The Ribosomal Intergenic Spacer and Domain I of the 23S rRNA Gene Are Phylogenetic Markers for *Chlamydia* spp.

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Current methods used to classify *Chlamydia* strains, including biological, morphological, and DNA hybridization techniques and major outer membrane protein (*omp1*) gene analysis, can be imprecise or difficult to perform. To facilitate classification, 2.8-kb partial ribosomal DNA (rDNA) segments from a *Chlamydia trachomatis* strain and a *Chlamydia psittaci* strain were amplified by PCR and sequenced. Subsequently, a 1,320-bp region in this segment, including both the 16S/23S intergenic spacer (232 ± 11 bp) and domain I (620 ± 2 bp) of the 23S gene, was sequenced from 41 additional strains and from the chlamydia-like organisms *Simkania* sp. strains "Z" and "Z1." When both parsimony and distance analyses were performed, these sequences were found to have variable regions that grouped the isolates into two lineages (*C. trachomatis* and non-*C. trachomatis*) and nine distinct genotypic groups. The *C. trachomatis* lineage included human, swine, and mouse-hamster groups. The non-*C. trachomatis* lineage included *Chlamydia pecorum*, *Chlamydia pneumoniae*, and *C. psittaci* abortion, avian, feline, and guinea pig groups. These nine groups were essentially equidistant from the genetic root and were congruent with groups identified previously by using DNA-DNA homology, genomic restriction endonuclease analysis, host specificity, tissue specificity, and/or disease production. Phylogenetic trees based on the intergenic spacer or on domain I were congruent with trees previously derived from *omp1* sequences. DNA sequence analysis of either the intergenic spacer or domain I provides a rapid and reproducible method for identifying, grouping, and classifying chlamydial strains.

Chlamydia spp. are obligately intracellular bacteria that replicate only in cytoplasmic inclusions of eukaryotic cells. Some 60 phenotypically distinguishable strains infect mammals and birds. *Chlamydia trachomatis* is taxonomically differentiated from *Chlamydia psittaci* by its capacity to synthesize glycogen and its sensitivity to sulfadiazine (50, 56). Although inconsistencies have been known to exist within this taxonomic system, initially it was thought to separate human strains from most animal strains. *C. psittaci*, containing predominantly animal strains, is a particularly heterogeneous taxon. Recently, some *C. psittaci* strains have been reclassified as members of the separate species *Chlamydia pneumoniae* (35) and *Chlamydia pecorum* (26). Current methods for identifying chlamydiae include DNA endonuclease restriction, PCR analysis, restriction fragment length polymorphism (RFLP) analysis, analysis of the sequence of the gene for the major outer membrane protein (*omp1*), identification of plasmids, histochemical staining, antigenicity analysis, serological techniques, infectivity analysis, and isolation (3, 11, 15, 18, 27, 38, 44, 80).

In 1995, Kahane et al. showed that a chlamydia-like organism, provisionally designated *Simkania* sp. strain "Z," is the closest known relative of the genus *Chlamydia* (43). The genus *Simkania* is morphologically a member of the order *Chlamydiales*. Analysis of 16S gene sequences, a method which is widely used to classify genera and higher taxa (76, 85, 96), clearly shows that the genera *Simkania* and *Chlamydia* are separate taxa in this order (1a, 30, 43, 62, 62a, 90, 92, 99). The usefulness of 16S genes in diagnosis and differentiation of *Chlamydia* species and strains is limited, however, as these genes are 93 to 97% identical.

In this study, the 16S/23S intergenic spacer and flanking ribosomal segments were subjected to a DNA sequence anal-

ysis to determine if they could be used to systematically identify and differentiate chlamydiae. This region was examined because several studies have revealed that there is some sequence diversity near the chlamydial 16S gene (19, 27, 72). A total of 43 *Chlamydia* isolates and the *Simkania* sp. strains "Z" and "Z1" were studied. Comparative analyses yielded phylogenetic trees that distinguished both previously established and new groups of chlamydiae.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 43 *Chlamydia* strains obtained from both animal and human hosts were used in this study (Table 1). Many of these organisms have been cultured in our laboratory by using Vero or McCoy cell cultures or developing chicken embryos (3) (Table 1). DNA samples from other isolates were provided by workers in laboratories which have been involved in characterization of these organisms (Table 1). All organisms were previously characterized by microimmunofluorescence, serology, *omp1* sequence analysis, RFLP analysis, DNA hybridization, and/or in vitro growth.

DNA preparation, amplification, and cloning. Genomic DNAs from intact chlamydiae were prepared by incubating the chlamydiae at 37°C in 50 to 100 μ l of 50 mM dithiothreitol–30 mM Tris–10 mM EDTA (pH 9.0) for 1 h, adding an equal volume of 1% Nonidet P-40, incubating the preparation for 1 h, adding RNase, incubating the preparation at 37°C for 1 h, extracting the preparation with phenol-chloroform (68), and finally extracting the preparation with chloroform. Chlamydial material that was shipped to us as DNA or as lysates was treated with RNase prior to PCR amplification. Approximately 0.25 μ g of template DNA was used in each 50- μ l PCR mixture; higher concentrations of template DNA were used for specimens of DNA that had not been freshly prepared. The PCR Reagent Kit (Perkin-Elmer, Foster City, Calif.) was used to amplify a single-band 2.8-kb PCR product from each strain in an OmniGene Temperature Cycler (Hybaid, Middlesex, England) with the following parameters: 30 cycles consisting of 25 s at 95°C, 15 s at 55°C, and 100 s at 72°C and a final 7-min extension at 72°C. The 16S oligonucleotide primer used in this amplification reaction mixture, #U16SF, matched three known 16S chlamydial sequences, the sequences of *C. psittaci* 6BC^T (T = type strain) (GenBank accession number M13769), *C. trachomatis* L2/434/BU (GenBank accession number M59178), and *C. pneumoniae* TW-183^T (GenBank accession number L06108), at approximately position 990 of the 1,540-bp gene. The 23S oligonucleotide primer used in this amplification mixture, #U23SR, complemented a highly conserved sequence at bacterial 23S gene position 2000, far enough into the 2,900-bp gene to obtain PCR products representing bona fide rRNA operons and to include a chlamydia-specific sequence, as well as possible intra-23S spacers. The primer sequences

