Restriction Fragment Length Polymorphisms of rRNA as Genetic Markers To Differentiate *Chlamydia* spp.

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Restriction fragment length polymorphisms (RFLPs) of rRNA genes of *Chlamydia* spp. were analyzed. A Southern analysis of chromosomal DNA digests with cloned rRNA gene probes revealed the presence of one locus for rRNA genes on chromosomal DNAs in *Chlamydia psittaci*, *Chlamydia pecorum*, and *Chlamydia pneumoniae* and two loci in *Chlamydia trachomatis*. The RFLPs of rRNA genes were characteristic for each *Chlamydia* sp. DNA probes cloned from flanking regions of the rRNA genes of avian *C. psittaci* hybridized either with only *C. psittaci* or with only the avian and ovine abortion strains of *C. psittaci*. The RFLPs of rRNA genes and the flanking regions provide suitable genetic markers for differentiation of *Chlamydia* spp.

Chlamydiae are obligatory intracellular bacteria which have a unique developmental cycle and manifest various pathogenicities (15). Currently, four species, *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae*, and *Chlamydia pecorum*, are recognized within the genus *Chlamydia* (7, 10, 15). *C. trachomatis* and *C. pneumoniae* are human pathogens, whereas *C. psittaci* and *C. pecorum* are animal pathogens, although human infection can occur by contact with infected animals. Immunological and genetic analyses have revealed differences among avian and mammalian strains of *C. psittaci* and *C. pecorum* (1, 2, 9, 14, 17, 18).

We have been examining the immunological and genetic relationships among strains of *C. psittaci* for purposes of proper classification (5, 6, 8). We previously described separation of *C. psittaci* strains into four groups and six types on the basis of DNA homology and restriction endonuclease digestion patterns. The DNA-DNA hybridization analysis showed that *C. psittaci* strains can be separated into two genetic groups, an avian-ovine abortion-feline group (group Av-Oa-Fe) and a nonovine abortion ruminant group (group Ru). These two groups exhibit less than 20% intergroup DNA-DNA homology. We proposed establishment of a separate species for the group Ru of *C. psittaci* and named this taxon *C. pecorum* (7). *C. pecorum* infects primarily sheep and cattle and causes pneumonia, polyarthritis, encephalomyelitis, and diarrhea, but not ovine abortion, whereas *C. psittaci* includes a large variety of strains that infect various animals, including birds, ruminants, cats, guinea pigs, and koalas, and causes a variety of pathogenic effects in the host animals, including ovine abortion. Cumulative data indicate that strains belonging to group Av-Oa-Fe of *C. psittaci* may be placed in a separate species on the basis of differences in genetic properties and immunogenicity. Similarly, *C. trachomatis* strains can be divided into a human group (group CtHu), which includes trachoma and lymphogranuloma venereum biovars, and a mouse group (group CtMo), which includes a mouse biovar. These two groups exhibit less than 40% intergroup homology (6, 11).

In this report, we describe cloning of a region of rRNA genes conserved among *Chlamydia* spp. Using these genes as probes of chromosomal restriction digests, we found that *C. psittaci*, *C. pecorum*, and *C. pneumoniae* have one rRNA operon locus, while *C. trachomatis* has two loci. The restriction fragment length polymorphisms (RFLPs) of these regions were also analyzed and correlated with the classification of strains based on the results of a genomic DNA analysis (6). Our results indicate that the RFLPs are useful markers for identification and typing of *Chlamydia* spp.

The chlamydiae studied were 13 strains of *C. psittaci*, 5 strains of *C. pecorum*, 1 strain of *C. pneumoniae*, and 3 strains of *C. trachomatis* (Table 1). These strains have been described previously (5-8). Two additional strains, a guinea pig inclusion conjunctivitis strain of *C. psittaci* (strain ATCC VR813) (16) and *C. pneumoniae* TW183^T (T = type strain) (10, 12), were also included. *C. psittaci* ATCC VR813 was shown to belong to group Av-Oa-Fe by Southern and DNA-DNA hybridization analyses (group Av-Oa-Fe-Gp) (data not shown). We used previously described procedures for cell culture growth of chlamydiae, purification of elementary bodies, and extraction of chlamydial DNA (5-7).

