Phylogenetic Analyses of *Chlamydia psittaci* Strains from Birds Based on 16S rRNA Gene Sequence

TATSUFUMI TAKAHASHI,[1]* MIKA MASUDA,[1] TOMOMI TSURUNO,[1] YUKARI MORI,[1]
IKUO TAKASHIMA,[2] TAKASHI HIRAMUNE,[1] AND NAOYA KIKUCHI[1]

Department of Epizootiology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069,[1] and Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060,[2] Japan

The nucleotide sequences of 16S ribosomal DNA (rDNA) were determined for 39 strains of *Chlamydia psittaci* (34 from birds and 5 from mammals) and for 4 *Chlamydia pecorum* strains. The sequences were compared phylogenetically with the gene sequences of nine *Chlamydia* strains (covering four species of the genus) retrieved from nucleotide databases. In the neighbor-joining tree, *C. psittaci* strains were more closely related to each other than to the other *Chlamydia* species, although a feline pneumonitis strain was distinct (98.3 to 98.6% similarity to other strains) and appeared to form the deepest subline within the species of *C. psittaci* (bootstrap value, 99%). The other strains of *C. psittaci* exhibiting similarity values of more than 99% were branched into several subgroups. Two pigeon strains and one turkey strain formed a distinct clade recovered in 97% of the bootstrapped trees. The other pigeon strains seemed to be distinct from the strains from psittacine birds, with 88% of bootstrap value. In the cluster of psittacine strains, three parakeet strains and an ovine abortion strain exhibited a specific association (level of sequence similarity, 99.9% or more; bootstrap value, 95%). These suggest that at least four groups of strains exist within the species *C. psittaci*. The 16S rDNA sequence is a valuable phylogenetic marker for the taxonomy of chlamydiae, and its analysis is a reliable tool for identification of the organisms.

Chlamydiae constitute a group of obligate intracellular organisms that are differentiated from other bacteria by their unique developmental cycles (43). The genus *Chlamydia* currently contains four species, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia pecorum*, and *Chlamydia psittaci* (16, 17, 22). *C. trachomatis* has been divided into three biotypes and 15 serovars (44). *C. pneumoniae*, which has been separated from *C. psittaci* based on DNA-DNA reassociation and the morphology of the elementary body (EB), is clearly discriminated by antigenic structures that react with monoclonal antibodies (MAbs) (32, 38). The genealogical characteristics of another newly classified species, *C. pecorum*, are also distinct (7, 16, 27). Strains of *C. psittaci*, which has a broader host range than other species, have been grouped by MAbs (5, 6, 19, 28, 39) and by genetic diversity (5, 15, 18, 20, 27, 41). We have reported that *C. psittaci* strains from pigeons and budgerigars can be grouped into six immunotypes by using MAbs to a pigeon strain and a budgerigar strain (40). Furthermore, Fukushi et al. (19) and Kikuta et al. (28) have demonstrated that extensive antigenic diversity exists not only between avian and mammalian *C. psittaci* strains but also within each of the groups. A comprehensive survey has been published by Anderson (6), who discriminated four avian serovars and three or more mammalian types by using serovar-specific MAbs.

The importance of genetic relationships among chlamydial species and strains has been established, and typing of the organisms has been performed by many investigators. DNA-DNA homology values, which have been measured and described by Cox et al. (11) and Fukushi and Hirai (15–17), are a basic taxonomic tool, although their analysis is not appropriate for routine strain identification. Other techniques, such as restriction endonuclease analysis (15, 18, 35), pulsed-field gel electrophoresis (14), and PCR assays (2, 4, 21, 33), made contributions toward characterizing the genetic diversity of the organisms. Nucleotide sequence analysis is also an effective method for determining phylogenetic relationships as well as for genealogical typing of microorganisms, due to its reproducibility. Recent improvements in direct sequencing from PCR-amplified DNA and in automated sequencing apparatuses have made the sequence comparison of over 1,000 nucleotides much easier to perform than DNA-DNA hybridization or ribotyping (36). Particularly, the integration of 16S rRNA gene (ribosomal DNA [rDNA]) sequence data has made possible the taxonomic assignment of any bacterial organism by the standard technique, i.e., nucleotide sequencing of small DNA fragments. On the other hand, efforts to type chlamydial strains have been concentrated on the major outer membrane protein (MOMP) gene (7, 27, 38, 44, 45). It is evident that the resolution power of 16S rDNA sequences is limited when closely related organisms are being examined (1, 13), although analysis based on the sequence is a valuable phylogenetic marker, as is DNA reassociation (36). Early studies of the taxonomy of species of the genus *Chlamydia*, using 16S rDNA sequence analyses, have suggested the existence of species-specific nucleotide stretches within the gene (3, 20), although the number of strains for which the sequence was determined is limited.

The aim of this study was to detect subtle differences between *C. psittaci* strains by comparing the nucleotide sequences of their 16S rDNA and to understand their phylogenetic relationships, particularly focusing on the strains of bird origin. We also examined interspecies relatedness by using corresponding sequences in other species of *Chlamydia*.

MATERIALS AND METHODS

Chlamydial strains. The strains used for the 16S rDNA analysis are shown with their hosts in Table 1. Two sequences of both *C. psittaci* and *C. pecorum* strains and three sequences of *C. trachomatis* and *C. pneumoniae* strains listed in the table were retrieved from the DNA Data Bank of Japan or from the GenBank database. Although the sequence of *C. psittaci* 6BC, ATCC VR 125[T] (the

* Corresponding author. Mailing address: Department of Epizootiology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069, Japan. Phone: 81(11)386-1112. Fax: 81(11)387-5890.

