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Genetic Diversity of Avian and Mammalian *Chlamydia psittaci* Strains and Relation to Host Origin

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Genetic relationships were reported for *Chlamydia psittaci* derived from psittacine birds, pigeons, turkeys, humans, cats, muskrats, cattle, and sheep and for *C. trachomatis*, including representative strains of the three biovars, through physical analysis of genomic DNA including DNA fingerprinting with restriction endonuclease *SalI*, DNA-DNA hybridization in solution with S1 nuclease, and Southern analysis with genomic DNA probes. A total of 26 strains were divided into four groups of *C. psittaci* and two groups of *C. trachomatis*, on the basis of DNA fingerprints. The six groups of *Chlamydia* spp. were related to host origin: two avian groups (Av1 and Av2), one feline and muskrat group (Fe1), one ruminant group (Ru1), one *C. trachomatis* biovars trachoma and lymphogranuloma group (CtHu), and one *C. trachomatis* mouse biovar group (CtMo), although an ovine abortion strain belonged to the avian group Av2. DNA-DNA hybridization assay and Southern analysis with genomic DNA probes indicated three DNA homology groups in the genus *Chlamydia*: an avian-feline group (groups Av1, Av2, and Fe1), a ruminant group (group Ru1), and a *C. trachomatis* group (groups CtHu and CtMo). Furthermore, the Southern analysis indicated that the homologous sequences (DNA homology of at least 14%) within the avian-feline group were distributed along the whole genome, whereas the homologous sequences (DNA homology of less than 24%) among the three DNA homology groups were localized in distinct regions of the genome DNA. These results suggest that *Chlamydia* spp. are derived from a common ancestor and have diverged into various groups showing restricted host ranges as a natural characteristic and that the species *C. psittaci* should be differentiated into groups related to host origin and DNA homology.

The genus *Chlamydia* comprises two species, *Chlamydia psittaci* and *Chlamydia trachomatis* (23). Differentiation of the two species is based on natural hosts, inclusion morphology, glycogen in inclusions, and folate biosynthesis (23). In the genus *Chlamydia*, interspecies DNA-DNA homology is less than 10%, while intraspecies DNA-DNA homology is almost 100% (12), although the mouse pneumonitis strain which is the mouse biovar of *C. trachomatis* showed only 30 to 60% DNA-DNA homology with other strains of the same species (29). The DNA-DNA homology of *C. psittaci* was, however, estimated for only two avian-related strains, California 10 (designated Frt-Hu/Cal10 in the present study) and 6BC (Prk/6BC in the present study) (12), despite the isolation of *C. psittaci* from a variety of animals and birds and despite a wide range of guanine and cytosine content (40 to 44 mol% G+C) (9). Immunological and biological analyses indicated phenotypic heterogeneity of *C. psittaci* (14, 27). Recent analyses of DNA fingerprints also showed differences among several strains of *C. psittaci* (1, 18, 28).

We have reported a series of studies for establishing a systematic classification of *C. psittaci*, such as immunological typing with monoclonal antibodies (8) and immunoblotting analysis with polyclonal antisera (7). We found at least two avian (major outer membrane protein types 1 and 2) and two mammalian (major outer membrane protein types 3 and 4) immunological types in *C. psittaci* through the immunoblotting analysis (7). Our preliminary analysis showed a large difference among avian and mammalian strains in DNA fingerprinting patterns (6). It is thus interesting to investigate genetic relationships among avian and mammalian *C. psittaci* strains.

The aim of this research is to find general genetic relationships among chlamydiae. We investigated genetic relationships among *Chlamydia* spp. from various origins by DNA fingerprinting with restriction endonucleases producing a simple pattern and by DNA-DNA hybridization. In this report, we show DNA divergence in *C. psittaci* and *C. trachomatis* and relatedness between the genetic groups and their host origins. The results indicate that the genus *Chlamydia* should be classified into several groups according to host origin.