Molecular cloning, including plaque hybridization, and plasmid subcloning were performed by the methods described by Maniatis et al. (13). The host and vectors for molecular cloning were *Escherichia coli* DP50^{supF} and TB1, phage lambda Charon 4A, and plasmid pUC19 (13). Genomic libraries were constructed with the lambda Charon 4A vector. Cloned DNAs were subcloned into plasmid pUC19 and sequenced with a Sequenase version 2 kit (USB, Toyobo, Inc., Osaka, Japan) as recommended by the manufacturer.

Total RNA was extracted with an RNA extraction kit (Amersham Japan, Inc., Tokyo, Japan) from chlamydia-infected cells harvested 48 h after infection. RNA was electrophoresed by using the formaldehyde method (13) and was blotted onto a Hybond-N membrane (Amersham Japan, Inc.) by using a vacuum (VacuGene; Pharmacia LKB Biotechnology, Tokyo, Japan).

Agarose gel electrophoresis of DNA, vacuum transfer of nucleic acids, hybridization, and labeling of DNA probes with digoxigenin-dUTP (Boehringer Mannheim-Yamanouchi, Tokyo, Japan) were done as described previously (4, 6). Immunological detection of Southern and Northern (RNA)

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TABLE 1. Chlamydial strains used in this study

Species	Fingerprint type ^a	Strain	Origin ^b
<i>C. psittaci</i>	Av1a	Prt/GCP-1	Parrot, systemic infection, Japan
		Hu/Itoh	Human, psittacosis, Japan
		Tk/CA	Turkey, ornithosis, United States
	Av1b	Frt-Hu/Cal10	Ferret-human, cold, United States
		Pgn/P1041	Healthy pigeon, Japan
		Pgn/PCM69	Pigeon, feces, Japan
	Av1c	Prk/6BC ^T (= ATCC VR125 ^T)	Parakeet, United States
		Ckt/Okame	Cockatiel, systemic infection, Japan
		Tk/NJ	Turkey, ornithosis, United States
	Av2a	Hu/Borg (= ATCC VR601)	Human, psittacosis, United States
	Av2b	Prk/Daruma	Parakeet, systemic infection, Japan
	Fe1	Ov/B577 (= ATCC VR656)	Sheep, abortion, United States
	<i>C. pecorum</i>	Fe1	Fe/Pn-1 (= ATCC VR120)
Fe/145			Cat, conjunctivitis, United States
Mu/M56 (= ATCC VR630)			Muskrat, systemic infection, Canada
Gp1		Gp/Ic (= ATCC VR813)	Guinea pig, inclusion conjunctivitis, United States
Ru1a		Bo/Yokohama	Cattle, enteric infection, Japan
		Bo/Maeda	Cattle, pneumonia, Japan
		Bo/Shizuoka	Cattle, encephalitis, Japan
Ru1b		Ov/IPA (= ATCC VR629)	Sheep, polyarthritis, United States
Ru1c		Bo/E58 ^T (= ATCC VR628 ^T)	Cattle, encephalitis, United States
<i>C. pneumoniae</i>		CnTW	TW183 ^T
<i>C. trachomatis</i>	CtHu	E/UW-5/Cx	Human, cervix, United States
	CtMo	L2/434/Bu ^T	Human, bubo, United States
		MoPn/Weiss-Nigg	Mouse, pneumonitis, United States

^a Fingerprint (genetic) types were determined previously (6).

^b Host (human, lower mammal, or bird), clinical condition or anatomic site, and/or geographic origin.

blots was performed with alkaline phosphatase-conjugated anti-digoxigenin antibody and the chemiluminescent substrate AMPPD (Boehringer Mannheim-Yamanouchi) by following the instructions in the manufacturer's manual.

Recombinant phages carrying conserved gene segments were cloned from genomic libraries of *C. psittaci* Prt/GCP-1 (genetic type Av1a), Frt-Hu/Cal10 (genetic type Av1b), and Prk/Daruma (genetic type Av2a) by plaque hybridization

with digoxigenin-labeled chromosomal DNA from *C. trachomatis* MoPn/Weiss-Nigg. The cloned region of the Frt-Hu/Cal10 DNA was 25.5 kb long and was derived from a single region (Fig. 1). Clones from Prt/GCP-1 and Prk/Daruma, which were 25 kb long, produced similar physical maps (data not shown) and were derived from the region homologous to the cloned region of the Frt-Hu/Cal10 library. Representative clones c1 and c14 from the Frt-Hu/Cal10

FIG. 1. Physical maps of the cloned regions carried by recombinant phages c1, c14, and c5. The cloned region was 25.5 kb long. The cross-hatched rectangle indicates the region hybridized with digoxigenin-dUTP-labeled chromosomal DNA of *C. trachomatis* Ct/MoPn. This region was bounded with *XhoI* and *HindIII* sites. The lines for probes P1 to P7 indicate the probe segments used for RFLP analysis in the experiments whose results are shown in Fig. 3 and Table 2. Probe 3 consisted of two separate fragments of the same size.