TABLE 1. Chlamydial strains used for sequence analysis of 16S rDNA

Species and strain	Immunotype[b]	Source	Accession no. of 16S rDNA sequence
C. psittaci			
6BC, ATCC VR 125[Ta]		Parakeet, psittacosis	M13769
6BC, ATCC VR 125[T]	B2	Parakeet, psittacosis	AB001778
Prk46	Ut[c]	Parakeet, psittacosis	AB001809
Prk48	Ut	Parakeet, psittacosis	AB001810
Prk49	Ut	Parakeet, psittacosis	AB001811
GCP-1	B2	Parrot, psittacosis	AB001786
Sugimoto-F	Ut	Cockatiel, psittacosis[d]	AB001812
Bud-1	B2	Budgerigar, psittacosis	AB001779
Bud-11F	B3	Budgerigar, subclinical	AB001780
Bud-16F	B3	Budgerigar, subclinical	AB001781
Bud-5695	B1	Budgerigar, psittacosis	AB001782
Izawa-1	B1	Budgerigar, psittacosis[d]	AB001788
Mizuno-1F	B2	Budgerigar, psittacosis[d]	AB001790
Sugawara		Budgerigar, psittacosis[d]	AB001813
P1015	P1	Pigeon, splenomegaly	AB001792
P1041	P1	Pigeon, splenomegaly	AB001793
P1305	P1	Pigeon, splenomegaly	AB001794
P1307	P3	Pigeon, splenomegaly	AB001795
P1313	P3	Pigeon, splenomegaly	AB001796
P1315	P3	Pigeon, splenomegaly	AB001797
P1321	P3	Pigeon, splenomegaly	AB001798
P1605	P3	Pigeon, subclinical	AB001799
P1646	P1	Pigeon, subclinical	AB001800
P1888	P1	Pigeon, subclinical	AB001801
PCM9	P1	Pigeon	AB001808
PCM27	P2	Pigeon	AB001804
PCM30	P2	Pigeon	AB001805
PCM44	P1	Pigeon	AB001806
PCM55	P1	Pigeon	AB001807
PCM131	P1	Pigeon	AB001803
Pgn-Au-46	P2	Pigeon	AB001802
T-3	P3	Dove, psittacosis	AB001814
T-4	P3	Dove, psittacosis	AB001815
Tk-CAL		Turkey, psittacosis	AB001816
Tk-NJ		Turkey, psittacosis	AB001817
Itoh	B1	Human, psittacosis[d]	AB001787
Ohmiya	P3	Human, psittacosis[d]	AB001791
Cal-10, ATCC VR 122	Ut	Ferret or human	AB001784
Koala		Koala, conjunctivitis	AB001789
OEA[a]		Sheep, abortion	Z49872
B577, ATCC VR 656	Ut	Sheep, abortion	AB001783
Fe-145	Ut	Cat, pneumonitis	AB001785
C. pecorum			
BE[a]		Koala	U73782
Bo/E8[a]		Cattle	U73785
Bo-1485	Ut	Cattle, pneumonitis	AB001774
Bo-Maeda	Ut	Cattle, pneumonitis	AB001775
Bo-Yokohama	Ut	Cattle, pneumonitis	AB001776
SPV-789	Ut	Sheep, subclinical	AB001777
C. pneumoniae			
TW-183, ATCC VR 2282[Ta]		Human, pneumonitis	Z49873
P1[a]		Human	U73783
N16[a]		Horse	U73784
C. trachomatis			
434, ATCC VR 902B[a]		Human, lymphogranuloma venereum	M59178
HAR-13[a]		Human	D89067
SFPD[a]		Hamster, ileitis	M83313

[a] Nucleotide sequence of the strain was retrieved from the database.
[b] Chlamydial strains were antigenically divided into six immunotypes, B1, B2, B3, P1, P2, and P3, by using MAbs.
[c] Ut, untypeable.
[d] Human psittacosis-related strain.

type strain of the species), has already been reported by Weisburg et al. (42), we determined the sequence of the same strain to confirm the reliability of the direct sequencing method employed in this study. A sequence from a *C. psittaci* strain which is not shown in the table, GPIC (guinea pig inclusion conjunctivitis), was also compared with other sequences. However, since the available sequence was too short to apply the analysis employed in this study, it was eliminated from the calculations for phylogenetic analysis and for determining similarity values. Undetermined nucleotides in retrieved sequences of the strains OEA (ovine enzo-

otic abortion) of *C. psittaci*, BE and Bo/E8 of *C. pecorum*, P1 and N16 of *C. pneumoniae*, and SFPD of *C. trachomatis* were changed to the predicted consensus nucleotides, based on the results of multiple alignment. These alterations possibly underestimate the nucleotide divergence of these strains from the others.

The strains used for the determination of 16S rDNA sequences included 13 strains from four species of Psittaciformes (*Amazona aestiva*, *Psittacula krameri*, *Nymphicus hollandicus*, and *Melopsittacus undulatus*), 19 strains from two species of Columbiformes (*Columba livia* and *Zenaida asiatica*), 2 strains from turkeys (*Meleagris gallopavo*), and 7 strains from mammals. Two human strains, Itoh and Ohmiya, were isolated from psittacosis patients. Four psittacine strains, Sugimoto-F, Izawa-1, Mizuno-1F, and Sugawara, were isolated from birds raised by psittacosis patients. Four *C. pecorum* strains were also used for the sequence determination in order to define the phylogenetic positions of *C. psittaci* strains within the genus *Chlamydia*. The sources and references for these strains were described previously (39), except for strains Koala, Sugawara, Tk-CAL, and Tk-NJ. The strain Koala was isolated from a koala (*Phascolarctos cinereus*) with clinical signs of severe conjunctivitis (40), and the strain Sugawara was isolated from a budgerigar (*Melopsittacus undulatus*) raised by a human psittacosis patient. The two turkey strains were isolated from individual ornithosis cases. Most of the strains were previously characterized and typed antigenically with MAbs by the authors (39). The results of immunotyping are shown in Table 1. All strains used for sequencing were propagated in the embryonic yolk sacs of hens' eggs. Ten-percent suspensions of the infected yolk sacs were prepared with a mortar and pestle, and they were stored at −80°C until use.