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TABLE 1. Strains, references, and sources

Strain	Reference(s)	Source ^a
<i>C. psittaci</i> strains		
CP3 (= VR 574)	57	NADC
MN (= VR 122)	23	NADC
MN Zhang	23, 98	Caldwell
6BC ^T (= VR 125 ^T)	34	NADC
M56 ^b	73	NADC
NJ1	54	NADC
CT1 ^c	53	NADC
GD	41	NADC
Par1	79	Storey
WC	55	NADC
EBA	60	NADC
A22	47, 77	Herring
B577 (= VR 656)	81	NADC
OSP	7	NADC
FP (= VR 120)	9	NADC
FP Cello	16	NVSL
FP Vaccine		Solvay
GPIC (= VR 813)	51	NADC
<i>C. pecorum</i> strains		
1710S	44, 45	Kaltenboeck
BP-1	24	NADC
E58 ^T (= VR 628 ^T)	49	NADC
IPA	58	NADC
L71	44, 45	Herring
Z	93	NADC
<i>C. pneumoniae</i> strains		
CM-1	12	Black
CWL-029	12	Black
CWL-1011	12	Black
FML-12	12	Black
FML-16	12	Black
N16	94	Storey
TW-183 ^T	46	Campbell
<i>C. trachomatis</i> strains		
A/Har-13 ^T	87	Caldwell
B/TW-5/OT	87	Caldwell
D/UW-3/CX	88	Caldwell
F/IC/CAL3	37	Caldwell
L2/434/BU	70	Caldwell
R22	5	NADC
R24	5	NADC
R27	5	NADC
H5	4	NADC
S45	44	Kaltenboeck
MoPn (= VR 123)	52	Caldwell
SFPD	78	Fox
Chlamydia-like organisms		
<i>Simkania</i> sp. strain "Z"	43	Kahane
<i>Simkania</i> sp. strain "Z1"		Kahane

^a NADC, Avian and Swine Respiratory Diseases Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa; Caldwell, H. D. Caldwell; Storey, C. Storey; Herring, A. J. Herring; NVSL, Diagnostic Virology Laboratory, National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, U. S. Department of Agriculture, Ames, Iowa; Solvay, Solvay Animal Health, Inc., Mendota Heights, Minn.; Kaltenboeck, B. Kaltenboeck; Black, C. M. Black; Campbell, L. A. Campbell; Fox, J. G. Fox; Kahane, S. Kahane.

^b Strain M56 was a yolk sac passage strain of the original preparation and was not VR 630, the isolate available from the American Type Culture Collection. VR 630 is a mixed culture which in Vero cells grows out as strain M56 and in McCoy host cells grows out as strain FP (4).

^c CT1 was called C1 by Page (53).

were as follows: #U16SF, GCATGTGGTTTAATTCGATGCAACGCGAAGA ACC; and #U23SR, GAATTTTCGCTACCTTAGGACCGTTATAGTTAC.

Clones of the 2.8-kb ribosomal PCR products were prepared for DNA sequencing by using a TA Cloning Kit (Invitrogen, San Diego, Calif.).

DNA sequence analysis. Oligonucleotide primer synthesis and DNA sequencing were performed by workers at the Iowa State University DNA Sequencing

and Synthesis Facility, Ames. The full-length, cloned, 2.8-kb PCR products from strains R22 and NJ1 were subjected to a double-stranded DNA sequence analysis. After comparison of these sequences, the homologous 2.8-kb PCR products were prepared from 41 additional strains (Table 1) and from *Simkania* sp. strains "Z" (43) and "Z1" (provided by S. Kahane as "ZV1" because it was a possible contaminant of "Z"). Clones and PCR products were characterized by cutting them with restriction enzyme *EcoRI*. *EcoRI* cut all 2.8-kb chlamydial PCR products at a single site, yielding 1.7- and 1.1-kb fragments. A number of the 2.8-kb cloned PCR products obtained during clone screening could not be cut with *EcoRI*, and a DNA sequence analysis of more than 500 bases of each product, performed by using a universal primer for the cloning vector, identified the products as a *Mycoplasma orale* ribosomal sequence. In addition, unlike the chlamydial products, the PCR products obtained from *Simkania* sp. strains "Z" and "Z1" were not cut by *EcoRI*.