FIG. 2. Northern analysis performed with probes from the cloned segments of genes coding for rRNA (rDNA). (A) Northern blots of cloned segments. Lane 1, preparation probed with 2.0-kb *Eco*RI segment (segment 1); lane 2, preparation probed with 4.2-kb *Hind*III segment (segment 2); lane 3, preparation probed with 7.5-kb *Eco*RI segment (segment 3). (B) Physical map of rRNA gene showing the region analyzed, the location of each probe, and the endonuclease cleavage sites. Segments 1 to 3 were the probes used for the Northern analysis shown in panel A.

genomic DNA library and c5 from the Prt/GCP-1 genomic DNA library were used for the preparation of probes and for RFLP analysis (Fig. 1). Probes P1 to P4 were included in c1 DNA. Probe P3 contained 2.7 kb of separate *Xho*I and *Xho*I-*Eco*RI fragments; probes P5 and P6 were isolated from c14; and probe P7, which was isolated from c5, was located next to probe P6.

The conserved region of Frt-Hu/Cal10 was located in an 8-kb segment, which contained a 2.0-kb *Eco*RI fragment (probe P4) in the middle of the region (Fig. 1). The portion of the P4 fragment that corresponded to the 16S rRNA gene was identical to the portion of the 3' end of the 16S rRNA gene described by Weisburg et al. (19) (data not shown). A Northern analysis showed that cloned segments P3, P4, and P5 hybridized with 16S and/or 23S rRNA (Fig. 2). These results indicated that the conserved region contained 16S and 23S rRNA genes.

We investigated the number of rRNA genes of *Chlamydia* spp., because Engel and Ganem (3) reported the presence of two rRNA genes in *C. trachomatis* Ct/MoPn, whereas the cloned ribosomal DNA segments of *C. psittaci* in this study were derived from one region. In addition, the restriction map of our cloned rRNA operon region was different from that of *C. trachomatis* Ct/MoPn (3). A Southern analysis of *Sal*I chromosomal digests with probe P4 revealed one hybridizing band, which was larger than 38 kb, for *C. psittaci*, *C. pecorum*, and *C. pneumoniae* DNAs and two hybridizing bands for *C. trachomatis* (15 and 17.5 kb for the group CtHu strain and 18 and 36 kb for the group CtMo strain) (Fig. 3A). Southern analyses of fingerprints performed with other enzymes, including *Kpn*I, which did not cut within the cloned 25- to 25.5-kb region of Frt-Hu/Cal10, Prt/GCP-1, and Prk/Daruma, and with probes of flanking segments (P3 and P5) also revealed one hybridizing band for *C. psittaci*, *C. peco-*

rum, and *C. pneumoniae* and two segments for *C. trachomatis* (data not shown).

RFLPs within the rRNA gene operon were also investigated. The sizes of the hybridizing segments correlated with the genetic types determined previously. In the case of *Eco*RI fingerprints, probe P4 hybridized with a 2.0-kb *Eco*RI restriction fragment of *C. psittaci*, *C. pecorum*, or *C. pneumoniae* DNA and with two *Eco*RI fragments of human and mouse strains of *C. trachomatis* (5.6 and 7.4 kb for the group CtHu strain and 4.6 and 7.0 kb for the group CtMo strain) (Fig. 3B and Table 2). Probes containing the 5' part of the 16S rRNA gene (probe P3) or the 3' part of the 23S rRNA gene (probe P5) hybridized with fragments consistent with the genetic types previously determined by DNA fingerprinting of chromosomal DNAs (Fig. 3C and Table 2). The *Eco*RI site in the 23S rRNA gene seemed to be conserved within the genus *Chlamydia*, whereas other restriction sites, including the *Eco*RI site in the 16S rRNA gene, showed variation.