Extraction of chlamydial DNA. Partially purified EBs were used for the extraction of chlamydial genomic DNA. The yolk sac suspension carrying chlamydiae was Dounce homogenized for 20 strokes and clarified by low-gravity centrifugation at 300 × *g* for 10 min. Chlamydial particles in the supernatant were sedimented at 5,000 × *g* for 60 min at 4°C. The pellet was suspended in 1 ml of 0.2 M Tris-Cl, pH 7.4, and sonicated at 130 W for 3 min at 10°C. The suspension was trypsinized with acetyltrypsin solution (0.2 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 20 min. The digested suspension sonicated by the above procedure was layered onto 3 ml of 25% sucrose in 33 mM Tris-Cl, pH 7.4, and centrifuged at 6,500 × *g* for 60 min at 4°C. The resultant white sediment containing EBs was resuspended in 50 µl of 10 mM Tris-Cl–1 mM EDTA, pH 7.4. The genomic DNA was extracted from the partially purified EB suspension with a DNA extraction kit (SMITEST; Sumitomo Kinzoku Kogyo Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions.

Sequence determination of 16S rDNAs. The chlamydial genomic DNA extracted from EBs was used for PCR amplification of the 16S rDNA. To amplify the DNA, PCR was performed with a pair of generic primers for chlamydiae, primer A(C) (5′ AGAATTTGATCTTRGTT 3′) and primer B(C) (5′ ggctaccttgttacgact 3′), and with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. During the PCR amplification, biotin was introduced into one of the strands of the PCR product, using one of the primers biotinylated in the 5′ end. The PCR products of the DNA were then converted into a single-stranded template by immobilization onto ferrous beads (Dynabeads; Dynal A.S., Oslo, Norway) with coupled streptavidin on the surface and denaturation with NaOH, as described by Hultman et al. (25, 26). The immobilized template was used for Sanger dideoxy DNA sequencing, as modified by Zimmermann et al. (46), with a panel of oligonucleotide primers designed for chlamydiae. The 5′ termini of the sequence primers were labeled with fluorescein isothiocyanate, and the procedures for polyacrylamide gel electrophoresis and determining DNA sequences were performed according to the manufacturer's instructions for the A.L.F. DNA Sequencer II (Pharmacia Biotech, Uppsala, Sweden).

Sequence alignment, calculation of similarity values, and phylogenetic tree construction. The nucleotide sequences of the 16S rDNA determined in this study were aligned manually along with the sequences retrieved from the DNA Data Bank of Japan or GenBank (Table 1). Evolutionary distance values were estimated by Kimura's two-parameter method (29) with the BioResearch (Fujitsu, Japan) SINCA program package. Percent similarity between individual sequences (A and B) was calculated with the Lasergene MegAlign program package (DNASTAR Inc., Madison, Wis.) as follows: similarity (A, B) = 100 × sum of the matches/[length − gap residues(A) − gap residues(B)]. The neighbor-joining method of Saitou and Nei (34) was employed to construct a phylogenetic tree with the BioResearch SINCA program. The topology of the tree was evaluated by a bootstrapping method (12).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession numbers listed in Table 1.

RESULTS

Nucleotide sequences of chlamydial 16S rDNAs. In this study, nucleotide sequences were determined for the 16S rDNAs of 44 strains of *C. psittaci* and 4 strains of *C. pecorum* (Table 1). They were analyzed along with the sequences appearing in previously published papers (33, 42) or in nucleotide databases. Two nucleotide sequences for *C. psittaci* 6BC, ATCC VR 125[T], were iden-

tical, indicating that the direct sequencing method is reliable. Alignment was performed from nucleotide residues 8 to 1,504 (the numbering system is based on that of *Escherichia coli* [10]). The 1,507-, 1,508-, 1,509-, and 1,507- to 1,508-base lengths of the sequences of *C. psittaci*, *C. pecorum*, *C. pneumoniae*, and *C. trachomatis* strains, respectively, correspond to 1,510 bases of the multiple alignment (including gapped residues).

Similarity values of chlamydial 16S rDNAs. Table 2 shows percent similarities and evolutionary distance values for combinations of the chlamydial 16S rDNA sequences. The similarities were quite high between sequences of the same species, except for *C. psittaci* Fe-145, from feline pneumonitis, and *C. trachomatis* SFPD, from hamster ileitis. Most combinations of strains in the same species exhibited a level of sequence similarity of 99.0% or more and 1.0 or fewer substitutions per 100 nucleotides. Although the strains Fe-145 and SFPD were more closely related to strains of their own species than to strains of other species, they were distinct within their species. The level of sequence divergence observed between the strain Fe-145 or SFPD and strains of different species ranged from 94.0 to 96.0%, while between strains of the same species, sequence divergence ranged from 98.3 to 98.7%. One hundred-percent similarities were observed in many combinations within the same species, although there was no apparent dependence on the hosts of the strains. Strains that were epidemiologically related showed high similarity values. For example, 100% similarity values were found among strains Prk46, Prk48, and Prk49 and between Bud-11F and Bud-16F. Ten isolates from feral pigeons, P1015 to P1888, exhibited sequence similarities of 99.8% or more.

Phylogenies of chlamydial 16S rDNAs. Close relatedness of each species of the genus of *Chlamydia*, as shown in Table 2, was also apparent in the neighbor-joining tree (Fig. 1). The clusters of all species had relatively deep sublines branching from a position close to the periphery of the genus. The most distant pair of strains within a species was *C. psittaci* Fe-145 and P1321, whose evolutionary distance was 1.85 per 100 bases (Table 2). The Fe-145 strain, which appeared distinct within the species, exhibited the deepest branch in the clade. Such deep branches were also observed in strain SFPD of *C. trachomatis* and strain N16 of *C. pneumoniae*, whose evolutionary distance values with other strains in the clade were 1.70 to 1.71 and 0.92 to 0.99 per 100 bases, respectively (Table 2). On the other hand, *C. pecorum* strains were similar to each other in that they produced a specific cluster (bootstrap value for the strains, 100%). Within the cluster of *C. psittaci* strains, except for Fe-145, several sublines were branched at positions that were relatively close to each other. Some of the subgroups were recovered in 80% or more of the bootstrap trees. A pigeon strain, a white-winged dove strain, and a turkey strain formed a distinct subgroup that was recovered in 97% of the bootstrapped trees. The other pigeon strains seemed to be distinct from the strains from psittacine birds, with a bootstrap value of 80%. The cluster formed by the 18 Columbiformes strains and two other strains was further branched into several subgroups. Nine pigeon strains (immunotype P1) and one turkey strain seemed to be related more to each other than to the other strains (immunotype P2 or P3), although bootstrap analysis did not reveal the specific association of these subgroups (less than 70%). The clusters of psittacine strains, which were made up of the strains from members of the order Psittaciformes (parrots and parakeets), humans, and a koala, were closely related to each other, except for three parakeet strains and two ovine abortion strains. These last five strains exhibited a specific association, with a bootstrap value of 95%.

