFGE band patterns show little restriction fragment length polymorphism in and around a repeated DNA sequence.** Variability in PFGE band

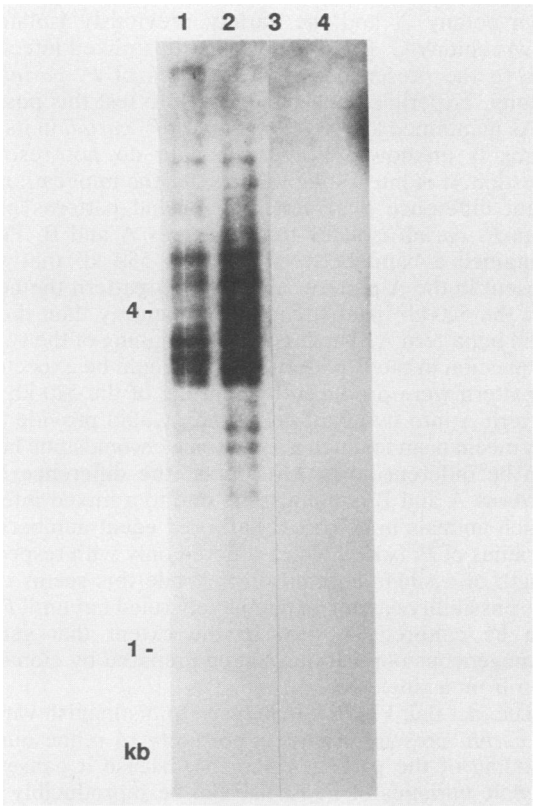

FIG. 10. Analysis of restriction fragments containing a sequence repeated in the *P. carinii* genome. Total DNAs from *P. carinii* samples SD(3) and L(7) and from cultured rat and human cell lines were digested to completion with *Eco*RI. Approximately 1 μg of each digested DNA sample was electrophoresed through 1% agarose. The DNA was transferred to a nylon membrane and hybridized to radioactive Rp3-1 DNA as described in Materials and Methods. Lanes: 1, SD(3); 2, L(7); 3, human; 4, rat.

patterns is common among members of the kingdom *Protista*. While the functional ramifications of such variability are generally unclear, studies on *S. cerevisiae* have established that a single species can display considerable variability in PFGE band patterns (4, 8). The variability displayed by *P. carinii* isolates may be a similar phenomenon. Analysis at the DNA sequence level should provide an independent indication of the genetic relatedness of *P. carinii* isolates that produce highly dissimilar PFGE band patterns.

To examine genetic relatedness at the DNA sequence level, we used the cloned *P. carinii* repeat Rp3-1 (see above; Fig. 3) to probe for restriction fragment length polymorphisms between a *P. carinii* sample that exhibited the B band pattern and a sample that exhibited the C band pattern. DNA was prepared from each sample, digested to completion with *Eco*RI, electrophoresed through a 0.7% agarose gel which was blot transferred onto a nylon membrane, and hybridized to the *P. carinii* repeat probe. The size distributions of the DNA fragments scanned by this repetitive probe were similar (Fig. 10).

Although the function of the repeated sequence family in question is not known, it seems likely that the *P. carinii* repeat does not encode essential gene products. Consistent with this supposition, preliminary experiments indicated that the repeat sequence is not represented in *P. carinii* RNA (data not shown). Since it is not highly expressed, the repeat might be expected to be among the more variable sequences in the *P. carinii* genome. However, the sizes of restriction fragments carrying copies of the Rp3-1 repeat appeared to be more similar than the PFGE band patterns. This suggests that the *P. carinii* isolates from colonies B and C are probably less divergent than they appear to be on the basis of karyotype comparisons.