On the other hand, probes of flanking regions hybridized with specific genetic types. Probe P2, containing segments upstream of the 16S rRNA gene, and probe P7, situated further downstream from the 23S rRNA gene, hybridized only with *C. psittaci* strains; probe P1, situated upstream of probe P2, and probe P6, situated between the 23S rRNA gene and probe P7, hybridized with avian and ovine abortion strains of *C. psittaci* but not with feline and guinea pig strains of *C. psittaci*, and strains of *C. pecorum*, *C. pneumoniae*, and *C. trachomatis*.

In summary, our results indicated that *C. psittaci*, *C. pecorum*, and *C. pneumoniae* have one copy of the rRNA operon and *C. trachomatis* has two copies. Previously, Engel and Ganem (3) and Palmer et al. (17) reported that *Chlamydia* spp. have two rRNA gene loci on the basis of the

FIG. 3. RFLP analysis of *Chlamydia* spp. Southern blots of *SalI* digests (A) and *EcoRI* digests (B and C) were hybridized with 2.0-kb *EcoRI* probe P4 (A and B) or 7.5-kb *EcoRI* probe P5 (C) of c14 cloned segments. The digested DNAs were electrophoresed in a 0.5% (A), 0.8% (B), or 0.7% (C) agarose gel and blotted onto a nylon membrane. The Southern blots prepared with P4 revealed one hybridized fragment for *C. psittaci*, *C. pecorum*, and *C. pneumoniae* and two fragments for *C. trachomatis*. The sizes of the hybridized fragments varied within each genetic type. (A and C) Lanes 1 to 6 and lane 9, *C. psittaci*; lanes 7 and 8, *C. pecorum*; lane 10, *C. pneumoniae*; lanes 11 to 13, *C. trachomatis*. Lane 1, genetic type Av1a strain Prt/GCP-1; lane 2, genetic type Av1b strain Frt-Hu/Cal10; lane 3, genetic type Av1c strain Prk/6BC^T (= ATCC VR125^T); lane 4, genetic type Av2a strain Prk/Daruma; lane 5, genetic type Av2b strain Ov/B577 (= ATCC VR656); lane 6, genetic type Fe1 strain Fe/Pn-1 (= ATCC VR120); lane 7, genetic type Ru1 strain Bo/Maeda; lane 8, genetic type Ru strain Bo/Shizuoka; lane 9, genetic type Gp1 strain Gp/Ic (= ATCC VR813); lane 10, genetic type CnTW strain TW183^T; lane 11, genetic type CtHu strain E/UW-5/Cx; lane 12, genetic type CtHu strain L2/434/Bu^T; lane 13, genetic type CtMo strain MoPn/Weiss-Nigg. (B) Lanes 1 to 7 and lane 10, *C. psittaci*; lanes 8 and 9, *C. pecorum*; lane 11, *C. pneumoniae*; lanes 12 to 14, *C. trachomatis*. Lane 1, genetic type Av1a strain Prt/GCP-1; lane 2, genetic type Av1b strain Frt-Hu/Cal10; lane 3, genetic type Av1c strain Prk/6BC^T (= ATCC VR125^T); lane 4, genetic type Av2a strain Prk/Daruma; lane 5, genetic type Av2b strain Ov/B577 (= ATCC VR656); lane 6, genetic type Fe1 strain Fe/Pn-1 (= ATCC VR120); lane 7, genetic type Fe1 strain Mu/M56 (= ATCC VR630); lane 8, genetic type Ru1 strain Bo/Maeda; lane 9, genetic type Ru1 strain Bo/Shizuoka; lane 10, genetic type Gp1 strain Gp/Ic (= ATCC VR813); lane 11, genetic type CnTW strain TW183^T; lane 12, genetic type CtMo strain MoPn/Weiss-Nigg; lane 13, genetic type CtHu strain E/UW-5/Cx; lane 14, genetic type CtHu strain L2/434/Bu^T. The arrowheads and numbers indicate the positions and sizes (in kilobases) of marker fragments. The marker fragments were digoxigenin-dUTP-labeled bacteriophage T4 DNA fragments restricted with *SalI* (A) and phage lambda DNA fragments restricted with *HindIII* (B and C).

observation that *C. trachomatis* MoPn and *C. psittaci* Gp/Ic produced two *EcoRI* bands which hybridized to the labeled 16S rRNA probe. Our results also showed that the probe containing the 16S rRNA gene hybridized with two *EcoRI* segments of *C. psittaci* DNA. However, this finding was expected, because an *EcoRI* site was present within the 16S rRNA genes of *C. psittaci*, *C. pecorum*, and *C. pneumoniae*, but not in *C. trachomatis*. These findings imply that rRNA genes are single-copy components in the *C. psittaci*, *C. pecorum*, and *C. pneumoniae* genomes, in contrast to the *C. trachomatis* genome, which has two copies. If the rRNA genes are multicopy genes in these three species, they must be within highly conserved repeated units more than 40 kb long. It is interesting that this genetic characteristic corresponded to phenotypic characteristics of the species, including the presence of glycogen deposits, the morphology of inclusions, and sulfadiazine resistance.