A 1,320-bp segment in each of the 41 2.8-kb products was subjected to double-stranded DNA sequence analysis. A total of 33 of the sequences were obtained from clones of the PCR product in vector pCRII (Invitrogen); other sequences were determined by directly sequencing 2.8-kb PCR products that had been extracted with phenol-chloroform and chloroform (68) and concentrated with Microcon 100 microconcentrators (Amicon Corp., Beverly, Mass.). The entire sequences of strains R27, CT1, and GPIC were determined from both PCR products and clones. Because *C. trachomatis* has been reported to have two ribosomal operons (19, 27), the sequences of the highly variable segments of *C. trachomatis* A/Har-13^T, B/TW-5/OT, D/UW-3/CX, F/IC/CAL3, L2/434/BU, and R22 were determined from both clones and PCR products to look for ambiguous sequence data. The sequences of 10 chlamydial strains (1710S, EBA, FP Cello, IPA, N16, M56, L71, Par1, S45, and TW-183^T) and of *Simkania* sp. strains "Z" and "Z1" were obtained from the PCR product template alone.

Several primers were found to be particularly useful for PCR amplification and sequencing, because they matched or complemented all of the chlamydial sequences. The locations of these primers are shown in Fig. 1. The forward primers used were primers #16SF2 (CGCCCGTCACATCATGG) and #16SF3 (TCGTAACAAGGTAGCCC). The complementary primers used were primers #23R (TACTAAGATGTTTCAGTTC), #23R2 (AAAAGGCACGCCGTCACAC), #23R3 (GATATCTCCAAGTTTGATT), and #23R4 (GAGTGTTCACGCATCTTT). Primer #16SF2 is located 150 bases before the 3' end of the 16S gene, primer #23R is located about 200 bases after the start of the 23S gene, primer #23R2 is located at the 3' end of domain I, primer #23R3 is located about 12 bases beyond the end of the 1,320-bp sequenced segment, and #23R4 is located about 1,200 bp into the 23S gene. Double-stranded DNA sequence data were compiled from approximately 70,000 bp of PCR products and/or clones. To gauge the accuracy of sequence analysis, more than 10,000 bp was examined by using both cloned and direct PCR templates.

Primer extension analysis. Total RNAs were prepared from lysed *C. psittaci* 6BC^T, *C. psittaci* NJ1, and *C. trachomatis* R22 by repeated phenol-chloroform extraction and DNase treatment and were used as templates in a primer extension analysis. Primer extension to identify the 5' end of the 23S rRNA was carried out by using a conserved primer complementing the chlamydial 23S rRNA (primer #23R [TACTAAGATGTTTCAGTTC]) and the GeneAmp ThermoStable rTth Reverse Transcriptase RNA PCR kit (Perkin-Elmer). [³²P]dCTP was incorporated into the rTth Reverse Transcriptase product with the following changes in the manufacturer's recommended conditions: the reverse transcription mixture was prepared by using unlabeled dCTP at one-half the recommended concentration, MnCl₂ and rTth enzyme were omitted, and the mixture was heated for 5 min in a boiling water bath. The heated mixture was cooled rapidly on ice, and 40 µCi of [³²P]dCTP (ICN, Irvine, Calif.), MnCl₂, and rTth enzyme were added. Extension was carried out for one cycle in a model 9600 GeneAmp PCR System (Perkin-Elmer) by using the following conditions: 10 min at 50°C, 15 min at 70°C, and 1 min at 95°C. For each chlamydial strain, one sequence ladder was prepared with [³²P]dCTP label and another sequence ladder was prepared with ³⁵S-labeled dATP (ICN) by using the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs, Beverly, Mass.).

DNA analysis programs and output. Sequence analysis programs described in the *Program Manual for the Wisconsin Package* (30a), Madison, were used for DNA and RNA analyses. These programs were the Reformat, SeqEd, BestFit, Gap, Reverse, Assemble, PileUp, Pretty, LineUp, Distances, FastA, RNAfold, and Squiggles programs. Phylogenetic distances were determined by Robin M. Bush and Walter Fitch, who performed a parsimony analysis with PAUP version 3.1 (83). The branching order reliability was evaluated by examining 100 bootstrap resampling replications (21). Sequence data were examined in the following four ways: (i) a PileUp multiple-sequence alignment, including appropriate gaps and substitutions, was prepared (Fig. 2 is a Pretty display of a PileUp alignment); (ii) by using the PileUp alignment, distance analysis tables containing Jukes-Cantor pairwise corrected difference values (42) were generated for the intergenic spacer and for domain I (Tables 2 through 4 summarize these data); (iii) outgroup sequence data were obtained from GenBank for *Pirellula marina* (accession number X07408) and by sequencing the DNAs of *Simkania* sp. strains "Z" and "Z1"; and (iv) the sequences were analyzed by using PAUP, the most parsimonious consensus trees were prepared, and branching order reliability was tested by examining 100 bootstrap sampling replications. Finally, biological, serological, and host range data were compiled and a rooted domain I tree was prepared.

FIG. 1. Ribosomal DNA sequence maps for *Chlamydia* spp. The vertical solid bars indicate sequence differences. The locations of primers that were conserved in all *Chlamydia* strains and that were particularly useful in the PCR and sequence analysis are indicated, as are conserved restriction sites. (A) DNA sequence map comparison for the 2.8-kb ribosomal segment from *C. psittaci* NJ1 and swine strain *C. trachomatis* R22. The shaded regions of the 16S and 23S ribosomal genes plus the intergenic spacer were used for a double-stranded sequence analysis performed with 43 *Chlamydia* strains and the chlamydia-like organism *Syngamia* sp. strain "71." (B) Sequence differences among representative strains of the nine groups of chlamydiae (strains A/Har-13^T, R22, MoPn, 6BC^T, BS77, FML-12, FP, GPLC, and E58^T). Each of the 43 *Chlamydia* strains clustered with one of these strains on the basis of the ribosomal sequence. Either a G, A, T, or C or a gap was scored by absolute difference from the consensus sequence (one, two, or three bases different out of nine sequences) or by a finding of no consensus sequence in the nine strains (i.e., the plurality for consensus was six). The baseline indicates absolute consensus in all nine strains; no consensus is shown by the tallest solid bars; three differences from the consensus are indicated by the next-highest bars; and one or two differences are indicated by the shortest bars. A bar marking three differences out of nine often coincided with a difference between the three *C. trachomatis*-like strains as compared to the other six strains, although this was not always the case.