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MATERIALS AND METHODS

Chlamydiae. The chlamydiae used were 23 strains of *C. psittaci* and 3 strains of *C. trachomatis* (Table 1). The host, geographic, and chronological origins of most strains were described in our previous study (7). Additional strains used in this work were Prk/6BC (20), Bo/E58 ATCC VR 628 (19), Bo/Maeda, Bo/Shizuoka (22), Ov/B577 ATCC VR 656 (26), *C. trachomatis* E/UW-5/Cx, and mouse pneumonitis Weiss-Nigg (21).

Cultivation and purification of chlamydiae. Most strains were propagated in L cells cultured in suspension form or in HeLa 229 cells as described previously (7). Bo/Maeda and Bo/E58 were adapted to HeLa 229 and Madin-Darby bovine kidney cells, respectively. Prk/Daruma, Ov/B577, and Bo/Shizuoka were adapted to L929 cells. DNAs of the strains were prepared from cultures of fifth to seventh passages in the line cells.

Procedures for cultivation and purification of elementary bodies were described previously (7), and a nuclease digestion step was included as follows. The harvested elementary bodies were treated with 20 μg of DNase I per ml and 10 μg of RNase A per ml in 0.25 M sucrose–0.01 M potassium phosphate–5 mM glutamate (pH 7.2) solution containing 10

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TABLE 1. DNA fingerprint groups of chlamydiae

Species and fingerprint type	Strain	Origin[a]	MOMP type[b]
C. psittaci			
Av1a	Prt/GCP-1	Parrot/systemic infection/Japan	1
	Bd/36	Budgerigar/healthy/Japan	1
	Bd/275	Budgerigar/healthy/Japan	1
	Bd/286	Budgerigar/healthy/Japan	1
	Hu/Itoh	Human, budgerigar/psittacosis/Japan	1
	Tk/CA	Turkey/ornithosis/United States	1
Av1b	Frt-Hu/Cal10	Ferret, human/cold/United States	1
	Pgn/P1041	Pigeon/healthy/Japan	1
	Pgn/PCM69	Pigeon/feces/Japan	1
Av1c	Prk/6BC	Parakeet/United States	2
	Ckt/Okame	Cockatiel/systemic infection/Japan	2
	Tk/NJ	Turkey/Ornithosis/United States	2
	Hu/Borg ATCC VR 601	Human/psittacosis/United States	2
Av2a	Prk/Daruma	Parakeet/systemic infection/Japan	3
Av2b	Ov/B577 ATCC VR 656	Sheep/abortion/United States	3
Fe1	Fe/Pn-1 ATCC VR 120	Cat/pneumonia/United States	3
	Fe/145	Cat/conjunctivitis/United States	3
	Mu/M56 ATCC VR 630	Muskrat/systemic infection/Canada	3
Ru1a	Bo/Yokohama	Cattle/enteric infection/Japan	4
	Bo/Maeda	Cattle/pneumonia/Japan	5
	Bo/Shizuoka	Cattle/encephalitis/Japan	5
Ru1b	Ov/IPA ATCC VR 629	Sheep/polyarthritis/United States	6
Ru1c	Bo/E58 ATCC VR 628	Cattle/encephalitis/United States	6
C. trachomatis			
CtHu	E/UW-5/Cx	Human//United States	Ct1
	L2/434/Bu	Human//United States	Ct1
CtMo	MoPn/Weiss-Nigg	Mouse/pneumonitis/United States	Ct2

[a] Origin is shown as follows: host animal or bird/clinical condition/geographic origin.
[b] MOMP, Major outer membrane protein.

mM $MgCl_2$ for 20 min at 37°C; then twice-crystallized trypsin was added to the solution at a concentration of 200 μg/ml, and the solution was incubated for 20 min at 37°C. The enzyme-treated elementary bodies were purified by sucrose density gradient ultracentrifugation. The purified elementary bodies were finally suspended in a small volume of 10 mM Tris hydrochloride (pH 7.4) and were used directly for DNA preparation or were stored at −80°C until use. The protein content of each sample was assayed by the method of Lowry et al. (16), using bovine serum albumin as the standard.