DISCUSSION

PFGE of *P. carinii* organisms prepared from infected lung tissue produced distinct band patterns. While PFGE is a standard technique that has been used to define the molecular karyotypes of numerous and diverse protists (2, 4, 12, 19, 27, 34, 35, 39), the vagaries of working with *P. carinii* engender circumspection in interpreting these data. The first issue to resolve is the identities of the PFGE bands. Do they originate from *P. carinii*? Several lines of evidence support the conclusion that the bands resolved by PFGE are *P. carinii* chromosomes. (i) The preparations subjected to PFGE contained no detectable eucaryotic microbes other than *P. carinii*. (ii) Previous sequence analysis of RNA isolated from *P. carinii* prepared by the methods used in this study showed that this RNA was from *P. carinii*. (iii) When total DNA prepared from *P. carinii* was inserted into a lambda phage vector, the resulting library was essentially free of rat sequences and four of four clones selected by hybridization to rRNA proved to contain *P. carinii* rRNA genes. (iv) Oligonucleotide probes specifically complementary to *P. carinii* 18S rRNA genes hybridized to a single band in three of the four PFGE band patterns observed and hybridized to two bands in the pattern that appeared to be derived from more than one type of *P. carinii*. (v) All of the bands that migrated between 300 and 700 kb hybridized to a cloned copy of a repeated sequence isolated from the *P. carinii* genomic library. This repeat did not hybridize to DNA from either rats or *S. cerevisiae*, indicating that the repeat was not something like a telomere that might not be specific for *P. carinii* DNA. (vi) When *P. carinii* samples did contain rat DNA, the contaminating host molecules migrated either faster or slower than the *P. carinii* bands and were readily distinguishable by hybridization to labeled rat DNA.

Another important consideration in discussing the results of PFGE analysis of *P. carinii* is that interpretation of such data must be qualified by recognition that the organism populations might not be composed of genetically identical *P. carinii* strains. The potential for heterogeneity in *P. carinii* preparations cannot be eliminated because the organism cannot be grown clonally in in vitro culture. Consequently, a PFGE band pattern produced from a given *P. carinii* preparation could represent either the chromosomal complement of a single genetic variety of the organism, i.e., the karyotype, or a composite karyotype composed of chromosomes from two or more genetic varieties of *P. carinii*. In addition, since the ploidy of the various morphological forms of *P. carinii* is not known, PFGE band patterns may represent a composite of the contents of haploid and diploid forms of the organism.

In view of the potential for heterogeneity in *P. carinii* preparations, the PFGE band patterns obtained were strikingly distinct and reproducible. Furthermore, with few exceptions, the staining intensities of the bands within each pattern were consistent with what would be expected from a collection of equimolar chromosomes. The distinctness, reproducibility, and stoichiometry of the PFGE band patterns from colonies A, B, and C support the notion that each of these patterns is an accurate indication of each of three

distinct *P. carinii* karyotypes. The alternative view, that the band patterns are each a composite formed by contributions from more than one karyotype, seems highly unlikely, because for such a situation to occur, it would be necessary for these hypothetical mixed infections to be both obligatory and invariant with respect to the relative amount of each contributing microbe.

If patterns A, B, and C are taken to be *P. carinii* karyotypes, it appears that the genome of this organism is composed of 15 to 19 chromosomes which, in the aggregate, contain approximately 10^7 base pairs of DNA. The largest chromosome detected was approximately 700 kb. Others have reported that *P. carinii* contains 20 chromosomes; 18 between 300 and 700 kb and 2 between 1 and 1.5 megabases (46). We too observed megabase-pair bands in some *P. carinii* preparations, but hybridization experiments determined that these bands were from the rat host. It is not clear whether the megabase-pair bands reported by Yoganathan and Buck (46) were from *P. carinii* or from the rat.

The distinct and reproducible band patterns produced from colonies A, B, and C suggest that in the immunosuppressed rat model, pathological infections often arise from a single dominant variety of *P. carinii*. By analogy with the results obtained with rat-derived *P. carinii*, it appears that the two *P. carinii* populations isolated from humans were each composed largely of a single variety of the organism but that each individual harbored a distinct type of *P. carinii*.