TABLE 2. Differences between chlamydial groups

Sequence	Group ^a	% Difference ^b								
		Human group	Swine group	Mouse-hamster group	<i>C. pneumoniae</i>	<i>C. pecorum</i>	Avian group	Abortion group	Feline group	Guinea pig group
Intergenic spacer	Human	1.0 (0.8)								
	Swine	5.3 (0.5)	1.9 (1.0)							
	Mouse-hamster	5.8 (0.4)	6.2 (0.9)	0.0 (0.0)						
	<i>C. pneumoniae</i>	24.2 (0.4)	22.9 (0.9)	23.0 (0.3)	0.3 (0.4)					
	<i>C. pecorum</i>	22.1 (0.4)	21.6 (0.7)	23.1 (0.3)	11.8 (0.5)	0.2 (0.2)				
	Avian	23.3 (0.9)	22.3 (0.8)	23.4 (0.7)	13.8 (0.5)	13.8 (0.4)	0.7 (0.5)			
	Abortion	23.6 (0.6)	22.1 (0.5)	23.0 (0.0)	12.9 (0.2)	12.9 (0.2)	1.8 (0.5)	0.0 (0.0)		
	Feline	20.3 (0.6)	19.8 (0.6)	18.6 (0.0)	13.4 (0.2)	13.4 (0.2)	5.6 (0.5)	5.6 (0.0)	0.0 (0.0)	
Guinea pig	22.9 (0.6)	22.7 (0.8)	21.5 (0.0)	13.4 (0.3)	13.9 (0.2)	5.6 (0.5)	5.6 (0.0)	5.6 (0.0)	0.0 (0.0)	
Domain I	Guinea pig	16.7 (0.1)	15.6 (0.4)	15.9 (0.0)	6.8 (0.2)	8.8 (0.2)	4.3 (0.1)	4.9 (0.1)	4.2 (0.1)	0.0 (0.0)
	Feline	16.8 (0.1)	16.6 (0.3)	16.3 (0.2)	6.1 (0.2)	7.8 (0.2)	3.5 (0.2)	3.9 (0.1)	0.1 (0.1)	
	Abortion	17.2 (0.1)	17.3 (0.1)	16.5 (0.2)	6.0 (0.2)	6.5 (0.2)	1.6 (0.2)	0.1 (0.1)		
	Avian	16.6 (0.1)	16.7 (0.1)	15.9 (0.1)	5.9 (0.3)	7.0 (0.2)	0.5 (0.3)			
	<i>C. pecorum</i>	18.0 (0.2)	18.0 (0.2)	17.8 (0.2)	6.1 (0.3)	0.2 (0.2)				
	<i>C. pneumoniae</i>	18.0 (0.2)	15.5 (0.3)	15.5 (0.3)	0.5 (0.4)					
	Mouse-hamster	15.9 (0.3)	5.7 (0.6)	0.0 (0.0)						
	Swine	4.7 (0.1)	1.2 (1.0)							
	Human	0.4 (0.2)								

^a The human, swine, and mouse-hamster groups are currently classified as *C. trachomatis* groups. The avian, abortion, feline, and guinea pig groups are currently classified as *C. psittaci* groups.

^b The values are means (standard deviations from the means) determined by using all of the strains in each group (Table 1).

Nucleotide sequence accession numbers. DNA sequence data for the ribosomal segments described in this paper have been deposited in the GenBank database under accession numbers U68419 through U68460, U76710, and U76711.

RESULTS

Characterization of sequence diversity in a 2.8-kb portion of the ribosomal operon. The 2.8-kb cloned ribosomal PCR products from two *Chlamydia* strains, swine strain R22 and turkey strain NJ1, contained approximately one-third of the 16S gene, the intergenic spacer, and two-thirds of the 23S gene. A comparison of the two sequences is shown in Fig. 1A. The 23S gene start site (TACAGACCAAGT. . .) was located four bases upstream of the homologous *Escherichia coli* site (data not shown). The 5' ends of the 2.8-kb segments each contained 563

bp of DNA homologous to 16S gene sequences that differed from each other by only 3%. A search of the GenBank database performed with the 16S gene segments indicated that R22 was most closely related to *C. trachomatis* and NJ1 was most closely related to *C. psittaci*. The 16S/23S intergenic spacer sequences of R22 and NJ1 were 243 and 224 bp long, respectively, and differed by 22.0%. The 1,960-base 23S gene segments differed by 9.6%. Much of the variation in the 23S segment was in the 5' end homologous to domain I of the *E. coli* 23S gene. The domain I sequences of R22 and NJ1 (622 and 621 bp, respectively) differed by 17%, while the remaining portions of the 23S segments (1,338 bp) varied by 6.4%. No intragenic spacers were identified within the chlamydial 23S genes.