Extraction of DNA. The purified elementary body suspension (4 to 5 mg of protein per ml) was added to final concentrations of 200 μg of proteinase K per ml, 20 mM EDTA (pH 8.0), 5% 2-mercaptoethanol, and 0.5% sodium *N*-laurylsarcosine. After digestion at 45°C for 1 h, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the proteinase K-digested elementary bodies solution, and the mixture was emulsified. After three extractions, the aqueous phase was treated with DNase-free RNase A (17) at a concentration of 1 μg/ml at 37°C for 30 min. The solution was again extracted three times with phenol-chloroform-isoamyl alcohol. Finally, the solution was dialyzed for 24 h at 4°C against a sufficient amount of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA.

Restriction endonucleases and DNA fingerprinting. The restriction endonucleases used were *Bam*HI, *Bgl*II, *Dpn*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Mbo*I, *Mlu*I, *Nar*I, *Nco*I, *Nru*I, *Pvu*II, *Sac*I, *Sau*3AI, *Sal*I, *Sma*I, *Stu*I, *Xba*I, and *Xho*I, which were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany; New England BioLabs, Inc., Beverly, Mass.; Nippon Gene Co., Ltd., Toyama, Japan; Takara Shuzo Co., Ltd., Kyoto, Japan; and Toyobo Co., Ltd., Osaka, Japan. They were used according to the instructions of the suppliers. DNA was completely digested at a concentration of 80 to 120 μg/ml with 4 U of restriction endonuclease per μg of DNA at an optimal temperature for 2 h. Submerged horizontal agarose gel electrophoresis was performed with SeaKem GTG agarose and Tris-acetate-EDTA or 0.5× Tris-borate-EDTA buffer (17), using restricted bacteriophage λ and T4dC DNAs (24) for the standards. The agarose gels were stained in ethidium bromide solution (0.5 μg/ml) for a few hours, and photographs were taken. For *Dpn*I, *Mbo*I, and *Sau*3AI digests, polyacrylamide gel electrophoresis was done by the method of Laemmli (15) followed by silver staining (Silver Stain Kit; Bio-Rad Laboratories, Richmond, Calif.).

Preparation of probe DNA. DNA was labeled by a random priming method (5) with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.) for free-solution DNA hybridization and with digoxigenin-dUTP (DNA labeling and detection kit, nonradioactive, Boehringer Mannheim-Yamanouchi, Japan) for Southern analysis. The specific activities of radioactive probes were from 7.7×10^7 to 9.0×10^7 dpm/μg of DNA. Each radioactive probe was used on the day it was prepared.

Free-solution DNA hybridization and S1 nuclease assay. DNA-DNA homology was estimated by free-solution hybridization assay with S1 nuclease by the method of Crosa et al. (3), with minor modifications. The unlabeled DNA (cold DNA) was sheared with a needle to a fragment size of about 500 base pairs. Probe DNA (0.15 ng) was mixed with 3 μg of cold DNA. Herring sperm DNA was also used as a control. The reaction volume was 30 μl in 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered 0.44 M NaCl (pH 7.0) with a 50-μl overlay of liquid

paraffin to prevent evaporation during the incubation at 65°C for 16 h. Reassociation of the DNA was stopped by quick chilling in ice water. A 20-μl portion was transferred into a tube containing 1 ml of S1 buffer (50 mM sodium acetate–200 mM NaCl–1 mM $ZnSO_4$, pH 4.6) and 25 μg of denatured herring sperm DNA per ml. Appropriate units of S1 nuclease were added to the tubes, and they were then incubated at 50°C for 1 h. Trichloroacetic acid-insoluble radioactivity was measured with a liquid scintillation counter. The percent reassociation was calculated by the following equation: {[(average counts of sample DNA) − (average counts of herring sperm DNA)]/[(average counts of homologous DNA) − (average counts of herring sperm DNA)]} × 100.