When these findings are considered, two questions immediately arise. What is the source of the dominant *P. carinii* variety found in a given colony, and how is the dominant variety maintained in a colony over time? While the data do not allow definitive answers, two factors probably contribute. The rats that were used to establish a given colony probably already carried a dominant variety of *P. carinii*. Low-level infection of rodents appears to be commonplace (16). Rats used in this study were obtained exclusively from specific commercial vendor colonies. With the exception of one experiment in which Lewis rats were housed with Sprague-Dawley rats, colonies in this study were always restocked with rats from the vendor colony used to found the colony. This practice is expected to minimize the possibility of heterogeneity in the *P. carinii* isolated from a given colony, provided that the animals residing in the vendor colonies also harbor a particular variety of *P. carinii*. Suggestive evidence that this is the case comes from the observation that the PFGE band patterns of *P. carinii* isolated from colonies A and B, which both housed Sprague-Dawley rats obtained from a specific colony at Harlan Industries, were nearly identical, although colony B was established years before colony A.

The second factor that may contribute to the maintenance of a distinct *P. carinii* variety in a colony is transmission of organisms from heavily infected animals to newcomers. Data supporting this idea include (i) previous reports that *P. carinii* pneumonia can be transmitted to uninfected rats kept either in close (cagemates) or distant (in the same room) contact (15, 44), (ii) our previous observation that maintenance of a reliably infected rat colony requires the presence of heavily infected animals in the colony at the time new animals are introduced, and (iii) the observation reported here that Lewis rats kept with Sprague-Dawley rats acquired *P. carinii* previously found only in a Sprague-Dawley colony (colony A). In the same experiment, a colony A Sprague-Dawley animal sampled after the introduction of Lewis animals was infected by what appeared to be a combination of two *P. carinii* varieties, the variety previously isolated from colony A and the variety previously isolated from Lewis colony C. It is possible that this mixed infection was due to the presence of two varieties of *P. carinii* in the colony. Experiments are under way to test this possibility.

As mentioned above, the ploidy of *P. carinii* in its various forms is unknown. While our data do not resolve this question, it is interesting to consider the implications of the slight difference observed in the band patterns produced from *P. carinii* isolates from colonies A and B. Pattern B contained a band between 520 and 550 kb that was not present in the A pattern, and in the B pattern the new band and the 520-kb band stained less intensely than the 520-kb band in pattern A. The less intense staining of the two bands in question in the B pattern is what would be expected if the B pattern were produced by splitting of the 520-kb band in pattern A into two bands. Diploidy would provide an obvious mechanism for such a split, since chromosome homologs can be different sizes. Of course, the difference between patterns A and B could also be due to a mixed infection in which animals in colony B harbored equal numbers of two varieties of *P. carinii* which differed only with respect to the length of a single chromosome. While this seems unlikely, this possibility cannot be completely ruled out until *P. carinii* can be cultured in vitro to the extent that genetically homogeneous populations can be prepared by clonal expansion from a single cell.

The fact that PFGE can be used to distinguish varieties of *P. carinii* presents a new opportunity to refine our understanding of the pathogen and the disease it causes. Since specific varieties of *P. carinii* can be reproducibly isolated from infected rat colonies over extended periods of time, it should be possible to distinguish between transmission of the organism and activation of a persistent resident strain. PFGE analysis of *P. carinii* isolated from humans holds similar promise as an epidemiological tool. Immunoblotting studies of humans with *P. carinii* pneumonia indicate that recurrent episodes of the disease are accompanied by changes in *P. carinii* antigens (29). A number of mechanisms could lead to these changes, including selective pressure exerted by chemotherapy and infection by more than one variety of *P. carinii*. It should be possible to use molecular karyotyping to examine possible correlations between pathogenicity and response to therapy, to distinguish relapses from reinfection, and to investigate outbreaks or clusters of cases.

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