TABLE 3. Differences within chlamydial groups

Taxon	% rDNA difference				Strain(s) ^c
	Intergenic spacer		Domain I		
	Mean ^a	Maximum ^b	Mean ^a	Maximum ^b	
<i>Chlamydia</i> spp.	13.56	24.91	9.39	18.43	
<i>C. trachomatis</i>	4.22	6.64	3.26	6.91	
Human group	1.00	2.10	0.38	0.64	A/Har13 ^T , B/TW-5/OT, D/UW-3/CX, F/IC/CAL3, L2/434/BU
Mouse-hamster group	0.0	0	0.0	0	MoPn, SFPD
Swine group	1.89	3.47	1.19	2.62	H5, R22, R24, R27, S45
<i>C. psittaci</i>	2.73	6.62	2.06	4.99	
Avian group	0.65	1.82	0.45	0.81	6BC ^T , CP3, CT1, GD, M56, MN VR 122, MN Zhang, NJ1, Parl, WC ^T
Abortion group (sheep, cattle)	0.0	0	0.11	0.16	A22, B577, EBA, OSP
Feline group	0.0	0	0.11	0.16	FP, FP Cello, FP Vaccine
Guinea pig group ^d					GPIC
<i>C. pneumoniae</i> (human, horse) ^e	0.30	0.91	0.54	1.30	CM-1, CWL-1011, CWL-029, FML-12, FML-16, N16, TW-183 ^T
<i>C. pecorum</i> (sheep, swine, cattle) ^e	0.15	0.45	0.16	0.49	1710S, BP-1, E58 ^T , IPA, L71, Z

^a Mean based on Jukes-Cantor corrected distances.

^b Maximum difference between any two strains in each group.

^c Isolated mammalian epizootics were associated with strains M56, MN VR 122, MN Zhang, and WC.

^d Only one guinea pig isolate is available.

^e Koala isolates were not available for this study.

TABLE 4. Variation within *omp1* and ribosomal sequences

Segment or gene ^a	% Identical positions for all strains	% Identical positions for two-thirds or more of the strains	No. of strains
<i>omp1</i> nucleotides	40 (47) ^b	72 (80)	34
<i>omp1</i> amino acids	50 (67)	70 (89)	34
Intergenic spacer	56	75	41
Domain I	74	91	41
16S ^c	91	97	4

^a Data for only five human *C. trachomatis* strains from the GenBank database or from previously published papers were included. 16S data for three of the four currently recognized species were included.

^b The values in parentheses are the values obtained when the variable domains were not included.

^c The strains used were *C. psittaci* 6BC^T, *C. trachomatis* L2/434/BU, *C. pneumoniae* TW-183^T, and *C. pecorum* E58^T.

Ribosomal sequence analysis of 43 *Chlamydia* strains. Based on the results of the sequence analysis of the 2.8-kb portion of the ribosomal operons of strains R22 and NJ1, a highly variable 1,320-bp ribosomal DNA (rDNA) segment flanked by conserved regions was identified (Fig. 1A and B). The sequence of this segment was determined for an additional 41 *Chlamydia* strains and two chlamydia-like organisms, *Simkania* sp. strains "Z" and "Z1" (Table 1). To establish whether the sequences of cloned PCR products and directly sequenced PCR products were comparable, more than 10,000 bp was examined by using both types of template. Only four base differences between cloned sequence and direct PCR product sequence were found. In each of these cases, the sequence of the PCR product, which represented a broader sample of ribosomal operons than a single clone, was selected as the final sequence. No ambiguous sequence data were obtained for the *C. trachomatis* sequences that were obtained from both cloned and direct PCR products. (If a high proportion of nonidentical ribosomal operons were present in any single isolate, ambiguous data would have been generated from the PCR product template.) A comparison of the sequences of the 43 strains indicated that the sequences clustered into nine groups. The differences among strains representing the nine groups are shown in Fig. 1B.

Analysis of the ribosomal intergenic spacer. The 16S/23S intergenic spacer sequences of the chlamydiae identified in Table 1 are shown as a multiple-sequence alignment in Fig. 2. The intergenic spacers spanned 232 ± 11 bases depending on the strain. Nine single-base differences were unique to only one or two strains, and approximately 80 sequence position differences were group clustered and conserved in species, subspecies, or several species. Among these 80 positions were three highly variable segments, each approximately 20 bp long (Fig. 2, α , β , and γ segments), between base 12 and base 115 that were distinctly group cluster specific. An RNAfold-Squiggles analysis of the intergenic spacer indicated that each of the α segments could form a hairpin structure with a 10-bp stem centered between bases 23 and 24 (analyses not shown). The *Simkania* sp. strain "Z1" intergenic spacer was 44 bases longer than the longest chlamydial intergenic spacer (Fig. 2).

To determine whether sequence conservation in the chlamydial intergenic spacer could be correlated with the *E. coli* model of rRNA processing, the rDNA sequence flanking the 5' end of the 16S gene from chlamydial strain MoPn was obtained from the GenBank database and spliced, by computer, to the MoPn intergenic spacer sequence that was determined in this study. In *E. coli*, homologous rRNA segments form a stem-loop structure to facilitate nucleolytic processing of the 16S

rRNA. An RNA sequence analysis of the spliced segments performed with the RNAfold and Squiggles programs showed that the two MoPn segments complemented each other, forming a 45-base stem (Fig. 3). The MoPn 5' flanking segment has been shown to contain rRNA start sites P1 and P2 (19). The complementary stem shown in Fig. 3 contained the P2 rRNA processing site and highly conserved segments of the intergenic spacer adjacent to the α segment.