TABLE 2. Percent similarity and evolutionary distance for the 16S rDNA sequences of chlamydial strains[a]

Percent similarity is shown in the upper right half of the chart; evolutionary distance (percent diversity) is shown in the lower left half. Boxes enclose values within a particular species.

Strain	6BC (10)	Prk46 (2)	Bud-1	Sugawara	P1015 (6)	P1307 (3)	P1321	PCM9 (2)	PCM27 (1)	Pgn-Au-46 (1)	T-4	Tk-NJ	Ohmiya	Cal-10	B577 (1)	Fe-145	BE	Bo-1485 (1)	Bo-Maeda	Bo-Yokohama	SPV-789	TW-183	P1	N16	434	HAR-13	SFPD
6BC (10)[b]	—	99.8	99.9	99.9	99.8	99.7	99.6	99.7	99.6	99.7	99.5	99.7	99.6	99.9	99.7	98.5	96.2	96.2	96.2	96.2	96.3	96.4	96.0	96.1	96.3	95.9	95.6
Prk46 (2)	0.21	—	99.7	99.7	99.6	99.5	99.4	99.4	99.5	99.5	99.4	99.4	99.4	99.7	99.6	98.3	96.2	96.2	96.2	96.2	96.3	96.2	95.8	95.9	96.2	95.8	95.5
Bud-1	0.07	0.28	—	99.9	99.7	99.6	99.7	99.6	99.7	99.4	99.6	99.5	99.9	99.7	99.7	98.5	96.2	96.2	96.2	96.2	96.3	96.2	95.9	96.0	96.2	95.8	95.5
Sugawara	0.07	0.28	0.14	—	99.9	99.7	99.9	99.7	99.9	99.6	99.7	99.7	99.9	99.7	99.7	98.5	96.2	96.2	96.2	96.2	96.3	96.2	95.9	96.0	96.2	95.8	95.5
P1015 (6)	0.21	0.42	0.28	0.14	—	99.9	99.8	99.9	99.8	99.9	99.3	99.9	99.5	99.9	99.7	98.4	96.2	96.2	96.2	96.2	96.3	96.3	96.0	96.1	96.3	95.9	95.6
P1307 (3)	0.35	0.56	0.42	0.28	0.14	—	99.9	99.7	99.9	99.2	99.9	99.4	99.9	99.6	99.4	98.3	96.2	96.2	96.2	96.2	96.3	96.3	96.0	96.1	96.3	95.9	95.6
P1321	0.42	0.64	0.28	0.14	0.21	0.07	—	99.8	99.9	99.2	99.2	99.8	99.2	99.8	99.5	98.3	96.2	96.1	96.1	96.0	96.2	96.3	96.0	96.0	96.2	95.8	95.5
PCM9 (2)	0.35	0.56	0.42	0.28	0.14	0.28	0.21	—	99.7	99.3	99.4	99.7	99.3	99.7	99.6	98.4	96.2	96.1	96.0	96.0	96.3	96.3	96.0	96.1	96.3	95.9	95.5
PCM27 (1)	0.42	0.64	0.28	0.14	0.21	0.07	0.35	0.28	—	99.3	99.3	99.8	99.3	99.9	99.5	98.5	96.2	96.1	96.0	96.0	96.2	96.2	95.8	96.0	96.2	95.7	95.5
Pgn-Au-46 (1)	0.35	0.56	0.56	0.42	0.14	0.85	0.21	0.28	0.35	—	99.3	99.9	99.3	99.9	99.3	98.5	96.4	96.3	96.2	96.3	96.4	96.4	96.1	96.2	96.4	96.0	95.6
T-4	0.49	0.71	0.42	0.28	0.71	0.14	0.92	0.78	0.85	0.85	—	99.3	99.2	99.3	99.2	98.3	96.2	96.1	96.0	96.0	96.1	96.1	95.8	95.9	96.1	95.7	95.3
Tk-NJ	0.35	0.56	0.49	0.35	0.14	0.64	0.21	0.14	0.21	0.07	0.78	—	99.9	99.9	99.6	98.5	96.2	96.1	96.0	96.0	96.2	96.2	95.9	96.0	96.2	95.8	95.4
Ohmiya	0.42	0.64	0.14	0.28	0.49	0.14	0.85	0.78	0.85	0.78	0.78	0.64	—	99.5	99.6	98.6	96.2	96.1	96.0	96.2	96.2	96.2	95.9	96.0	96.2	95.8	95.4
Cal-10	0.07	0.28	0.28	0.28	0.14	0.42	0.21	0.28	0.35	0.14	0.21	0.14	0.28	—	99.7	98.6	96.2	96.1	96.0	95.9	96.2	96.3	95.8	96.2	96.3	95.8	95.4
B577 (1)	0.28	0.42	0.35	0.35	0.28	0.64	0.49	0.35	0.64	0.64	0.92	0.78	0.42	0.35	—	98.3	96.3	96.1	96.0	95.9	96.2	96.1	95.8	95.9	96.1	95.7	95.5
Fe-145	1.42	1.64	1.49	1.49	1.64	1.78	1.85	1.64	1.64	1.64	1.78	1.71	1.49	1.49	1.71	—	95.9	95.8	95.7	95.9	96.0	95.6	95.2	95.2	95.3	94.7	95.3
BE	3.54	3.62	3.62	3.62	3.69	3.69	3.76	3.69	3.69	3.47	3.84	3.54	3.62	3.62	3.69	3.99	—	99.9	99.7	99.7	99.8	95.4	95.4	95.5	95.0	95.0	95.0
Bo-1485 (1)	3.62	3.69	3.69	3.69	3.77	3.77	3.84	3.77	3.77	3.54	3.91	3.62	3.69	3.69	3.77	4.07	0.07	—	99.7	99.9	99.9	95.4	95.5	95.5	95.0	95.0	95.1
Bo-Maeda	3.76	3.84	3.84	3.84	3.91	3.91	3.99	3.91	3.91	3.69	4.06	3.76	3.84	3.84	3.91	4.21	0.35	0.28	—	99.7	99.9	95.4	95.4	95.4	95.0	94.8	95.0
Bo-Yokohama	3.54	3.62	3.62	3.62	3.62	3.62	3.76	3.62	3.62	3.47	3.84	3.54	3.62	3.62	3.69	3.99	0.14	0.07	0.35	—	99.6	95.5	95.4	95.6	95.0	94.9	95.0
SPV-789	3.47	3.54	3.54	3.54	3.69	3.69	3.76	3.69	3.69	3.47	3.84	3.47	3.54	3.54	3.69	3.91	0.21	0.14	0.42	0.07	—	95.4	95.5	95.6	95.0	95.0	95.1
TW-183	3.98	4.21	4.06	4.06	4.29	4.36	4.51	4.36	4.36	4.13	4.36	4.06	4.06	4.06	4.28	4.89	4.51	4.58	4.73	4.58	4.74	—	99.9	99.0	95.4	94.6	95.0
P1	4.06	4.29	4.13	4.13	4.36	4.44	4.43	4.44	4.44	4.21	4.43	4.13	4.13	4.13	4.36	4.96	4.58	4.66	4.74	4.66	4.81	0.07	—	99.1	95.4	94.6	94.1
N16	3.98	4.21	4.06	4.06	4.21	4.36	4.43	4.36	4.36	4.13	4.36	4.06	4.06	4.06	4.28	4.96	4.58	4.66	4.74	4.66	4.81	0.92	0.99	—	95.4	94.7	94.0
434	4.88	5.00	5.00	5.00	5.03	5.18	5.26	5.18	5.18	4.80	5.18	4.88	5.00	5.00	5.03	4.95	4.80	4.88	4.95	4.96	4.96	4.95	4.96	5.03	—	99.5	98.3
HAR-13	5.11	5.04	5.19	5.19	5.27	5.42	5.49	5.42	5.42	5.03	5.42	5.11	5.19	5.19	5.11	5.18	4.96	5.04	5.19	5.11	5.19	5.03	5.03	5.19	0.42	—	98.3
SFPD	4.65	4.58	4.73	4.73	4.80	4.96	5.03	4.96	4.80	4.58	4.96	4.65	4.73	4.73	4.65	4.72	4.65	4.73	4.88	4.81	4.88	5.89	5.97	6.18	1.70	1.71	—