Southern hybridization analysis of genomic DNA. DNA was transferred to a sheet of nylon membrane (Hybond N; Amersham Japan Corp., Tokyo, Japan) by using the vacuum transfer method with VacuGene (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden) without depurination, following the instructions of the manufacturer. Hybridization was carried out at 65°C for 16 to 20 h in 0.75 M sodium ion buffer. After hybridization, the sheets were washed two times at room temperature for 5 min in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate, followed by two 15-min washes at 65°C in 0.1× SSC containing 0.1% sodium dodecyl sulfate. Immunological detection of hybridized digoxigenin-labeled probe was performed with the commercial kit.

RESULTS

Fingerprinting of strain Frt-Hu/Cal10 DNA with various restriction endonucleases. We tested the susceptibility of chlamydial DNA to a variety of restriction endonucleases as an initial step in characterizing the DNA. Frt-Hu/Cal10 DNA was examined with a total of 20 restriction endonucleases (Fig. 1). The number of restriction fragments was consistent with values predicted from 41 mol% G+C of the DNA and recognition sequences of the enzymes. Two of the three isoschizomers, *Mbo*I and *Sau*3AI, restricted more than 200 fragments of less than 2.0 kilobases (kb), but *Dpn*I did not digest the DNA of Frt-Hu/Cal10. Digestion with *Sal*I presented a fingerprint simple enough to use for comparing the overall chromosomal DNA structures of various strains. We selected this restriction endonuclease, *Sal*I, for the present study.

Comparison of *Chlamydia* spp. with the restriction endonuclease *Sal*I. The fingerprints with *Sal*I exhibited four completely different patterns for *C. psittaci* and two patterns for *C. trachomatis* (Fig. 2). Similar results were obtained by *Eco*RI digestion of the DNAs and the other enzymes. We thus classified the 26 *Chlamydia* strains into four groups of *C. psittaci*, which are designated Av1, Av2, Fe1, and Ru1 and into two groups of *C. trachomatis*, which are designated CtHu and CtMo (Table 1).

Except for one strain, Prk/Daruma, the fingerprints of avian and human psittacosis strains were similar to each other, with minor differences of several fragments (Fig. 2). These avian and human psittacosis strains were in group Av1. Prk/Daruma was in group Av2, which included an ovine abortion strain (Fig. 2). These two strains showed greater homology with the other avian strains, as described below. Both feline strains and the muskrat-derived Mu/M56 ATCC VR 630 formed group Fe1, showing identical fingerprints (Fig. 2). Similar but not identical fingerprints were observed for bovine and ovine strains, which were classified as Ru1 (Fig. 2; Table 1). Strains of *C. trachomatis* biovars

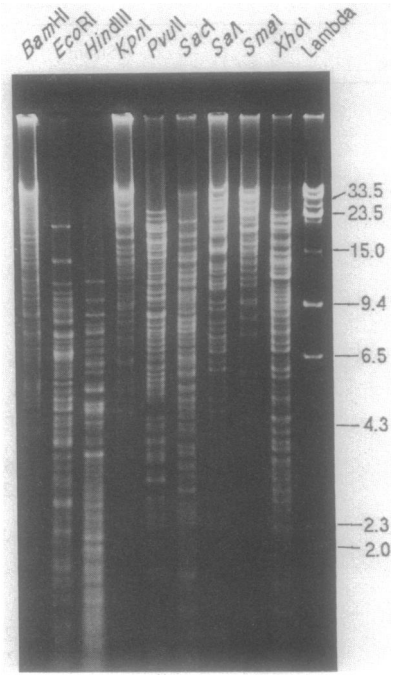

FIG. 1. DNA restriction endonuclease susceptibility of chlamydial DNA. DNA from strain Frt-Hu/Cal10 was digested with the indicated restriction endonucleases. The digests were electrophoresed in 0.6% agarose gel. The rightmost lane was a *Hin*dIII digest of bacteriophage λ DNA for fragment length standards (shown in kilobases).

trachoma and lymphogranuloma had similar fingerprints, whereas the strain of mouse biovar showed fingerprints different from those of the other *C. trachomatis* strains.