A parsimony analysis by heuristic search performed with PAUP, version 3.1, was used to construct a phylogenetic tree based on the intergenic spacers (Fig. 4). The shortest tree required 135 steps for the 254 bases. The intergenic spacer tree was midpoint rooted for appearance. This analysis separated the chlamydiae into the following two distinct lineages: the *C. trachomatis* lineage, which included strains isolated from humans, swine, a mouse, and a hamster; and the non-*C. trachomatis* lineage, which included *C. psittaci*, *C. pecorum*, and *C. pneumoniae*. The *C. trachomatis* sequences clustered in a host-specific manner, with the human isolates the greatest distance (greatest number of sequence changes) from the common node. The non-*C. trachomatis* sequences were subdivided into six groups, and within each group the strains at the tips of the branches were essentially indistinguishable. Two of the six groups, *C. pneumoniae* and *C. pecorum*, diverged from a common node, while the remaining four groups (all *C. psittaci*) diverged from a separate node. The members of two of the *C. psittaci* groups (abortion and feline) exhibited no sequence variation, while the members of the avian *C. psittaci* group exhibited a small amount of sequence variation. Bootstrap resampling of the data revealed a high degree of confidence in the branching order.

Distance analysis of the intergenic spacer and the 23S domain I segment. A comparison of the intergenic spacers showed that there were 143 positions (56.1% of the sequence positions) at which the bases were identical in all chlamydial strains; 28 or more strains had the same base at 75.1% of the positions in the sequence. The *C. trachomatis* intergenic spacers differed by $\geq 18\%$ from those of all of the other strains (Table 2). *C. pneumoniae* and *C. pecorum* intergenic spacers differed from *C. psittaci* intergenic spacers by $13.3\% \pm 1\%$ (mean $\pm$ standard error of the mean) and by $11.8\% \pm 0.5\%$ from each other. When the intergenic spacer sequences were used to cluster the organisms into the nine genetically related groups, the standard deviation from the mean within each group or between any two groups was $\leq 1.0\%$. The mean of the pairwise differences within most of the groups was also $\leq 1.0\%$; the only exception was the swine group (swine strain S45 differed by $3.0\% \pm 0.5\%$ from other swine strains) (Tables 2 and 3). The difference between the abortion and avian groups was $1.8\% \pm 0.5\%$, a value slightly less than the $1.9\% \pm 1.0\%$ difference within the swine group. This closeness indicated that, for the intergenic spacer, an approximately 2% mean difference could be considered a threshold between group and subgroup designations. All other groups were at least 5.3% different from each other. The mean of intergenic spacer differences overall was 13.56%, with the largest difference being 24.91% and the smallest difference being 0% (Table 3). The *Simkania* intergenic spacer differed from *Chlamydia* intergenic spacers by 89 to 96%. The chlamydial distance data were used to produce an intergenic spacer tree by the neighbor-joining method (data not shown). This tree preserved the relationships between the clusters that were present in the parsimony tree in Fig. 4.

For the domain I segments, the bases at 460 positions (74.2% of the sequence positions) were identical in all chlamydial strains. At least 28 strains were identical at 90.7% of the

FIG. 3. Sequence complementarity between the intergenic spacer and the 16S promoter in strain MoPn. The rRNA promoter sequence for the MoPn 16S gene (accession number M18268) was spliced, by computer, to the MoPn intergenic spacer sequence. The complementary 40-bp stem was identified by using the Genetics Computer Program Squiggles and is similar to the complementary homologous *E. coli* sequences involved in nucleolytic processing of 16S rRNA (74). P1 and P2 are rRNA start sites identified by Engel and Ganem (19). P2 is probably the RNase III nucleolytic processing site.

FIG. 4. Most parsimonious consensus tree based on the intergenic spacers of chlamydial strains (232 $\pm$ 11 bp). The numbers are bootstrap values, which were determined by 100 bootstrap resampling replications (values approaching 100 indicate high branching order reliability). The horizontal branch lengths are proportional to the numbers of nucleotides that differ between nodes or clusters. Tree bisection-reconnection branch swapping was employed. This unrooted tree is a consensus tree based on the seven most parsimonious trees, which differed only in the specific arrangement of strains R22, R24, R27, and H5 within the swine cluster. Strain FML-16 was identical to TW-183^T. Strain EBA was identical to A22 and OSP.

positions in the sequence. The domain I segments of the *C. trachomatis* strains differed by $\geq 15\%$ from the domain I segments of all of the other isolates (Table 2). *C. pecorum* and *C. psittaci* differed from *C. pneumoniae* by $6.7\% \pm 1.3\%$ and differed from each other by $7.6\% \pm 1.4\%$. When the domain I sequences were clustered into the nine genetically distinct groups, the standard deviation from the mean within each group or between any two groups was $\leq 1.0\%$. The mean differences within most groups were $\leq 0.5\%$; the only exception was the swine group (swine strain S45 differed by $2.5\% \pm 0.2\%$ from other swine strains) (Tables 2 and 3). The difference between the abortion and avian groups was $1.6\% \pm 0.2\%$, a value only slightly greater than the $1.2\% \pm 1.0\%$ difference within the swine group. This closeness indicated that an approximately 1.5% difference in domain I could be considered a threshold between group and subgroup designations. All other groups were at least 3.5% different from each other. The mean of the domain I differences overall was 9.39%, with the largest difference being 18.43% and the smallest difference being 0% (Table 3). The difference between domain I of a *Simkania* strain and domain I of any single *Chlamydia* strain was $\geq 26\%$. The differences between domain I of an outgroup, *Pirellula marina*, which has an unlinked operon, and domain I of *Chla-*

mydia and *Simkania* strains were 39 to 46%. These distance data were used to produce trees by the neighbor-joining method, by Fitch analysis, and by maximum-likelihood analysis (data not shown). These trees preserved the relationships between the clusters that are shown in the parsimony tree in Fig. 5.