[a] Boxes enclose values within a particular species. Percent similarity is shown in the upper right half of the chart. Evolutionary distance, expressed as percent diversity, is shown in the lower left half.

[b] The number in parenthesis is the number of chlamydial strains which have identical 16S rDNA sequences. Identical strains are as follows: with 6BC, GCP-1, Sugimoto-F, Bud-1, Bud-11F, Bud-16F, Bud-5695, Izawa-1, Mizuno-IF, Itoh, and Koala; with Prk46, Prk48 and Prk49; with P1015, P1041, P1305, P1646, P1888, PCM131, and Tk-CAL; with P1307, P1313, P1315, and P1605; with PCM9, PCM44 and PCM55; with PCM27, Pgn-Au-46, B577, and Bo-1485, PCM30, T-3, OEA, and Bo/E8, respectively.

0.03 0.00

100 HAR-13
100 434 — *C. trachomatis*
SFPD[a]

Bo-Maeda
Bo/E8[a]
Bo-1485
Bo-Yokohama — *C. pecorum*
100 BE[a]
SPV-789

100 P1[a]
100 TW-183 — *C. pneumoniae*
N16[a]

Fe-145
98 Pgn-Au-46(P2)
97 T3(P3)
Tk-NJ
Cal-10
PCM27(P2)
PCM30(P2)
T4(P3)
P1605(P3)
72 P1315(P3)
P1313(P3)
P1307(P3)
P1321(P3)
87 PCM55(P1)
PCM9(P1)
PCM44(P1)
Tk-CAL
PCM131(P1)
P1888(P1)
P1646(P1)
P1305(P1)
P1015(P1)
P1041(P1)
GCP-1(B2)
6BC(B2)
6BC
Bud-5695(B1)
Koala
Izawa-1(B1)
Sugimoto-F
Bud-1(B1)
Mizuno-1F(B2)
Bud-11F(B3)
Sugawara
Itoh(B1)
Bud16F(B3)
Ohmiya(P3)
95 Prk49
Prk48
Prk46
B577
OEA[a]

72 99 97 80 88 — *C. psittaci*

FIG. 1. Unrooted neighbor-joining tree based on 16S rDNA sequences in the strains of four species of the genus *Chlamydia*. The numbers indicate the estimated confidence levels, expressed as percentages, for the positions of the branches as determined by bootstrap analysis (12) with 1,000 replications. Bootstrapping values less than 70% are not shown. The scale bar indicates the evolutionary distance value (K_{nuc}) between sequences, determined by measuring the lengths of the horizontal lines connecting two organisms. The designation B1, B2, B3, P1, P2, or P3 shown in brackets indicates an immunotype determined previously by using MAbs (39). In the strains marked "a", to minimize the branch length, undetermined residues in the sequence were changed to match the nucleotides conserved in other sequences.