The classification data based on RFLP and DNA hybrid-

ization results obtained with probes derived from rRNA flanking regions were in agreement with our previous genetic grouping and typing data. In particular, the hybridization patterns for probes P2 and P7 from the flanking regions are consistent with the genetic differentiation of *C. pecorum* from avian, ovine abortion, feline, and guinea pig (group Av-Oa-Fe-Gp) strains of *C. psittaci*.

These results suggest that the RFLPs of rRNA gene loci may be used as genetic markers for identification of *Chlamydia* species. Furthermore, it should be possible to choose genus-, species-, or type-specific probes and primer sequences from these rRNA gene loci and their flanking regions for hybridization and polymerase chain reaction assays for detection and determination of genetic types of chlamydiae.

Sequencing of the rRNA operons of ovine abortion, feline, and guinea pig strains of *C. psittaci* and strains of *C. pecorum* may provide information regarding the genetic

TABLE 2. *EcoRI* fragments of genomic DNAs of *Chlamydia* spp. hybridized with cloned ribosomal DNA probes of avian *C. psittaci* containing conserved and flanking regions

Species	Genetic type	Strain	Fragment sizes (kb) with the following probes ^a :						
			P1	P2	P3	P4	P5	P6	P7
<i>C. psittaci</i>	Av1a	Prt/GCP-1	21.0	21.0	21.0	2.0	12.5	12.5	3.3
	Av1b	Frt-Hu/Cal10	11.5	11.5	11.5	2.0	7.5	5.0, 7.5	3.3
	Av1c	Prk/6BC ^T	21.0	21.0	21.0	2.0	12.5	12.5	3.3
	Av2a	Prk/Daruma	21.0	21.0	21.0	2.0	12.5	12.5	2.4
	Av2b	Ov/B577	21.0	21.0	21.0	2.0	12.5	12.5	2.4
	Fe1	Fe/Pn-1	— ^b	8.6	8.6, 2.05	2.0	7.0	—	7.0, 6.2
	Gp1	Gp/Ic	—	2.6	2.6	2.0	11.0	—	2.4
<i>C. pecorum</i>	Ru1a	Bo/Shizuoka	—	—	4.0	2.0	4.0	—	—
	Ru1c	Bo/E58 ^T	—	—	4.0	2.0	3.8	—	—
<i>C. pneumoniae</i>	CnTW	TW183 ^T	—	—	2.8	2.0	5.4	—	—
<i>C. trachomatis</i>	CtMo	MoPn/Weiss-Nigg	—	—	7.0, 4.6	7.0, 4.6	8.7, 3.85	—	—
	CtHu	E/UW-5/Cx	—	—	7.4, 5.6	7.4, 5.6	3.2	—	—

^a The probes were a 3.8-kb *XhoI-EcoRI* fragment (P1), a 2.3-kb *XhoI* fragment (P2), a mixture of a 2.7-kb *XhoI* fragment and a *XhoI-EcoRI* fragment (P3), a 2.0-kb *EcoRI* fragment (P4), a 7.6-kb *EcoRI* fragment (P5), a 5.0-kb *BamHI* fragment (P6), and a 3.3-kb *EcoRI* fragment (P7). Probes P1 through P6 were cloned from a genetic type Av1b strain Frt-Hu/Cal10 library; probe P7 was cloned from a genetic type Av1a strain Prt/GCP-1 library.

^b —, no signal was detected.

distances of these chlamydial strains from each other. The results of analyses of rRNA genes, especially 16S and 5S rRNAs genes, have been used as indicators of phylogenetic relatedness among bacteria (20). Therefore, the sequences of the rRNA genes and other genes of these strains may be useful for determining the phylogenetic relationships of these organisms within the genus *Chlamydia*.

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