The variations in chlamydial intergenic spacers, domain I segments, 16S genes (from GenBank), and *omp1* sequences (from GenBank or from previously published papers) are shown in Table 4. Domain I and the intergenic spacer were each more conserved than *omp1*, even when *omp1* variable domains were eliminated from the analysis. The 16S gene was more conserved than the intergenic spacer or domain I.

Phylogenetic analysis of domain I of the 23S gene. Domain I was the most variable 23S gene segment, and differences grouped chlamydial strains into species- or subspecies-specific groups. Domain I, which spanned 620 ± 2 bp depending on the strain, was used for a phylogenetic analysis by a heuristic search in which PAUP, version 3.1, was used (Fig. 5). The shortest tree required 493 steps over 638 nucleotides. The domain I tree was rooted with the genus *Simkania* and the planctomycete *P. marina* (accession number X07408). *P. marina* was more distantly related than the genus *Simkania*. This analysis showed that the chlamydiae are separated into two lineages, a *C. trachomatis* lineage and a non-*C. trachomatis* lineage. The non-*C. trachomatis* lineage diverged furthest from the common node. The *C. trachomatis* lineage included three host-specific groups: human, swine, and mouse-hamster groups. The mouse-hamster group was the group that diverged the least from the common *C. trachomatis* node, while the swine group had the most sequence differences compared with the common node. The non-*C. trachomatis* lineage diverged into six groups: a *C. pneumoniae* group, a *C. pecorum* group, and *C. psittaci* abortion, avian, feline, and guinea pig groups. *C. psittaci* avian isolates included epizootic isolates from mammalian hosts. Bootstrap resampling of the data revealed a high degree of confidence in the branching order.

DISCUSSION

In this study we established that PCR and DNA sequence analysis of the intergenic spacer and domain I in the chlamydial ribosomal operon is a reliable and easy way to classify and identify species and other groups of *Chlamydia* spp. The following conclusions were drawn: (i) the genus *Chlamydia* is a monophyletic group with two branched lineages; (ii) the *C. trachomatis* lineage includes three distinct groups, and the non-*C. trachomatis* lineage includes six distinct groups; (iii) most *Chlamydia* groups are specifically adapted to certain hosts; (iv) chlamydial groups identified by using other techniques are consistent with the two lineages and nine groups; (v) the intergenic spacer and/or domain I of *Chlamydia* strains may be used as a foundation for phylogenetic analysis because each sequence is functionally conserved and subject to systematic selective pressures; and (vi) sequence analysis of the segments provides a legitimate and unambiguous way to taxonomically identify and classify chlamydial strains.

Conserved oligonucleotide primers for the 16S gene and for the 23S gene were used to PCR amplify a 2.8-kb segment of the ribosomal operon from 43 *Chlamydia* strains and from *Simkania* sp. strains "Z" and "Z1" for sequence analysis. A complete DNA sequence analysis of this segment from two strains, *C. trachomatis* swine strain R22 and *C. psittaci* turkey strain NJ1, revealed that the 16S/23S intergenic spacer and domain I of the 23S gene (homologous to domain I of the *E. coli* 23S gene) had potential for differentiating species, as they exhibited 22

FIG. 5. Most parsimonious, rooted, consensus tree based on domain I of the *Chlamydia* 23S gene (620 ± 2 bp). Two equally parsimonious trees were identified; the only difference between these trees was a positional exchange of the abortion group with the feline and guinea pig groups. The numbers are bootstrap values, which were determined by 100 bootstrap resampling replications (values approaching 100 indicate high branching order reliability). The horizontal branch lengths are proportional to the number of nucleotide differences between nodes or clusters. Domain I sequences from *Sinkania* sp. and *P. murina* were used to root the tree. The tree was correlated with available information for each strain. Subtree pruning-regrating branch swapping was employed. Strain FML-16 was identical to TW-183¹. Strain EBA was identical to A22 and OSP. Serovars are generally host or disease specific; established serovars are indicated with letters, as immunotypes (I), or as groups (Gp) (3-5, 60, 88). The dates are dates of isolation or earliest publication. Strains R22, R24, and R27 were isolated from multiple sites associated with respiratory disease, conjunctivitis, and enteritis. STD, sexually transmitted disease.

and 17% diversity, respectively. The sequence of a 1,320-bp segment containing the intergenic spacer (232 bp) and domain I (620 bp) was determined for each of the strains. Separate analyses of the intergenic spacer and domain I were performed because the two sequences coded for different gene functions, each sequence exhibited substantial genetic diversity, and each region was a short, concise segment whose sequence was easily determined. The intergenic spacer and domain I sequences were subjected to a character analysis by using the PAUP program, and the most parsimonious consensus trees were produced (Fig. 1 and 4). The groups identified on the tree based on the intergenic spacer were nearly identical to the groups on the rooted tree based on domain I. When the same sequences were subjected to a distance analysis (Tables 2 and 3), the results were essentially identical to the results obtained when PAUP was used. These ribosomal trees were congruent with phylogenetic trees based on *omp1* (15, 22, 44, 80, 97), 16S gene (1a, 43, 62a), and DNA reassociation (44) data.