DISCUSSION

Our treeing analysis based on the 16S rDNA sequence exhibited specific associations within species of the genus *Chlamydia*. This indicates that 16S rDNA is a suitable marker for the identification of *Chlamydia* to the species level. Other treeing analyses in chlamydial phylogeny have been performed comprehensively by Kaltenboeck et al. (27) and Zhang et al. (45) based on the MOMP gene, which contains variable domains and conserved regions, as does 16S rDNA (37). Furthermore, MAbs recognizing surface-exposed epitopes of the MOMP have been used to type strains of chlamydiae (5, 6, 19, 28, 30, 31, 39). Therefore, sequence analysis with the MOMP

gene may have an advantage over analysis with 16S rDNA in terms of correlation with antigenic structures of the organisms. Nevertheless, phylogenetic trees based on the MOMP gene sequence (27, 45) are close in detail to our tree in their branching patterns. Particularly, interspecies relatedness and the phylogenetic position of a feline pneumonitis strain are nearly identical to those of our tree. The difference in the percent similarity between two genes reflects the difference in the length of sublines within a certain species cluster. For example, 99.7% similarity between strains 6BC, of parrot origin, and B577, from an aborted ewe, in their 16S rDNAs (Table 2) is equivalent to more than 79.8% similarity in their MOMP genes

(27). Such dissimilarity causes shorter sublines for both strains in our tree. 16S rDNA is remarkably conserved in the genus *Chlamydia* (7, 20), while the MOMP gene is highly variable even within a particular species. This may be because strains that are closely related in terms of phylogeny have been adapting to distinct host animals such as birds and mammals and suffering immunological pressures in the animal body. The modes of immunological response are so different that gene mutations induced in regions coding surface-exposed epitopes of the MOMP may be diverse.

The robustness of distant relatedness between a feline pneumonitis strain and other "authentic" *C. psittaci* strains was confirmed in the 16S rDNA sequence analyses. This was consistent with reported data based on chromosomal DNA-DNA relatedness (11, 15), restriction fragment length polymorphism analysis of rDNA (18), and serovar-specific MAbs (6). Recently, Herring summarized diversity among *C. psittaci* strains in his review (24) and concluded that four groups exist within the species: avian strains, ruminant abortion strains, feline pneumonitis, and GPIC. From the low level of DNA-DNA relatedness between the former two groups and the latter two groups (11, 15), discrimination of at least two taxa has been confirmed. In the present study, a region of 570 nucleotides in the GPIC 16S rDNA was used for sequence comparison (data not shown). A nucleotide change was found at four residues from the corresponding region of strain 6BC, while nine changes exist in the regions of strain Fe-145. Since the phylogenetic distinctiveness of GPIC in the species *C. psittaci* is still unclear, it is necessary to determine the full length of the sequence.

Except for the feline pneumonitis group, *C. psittaci* strains, including ovine abortion strains, exhibit a relatively low level of divergence in 16S rDNA. The phylogenetic positions of strains B577 and OEA were closer to those of psittacine strains than to two other clusters formed by strains from members of the order Columbiformes and turkeys. Although the ovine abortion strains specifically formed a monophylic clade with three parakeet strains, the phylogenetic independence of the ruminant abortion group was not clear in the 16S rDNA. On the other hand, one of the clades formed by strains Tk-NJ, from a turkey with ornithosis, Pgn-Au-46, from a pigeon (9), and T3, from a white-winged dove (23), could be clearly discriminated from the others. This indicates that substantial divergence exists within the avian strains of *C. psittaci.*

The other Columbiformes strains also formed a monophylic cluster, which was recovered in 80% of bootstrapped trees. In each clade further branched from the Columbiformes cluster, strains tended to share the same profile of reactivities with a panel of MAbs against a pigeon strain of *C. psittaci* (39). On the other hand, immunotypes of the psittacine strains (B1 to B3) did not seem to correspond to particular clusters in the phylogenetic tree. This difference observed in the strain groups is probably due to the degree of antigenic variability in pigeon strains. The pigeon immunotypes were discriminated by 9 of 13 MAbs tested, while the psittacine strains were typed by 3 of 9 clones. This is consistent with the uniformity of 16S rDNA in psittacine strains, except for three that are closely related to ovine abortion strains.

We encountered two strains in which phylogenetic positions were not consistent with previous reports. Strain Ohmiya, from a human psittacosis patient who raised pigeons, was immunotyped as P3 in a previous study and was expected to belong to the Columbiformes group, although it was rather close to psittacine strains. This may be explained by the fact that the P3 (and P2) immunotype has been characterized by its low reactivities with group-specific MAbs (39). Strain Koala, from a

koala with conjunctivitis, has been classified as *C. pneumoniae* because of its MOMP gene sequence (17, 27, 38). However, it showed 100% similarity in 16S rDNA with eight strains from psittacine birds and one strain from a human psittacosis patient. More strains from the koala must be examined to explain this result.

The two turkey strains used in this study were also distant from each other in the phylogenetic tree, suggesting that at least two genotypes exist within strains from the host animal. However, it is possible that the turkey infections caused by these organisms were transmitted by contact with carrier pigeons or other free-living birds. If so, analyses based on the 16S rDNA sequence of other turkey strains should be able to clarify the epizootiology of these taxa.

Kaltenboeck et al. (27) drew dendrograms of chromosomal DNA reassociation based on the data sets of DNA-DNA hybridization (11, 15) for comparison with their MOMP tree. Most of the branching patterns revealed in our phylogenetic tree agreed with the above-mentioned dendrogram. Stackebrandt and Goebel (36) reported that organisms that have less than 97.0% homology in rRNA will not reassociate to more than 60% genomic relatedness, regardless of which hybridization method is applied. However, the reverse is not true; several combinations of strains of either mycobacteria or *Fibrobacter* with 99% or more homology in rRNA sequences showed less than 50% relatedness in DNA reassociation (1, 8). These findings indicate that highly homologous 16S rDNAs do not always reflect genealogically close relatedness. However, branching patterns in a phylogenetic tree based on the 16S rDNA sequence were more reliable than those produced by other genetic typing analyses, if the bootstrap value was sufficiently high. In the present study, the degree of nucleotide change within a particular species of the genus *Chlamydia* was relatively small, but the bootstrapping tree of the species was recovered with high probabilities. This suggests that the sequence diversity of the gene is stable and that such a stretch may be species- or taxon-specific.