*Sal*I fingerprints of groups Av1, Av2, and Ru1 displayed several subpatterns (Fig. 2). Hence, group Av1 was further classified into the three subgroups of parrot, budgerigar, and turkey strains (Av1a), pigeon strains (Av1b), and turkey and parakeet strains (Av1c), whereas group Av2 was subgrouped into the parakeet strain (Av2a) and the ovine strain (Av2b) (Table 1). Each of the three human psittacosis strains belonged to a different Av1 subgroup. Group Ru1 was also subdivided into three subtypes, Ru1a to Ru1c (Table 1).

DNA-DNA homology among the representative strains of each group of fingerprint patterns. The DNA fingerprinting revealed unexpected diversity of the DNA of *C. psittaci*. Accordingly, we evaluated the DNA-DNA homology among the *Chlamydia* spp. (Table 2). Members of Av1 showed over 70% DNA-DNA homology, and they shared 27 to 85% homology with the strains of Av2. Fe1 also exhibited 14 to 33% homology to Av1 and Av2, whereas Ru1, CtHu, and CtMo showed less than 28% homology with other strains examined.

The DNA-DNA homology evaluated by Southern analysis with genomic probes revealed two kinds of distribution of the homologous sequences on chromosomal DNA among the strains (Fig. 2, 3, and 4). One is the averaged distribution along the entire genome as seen among Av1, Av2, and Fe1 (Fig. 2B and 4). The other is the localized distribution on distinct regions of the genome as seen among a group of avian and feline strains, a group of ruminant strains, and a group of *C. trachomatis* strains (Fig. 2C and 3B). The conserved regions were observed mainly as *Sal*I fragments

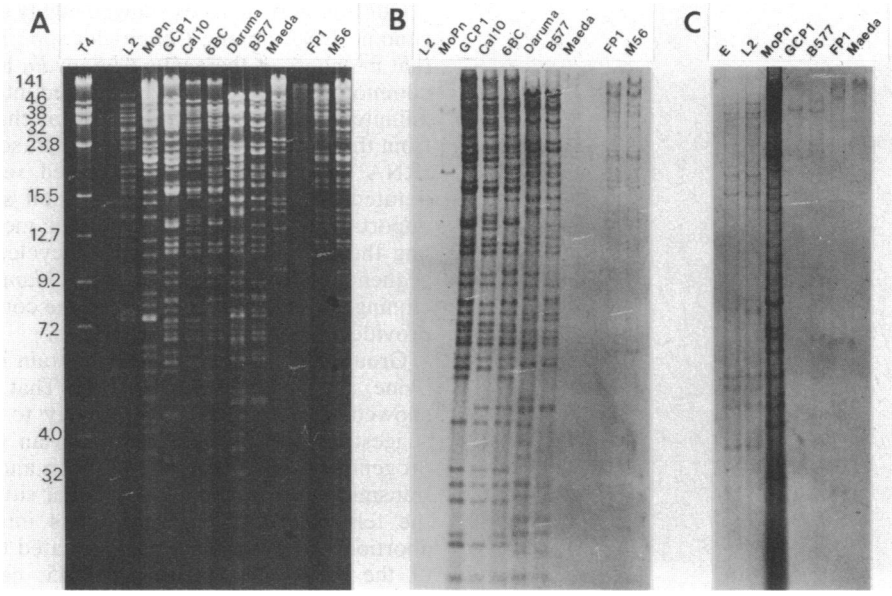

FIG. 2. DNA fingerprints and Southern blot of chlamydial DNA digested with restriction endonuclease *Sal*I. DNAs were digested with *Sal*I and electrophoresed in 0.5% agarose gel. (A) Gel stained with ethidium bromide. (B) Southern blot of the gel in panel A probed with digoxigenin-labeled Prt/GCP-1 genomic DNA. (C) Southern blot probed with digoxigenin-labeled Ct/MoPn genomic DNA. The leftmost lane is a mixture of *Kpn*I, *Sal*I, and *Sma*I digests of bacteriophage T4dC DNA for fragment length standards (shown in kilobases).