In this study, we showed species specificity of the 16S rDNA of the genus *Chlamydia*. However, due to the limited number of strains used in the analysis, the branching pattern in the phylogenetic tree may change. Therefore, it is necessary to complete the 16S rDNA sequence database for all of the typical strains of the undetermined taxa of the genus *Chlamydia* and to determine the sequences for more strains in each taxon. Detecting a specific sequence in order to identify pathogenic agents such as chlamydiae is important because of the possibility of identification of the organisms directly from clinical specimens without the fastidious procedures required for isolation. In addition, the lability of rRNA molecules can be useful for detecting active infection with chlamydiae rather than the persistence of dead organisms. Sequence analysis of about 1,500 nucleotides has become rapid and inexpensive (13, 36). This improvement has made the sequence comparison of 16S rDNA much easier to perform than DNA-DNA hybridization or ribotyping. Additional analyses, such as the sequencing of the MOMP gene or 16S-23S intergenic spacer regions, which are more variable than 16S rDNA, should be employed for more precise identification.

The present phylogenetic analysis based on 16S rDNA sequences indicates that at least the following four groups exist within the species *C. psittaci*: (i) a feline pneumonitis strain, (ii) Pgn-Au-46 and two other strains, (iii) ovine enzootic abortion strains and three parakeet strains, and (iv) other strains from members of the orders Columbiformes and Psittaciformes, including strains related to human psittacosis. The fourth group of strains can probably be subdivided into several subgroups,

such as psittacine strains and pigeon strains. If the node for a feline pneumonitis strain is regarded as an independent species or subspecies, strains of mouse pneumonitis and SFPD in the clade of *C. trachomatis* and a nonhuman strain(s) of *C. pneumoniae*, respectively, may also need to be considered for reclassification.

REFERENCES

1. **Amann, R. I., C. Lin, R. Key, L. Montgomery, and D. A. Stahl.** 1992. Diversity among *Fibrobacter* strains: towards a phylogenetic classification. Syst. Appl. Microbiol. **15:**23–32.
2. **An, Q., J. Liu, W. O'Brien, G. Radcliffe, D. Buxton, S. Popoff, W. King, M. Vera-Garcia, L. Lu, J. Shah, J. Klinger, and D. M. Olive.** 1995. Comparison of characteristics of Qβ replicase-amplified assay with competitive PCR assay for *Chlamydia trachomatis.* J. Clin. Microbiol. **33:**58–63.
3. **An, Q., and D. M. Olive.** 1994. Molecular cloning and nucleic acid sequencing of *Chlamydia trachomatis* 16S rRNA genes from patient samples lacking the cryptic plasmid. Mol. Cell. Probes **8:**429–435.
4. **An, Q., G. Radcliffe, R. Vassallo, D. Buxton, W. J. O'Brien, D. A. Pelletier, W. G. Weisburg, J. D. Klinger, and M. Olive.** 1992. Infection with a plasmid-free variant chlamydia related to *Chlamydia trachomatis* identified by using multiple assays for nucleic acid detection. J. Clin. Microbiol. **30:**2814–2821.
5. **Andersen, A. A.** 1991. Comparison of avian *Chlamydia psittaci* isolates by restriction endonuclease analysis and serovar-specific monoclonal antibodies. J. Clin. Microbiol. **29:**244–249.
6. **Andersen, A. A.** 1991. Serotyping of *Chlamydia psittaci* isolates using serovar-specific monoclonal antibodies with the microimmunofluorescence test. J. Clin. Microbiol. **29:**707–711.
7. **Anderson, I. E., S. I. F. Baxter, S. Dunbar, A. G. Rae, H. L. Philips, M. J. Clarkson, and A. J. Herring.** 1996. Analyses of the genomes of chlamydial isolates from ruminants and pigs support the adoption of the new species *Chlamydia pecorum.* Int. J. Syst. Bacteriol. **46:**245–251.
8. **Baess, I.** 1983. Deoxyribonucleic acid relationships between different serovars of *Mycobacterium avium, Mycobacterium intracellulare* and *Mycobacterium scrofulaceum.* Acta Pathol. Microbiol. Immunol. Scand. Sect. B **91:**260–296.
9. **Banks, J., B. Eddie, M. Sung, N. Sugg, J. Schachter, and K. F. Meyer.** 1970. Plaque reduction technique for demonstrating neutralizing antibodies for *Chlamydia.* Infect. Immun. **2:**443–447.
10. **Brosius, J., J. L. Palmer, J. P. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli.* Proc. Natl. Acad. Sci. USA **75:**4801–4805.
11. **Cox, R. L., C.-C. Kuo, J. T. Grayston, and L. A. Campbell.** 1988. Deoxyribonucleic acid relatedness of *Chlamydia* sp. strain TWAR to *Chlamydia trachomatis* and *Chlamydia psittaci.* Int. J. Syst. Bacteriol. **38:**265–268.
12. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using bootstrap. Evolution **39:**783–791.
13. **Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk, Jr.** 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. **42:**166–170.
14. **Frutos, R., M. Pages, M. Bellis, G. Roizes, and M. Bergoin.** 1989. Pulsed-field gel electrophoresis determination of the genome size of obligate intracellular bacteria belonging to the genera *Chlamydia, Rickettsiella,* and *Porochlamydia.* J. Bacteriol. **171:**4511–4513.
15. **Fukushi, H., and K. Hirai.** 1989. Genetic diversity of avian and mammalian *Chlamydia psittaci* strains and relation to host origin. J. Bacteriol. **171:**2850–2855.
16. **Fukushi, H., and K. Hirai.** 1992. Proposal of *Chlamydia pecorum* sp. nov. for *Chlamydia* strains derived from ruminants. Int. J. Syst. Bacteriol. **42:**306–308.
17. **Fukushi, H., and K. Hirai.** 1993. *Chlamydia pecorum:* the fourth species of genus *Chlamydia.* Microbiol. Immunol. **37:**512–522.
18. **Fukushi, H., and K. Hirai.** 1993. Restriction fragment length polymorphisms of rRNA as genetic markers to differentiate *Chlamydia* spp. Int. J. Syst. Bacteriol. **43:**613–617.
19. **Fukushi, H., K. Nojiri, and K. Hirai.** 1987. Monoclonal antibody typing of *Chlamydia psittaci* strains derived from avian and mammalian species. J. Clin. Microbiol. **25:**1978–1981.
20. **Gaydos, C. A., L. Palmer, T. C. Quinn, S. Falkow, and J. J. Eiden.** 1993. Phylogenetic relationship of *Chlamydia pneumoniae* to *Chlamydia psittaci* and *Chlamydia trachomatis* as determined by analysis of 16S ribosomal DNA sequences. Int. J. Syst. Bacteriol. **43:**610–612.
21. **Gaydos, C. A., T. C. Quinn, and J. J. Eiden.** 1992. Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. J. Clin. Microbiol. **30:**796–800.
22. **Grayston, J. T., C.-C. Kuo, L. A. Campbell, and S.-P. Wang.** 1989. *Chlamydia pneumoniae* sp. nov. for *Chlamydia* sp. strain TWAR. Int. J. Syst. Bacteriol. **39:**88–90.
23. **Grimes, J. E., T. D. Sullivan, and J. V. Irons.** 1966. Recovery of ornithosis agent from naturally infected white-winged doves. J. Wildl. Manag. **30:**594–598.
24. **Herring, A. J.** 1993. Typing *Chlamydia psittaci:* a review of methods and recent findings. Br. Vet. J. **149:**455–475.
25. **Hultman, T., S. Bergh, T. Moks, and M. Uhlén.** 1991. Bidirectional solid phase sequencing of *in vitro*-amplified plasmid DNA. BioTechniques **10:**84–93.
26. **Hultman, T., S. Ståhl, E. Hornes, and M. Uhlén.** 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. Nucleic Acids Res. **17:**4937–4946.
27. **Kaltenboeck, B., K. G. Kousoulas, and J. Storz.** 1993. Structures of and allelic diversity and relationships among the major outer membrane protein (*ompA*) genes of the four chlamydial species. J. Bacteriol. **175:**487–502.
28. **Kikuta, A., N. Furukawa, T. Yoshida, H. Fukushi, T. Yamaguchi, and K. Hirai.** 1991. Antigenic analysis of avian *Chlamydia psittaci* using monoclonal antibodies to the major outer membrane protein. J. Vet. Med. Sci. **53:**385–389.
29. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
30. **Kuroda-Kitagawa, Y., C. Suzuki-Muramatsu, T. Yamaguchi, H. Fukushi, and K. Hirai.** 1993. Antigenic analysis of *Chlamydia pecorum* and mammalian *Chlamydia psittaci* by use of monoclonal antibodies to the major outer membrane protein and a 56- to 64-kd protein. Am. J. Vet. Res. **54:**709–712.
31. **Olsen, G. J., and C. R. Woese.** 1993. Ribosomal RNA: a key to phylogeny. FASEB J. **7:**113–123.
32. **Puolakkainen, M., J. Parker, C.-C. Kuo, J. T. Grayston, and L. A. Campbell.** 1995. Further characterization of *Chlamydia pneumoniae*-specific monoclonal antibodies. Microbiol. Immunol. **39:**551–554.
33. **Roosendaal, R., J. M. M. Walboomers, O. R. Veltman, I. Melgers, C. Burger, O. P. Bleker, D. M. Maclaren, C. J. L. M. Meijer, and A. J. C. Van den Brule.** 1993. Comparison of different primer sets for detection of *Chlamydia trachomatis* by the polymerase chain reaction. J. Med. Microbiol. **38:**426–433.
34. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
35. **Scieux, C., F. Grimont, B. Regnault, and P. A. D. Grimont.** 1992. DNA fingerprinting of *Chlamydia trachomatis* by use of ribosomal RNA, oligonucleotide and randomly cloned DNA probes. Res. Microbiol. **143:**755–765.
36. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
37. **Stephens, R. S., R. Sanchez-Pescador, E. A. Wagar, C. Inouye, and M. S. Urdea.** 1987. Diversity of *Chlamydia trachomatis* major outer membrane protein genes. J. Bacteriol. **169:**3879–3885.
38. **Storey, C., M. Lusher, P. Yates, and S. Richmond.** 1993. Evidence for *Chlamydia pneumoniae* of non-human origin. J. Gen. Microbiol. **139:**2621–2626.
39. **Takahashi, T., I. Takashima, and N. Hashimoto.** 1988. Immunotyping of *Chlamydia psittaci* by indirect immunofluorescent antibody test with monoclonal antibodies. Microbiol. Immunol. **21:**251–263.
40. **Ueno, H., S. Mizuno, I. Takashima, R. Osawa, W. Blanshard, P. Timms, N. White, and N. Hashimoto.** 1991. Serological assessment of chlamydial infection in the koala by a slide EIA technique. Aust. Vet. J. **68:**393–396.
41. **Viale, A. M., A. K. Arakaki, F. C. Soncini, and R. G. Ferreyra.** 1994. Evolutionary relationships among eubacterial groups as inferred from GroEL (chaperonin) sequence comparisons. Int. J. Syst. Bacteriol. **44:**527–533.
42. **Weisburg, W. G., T. P. Hatch, and C. R. Woese.** 1986. Eubacterial origin of chlamydiae. J. Bacteriol. **167:**570–574.
43. **Wyrick, P. B., and S. J. Rickmond.** 1989. Biology of chlamydiae. J. Am. Vet. Med. Assoc. **195:**1507–1512.
44. **Yuan, Y., Y.-X. Zhang, N. G. Watkins, and H. D. Caldwell.** 1989. Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. Infect. Immun. **57:**1040–1049.
45. **Zhang, Y.-X., J. G. Fox, Y. Ho, L. Zhang, H. F. Stills, Jr., and T. F. Smith.** 1993. Comparison of the major outer-membrane protein (MOMP) gene of mouse pneumonitis (MoPn) and hamster SFPD strains of *Chlamydia trachomatis* with other *Chlamydia* strains. Mol. Biol. Evol. **10:**1327–1342.
46. **Zimmermann, J., H. Voss, C. Schwager, J. Stegemann, H. Erfle, K. Stucky, T. Kristensen, and W. Ansorge.** 1990. A simplified protocol for fast plasmid DNA sequencing. Nucleic Acids Res. **18:**1067.