of 45 kb (Av1); 56 and 42 kb (Av2); 58 kb (Fe1); 52 kb (Ru1); 16.5 and 14.3 kb (CtHu); and 37, 17.5, and 11.5 kb (CtMo), and as *Eco*RI fragments of 2.0, 13.5, and 22 kb (Av1a, Av1c, and Av2); 2.0, 7.6, and 12.1 kb (Av1b); 2.0, 2.8, 3.5, 4.1, and 7.0 kb (Fe1); 2.0 and 4.0 kb (Ru1a); 2.0 and 3.9 kb (Ru1b); 3.0, 3.5, 3.8, 5.6, and 7.0 kb (CtHu); and 3.4, 3.7, 4.6, 6.6, 7.2, 7.6, and 8.0 kb (CtMo). These results indicate that the genus *Chlamydia* includes at least three groups defined by DNA homology: an avian and feline group, a ruminant group, and a *C. trachomatis* group.

TABLE 2. DNA homology among representative strains of each fingerprint type

Chlamydial DNA source	% Homology with probe DNA from strain[a]:									
	GCP-1	Cal10	6BC	Daruma	B577	FP-1	Maeda	L2	E	MoPn
Av1a										
Prt/GCP-1	100	79	100	44	40	31	3			3
Hu/Itoh	82	73	87							
Av1b										
Frt-Hu/Cal10	89	100	86	50	27	31	1	33	23	3
Pgn/P1041	104	94	95							
Av1c										
Prk/6BC	90	93	100	65	34	31	10	15		4
Hu/Borg	78	94	82							
Av2a Prk/Daruma	68	50	80	100	100					
Av2b Ov/B577	68	46	85	61	100	33	1		15	2
Fe1										
Fe/Pn-1		33		23	14	100	12	24	18	1
Fe/145						100				
Mu/M56						100				
Ru1a Bo/Maeda		19		7	1	9	100	32	4	1
Ru1b Ov/IPA							100			
Ru1c Bo/E58							88			
CtHu										
L2/434/Bu		28		7	5	24	10	100	100	24
E/UW-5/Cx		27						92	100	20
CtMo MoPn		20		2	6	10	4	29	52	100

[a] Abbreviations: GCP-1, Prt/GCP-1; Cal10, Frt-Hu/Cal10; 6BC, Prk/6BC; Daruma, Prk/Daruma; B577, Ov/B577; FP-1, Fe/Pn-1; Maeda, Bo/Maeda; L2, L2/434/Bu; E, E/UW-5/Cx; MoPn, MoPn/Weiss-Nigg.

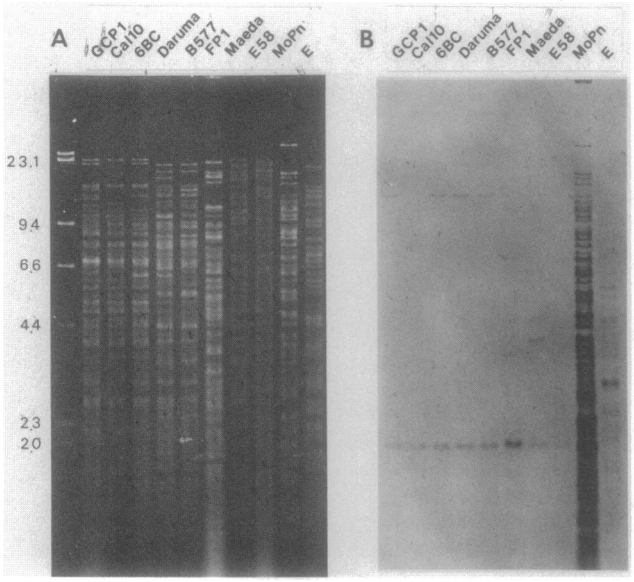

FIG. 3. Hybridization of genome DNA probes to the DNAs of *C. psittaci* and *C. trachomatis* digested with *Eco*RI. (A) Gel stained with ethidium bromide. (B) Southern blot of the gel in panel A probed with Ct/MoPn genomic DNA. The leftmost lane is bacteriophage λ DNA digested with *Hin*dIII for fragment length standards (shown in kilobases).

DISCUSSION

We have identified the four DNA fingerprint groups in *C. psittaci* and two groups in *C. trachomatis* with restriction endonuclease *Sal*I. The DNA-DNA hybridization confirmed the heterogeneity and defined at least three genetic groups of chlamydiae. Each of the DNA fingerprint types except Av2 was related to the host origin.

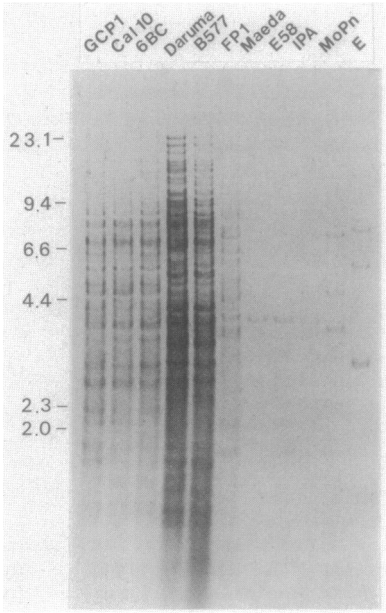

FIG. 4. Hybridization of Ov/B577 genome DNA probes to the DNAs of *C. psittaci* and *C. trachomatis* digested with *Eco*RI. Sizes (in kilobases) of a *Hin*dIII digest of bacteriophage λ DNA are shown at the left.

Our Southern analysis showed highly conserved regions of genome DNA among *Chlamydia* spp. This result indicates that members of the genus *Chlamydia* have evolved from a common ancestor and have diverged into groups possessing a limited host specificity as a natural characteristic. Judging from the size of the *Eco*RI fragments, some of them may be rRNA genes (4). Other conserved regions may contain iterated sequences such as insertion sequences or more-important genes related to the basic mechanism of controlling the chlamydial development cycle, although insertion sequences have not been found in *Chlamydia* spp. Molecular cloning and sequence analysis of the conserved regions may provide answers to these questions.

Group Av2 included the avian strain Prk/Daruma and the ovine abortion strain Ov/B577. That these two strains showed high DNA-DNA homology to other avian strains suggests the transmission of an avian *C. psittaci* strain, a progenitor of Prk/Daruma, to sheep and the fixation of the transmitted agent to sheep. A similar situation is possible for the feline strains examined. It is interesting that ovine abortion *C. psittaci* genetically related to Ov/B577 and one of the feline strains used, Fe/145, caused abortion and conjunctivitis, respectively in humans (10, 25). Therefore, human pathogenic *C. psittaci* would possess the genetic background of avian *C. psittaci*.

Our result indicating almost no genetic relatedness among several strains of *C. psittaci* disagrees with the earlier observations that intraspecies DNA-DNA homology of *C. psittaci* was almost 100% (12, 29), but the earlier data were obtained from experiments with only two strains of *C. psittaci*, Frt-Hu/Cal10 and Prk/6BC, both of which belonged to the same DNA fingerprinting group, Av1. Therefore, failure to find any intraspecies heterogeneity of DNA-DNA homology was to be expected. Recent data by Cox et al. (2) showed values of DNA-DNA homology among avian, ovine abortion, and feline strains similar to those presented in this study.

We have been classifying the strains of *C. psittaci* (6–8). The results of immunological and genetic classifications are in good agreement, as shown in Table 1. The classification of bacterial species has been reevaluated at the level of DNA-DNA homology, rRNA-DNA homology, and sequencing of rRNA as taxonomic tools in this decade (13, 30). Our data showing less than 30% DNA-DNA homology among each genetic group of *C. psittaci* suggest that the genetic groups should be regarded as species (11). In conclusion, the current members of *C. psittaci* should be evaluated for reconsideration of classification criteria, taking into account various phenotypic and molecular properties such as host range, immunological specificity of the major outer membrane protein, chromosomal DNA fingerprint patterns, DNA-DNA homology, and rRNA sequences.

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