Rooting the *Chlamydia* domain I tree with *P. marina* (the most closely related planctomycete) and with the genus *Simkania* showed that all *Chlamydia* strains evolved from a common ancestor and diverged at a node located between the *C. trachomatis* lineage and the non-*C. trachomatis* lineage (Fig. 5). This early separation of *C. trachomatis* is supported by biological evidence (50, 56). Similarities between the *C. trachomatis* and non-*C. trachomatis* lineages indicate that the common ancestor would have had the typical chlamydial group antigen (lipopolysaccharide), rough phenotype, cell wall structure, genome size (20), ribosomal operon structure, and obligately host-dependent growth cycle. Rooting of the 16S gene has provided further evidence that *C. trachomatis* diverged from non-*C. trachomatis* taxa (43). This bipolar divergence was also evident in phylogenetic studies based on *omp1* (22, 44, 97), *omp2* (*envB*) (22), and the intergenic spacer (Fig. 4); however, confirmation by rooting has not been possible because out-group sequences with significant homology have not been identified. A lack of DNA-DNA homology (17, 25, 44) indicates that these lineages differ sufficiently to be considered separate genera (71).

The genus *Simkania* is a bacterial taxon that shares characteristics with the chlamydial common ancestor; it has a similar ribosomal operon, morphology, and obligately host-dependent growth cycle. This makes the genus *Simkania* an appropriate root for a phylogenetic investigation of the genus *Chlamydia*. *Simkania* sp. strain "Z," a cell culture contaminant whose 16S gene (accession number L27666) is only 18% different from the chlamydial 16S gene (43), has inner and outer membranes which morphologically resemble membranes of *Chlamydia* spp. *Simkania* sp. strain "Z1," which was isolated by Kahane as a possible subgroup of "Z," was indistinguishable from "Z" in our study. Identifying a next-closest root for the genera *Chlamydia* and *Simkania* is more difficult because the branching order within the domain *Bacteria* is largely unresolved (1, 10). However, Van de Peer et al. have suggested that the genus *Planctomyces* probably diverged prior to the branching of the genus *Chlamydia* and the other 15 eubacterial lineages (86). This suggests that *P. marina* shares an ancestor with the genera *Simkania* and *Chlamydia* and nearly all other eubacteria. A relationship between the genera *Planctomyces* and *Chlamydia* was supported by the results of a higher-order structure analysis of the 16S gene (90). Unlike the chlamydiae, *P. marina* has an unlinked ribosomal operon with more than 7 kb separating the 16S and 23S genes (75). The 16S gene sequence of *P. marina* differs by 35% from the sequences of *Simkania* and *Chlamydia* strains, and the 23S genes of *P. marina* and *Chlamydia* spp. are 40% different (91). Planctomycetes are free-

living organisms, and at least one has a genome that is five times larger than the chlamydial genome (89).

On the basis of the results of an analysis of the intergenic spacer and domain I we identified nine phylogenetic groups in the two chlamydial branches, many of which could be readily distinguished by host specificity. The *C. trachomatis* taxon included three groups, one specific for human hosts, one specific for mouse-hamster hosts, and one specific for swine hosts. *C. trachomatis* human strains have been isolated from humans with trachoma or sexually transmitted disease. They are clustered into 18 serovars (11) and are closely related as determined by *omp1* analyses (22, 97), *omp2* analysis (22), intergenic spacer analyses (Tables 2 and 3 and Fig. 4), and domain I sequence analyses (Tables 2 and 3 and Fig. 5). Worldwide, these serovars exhibit only minor epidemiological differences, which are identifiable by *omp1* analysis. The *C. trachomatis* mouse-hamster group is also host specific, having been isolated only from members of the family *Muridae*. Members of this group have been isolated from animals with respiratory infections or proliferative ileitis, and their *omp1* sequences are divergent (97). The *C. trachomatis* swine group has only recently been characterized and appears to be specific for swine. The strains in this group have been associated with conjunctivitis, enteritis, and pneumonia (5, 44, 66, 67).

The non-*C. trachomatis* taxon is comprised of six genotypic groups that are currently designated *C. psittaci*, *C. pneumoniae*, and *C. pecorum*. The *C. psittaci* feline pneumonitis group is endemic in cats, causing mucosal infections with predominantly upper respiratory tract symptoms (28, 69). Whether they were identified in the 1940s, in the 1990s, or in different parts of the world, feline pneumonitis isolates are serologically the same and have identical *omp1* restriction endonuclease patterns (69). The *C. psittaci* guinea pig inclusion conjunctivitis group, for which only a single isolate has been identified, has not been recovered from hosts other than the guinea pig. The *C. psittaci* avian group includes eight serovars. Serovars A and B are endemic in psittacine birds and pigeons, respectively. The other avian serovars have been isolated from members of more than one avian family or include only a limited number of isolates, and so the natural hosts of these serovars are not yet known. *C. psittaci* avian infections are often systemic and are either acute, inapparent, severe, or chronic with intermittent shedding. All *C. psittaci* avian isolates are similar in virulence and growth characteristics in cell culture. They appear to be uniquely susceptible to bacteriophage Chp1 (79). Sporadic human infection and illness following exposure to birds harboring *C. psittaci* avian strains have been widely documented. Isolates WC, M56, MN VR 122, and MN Zhang were obtained from sporadic infections of humans or other mammals (23, 55, 73, 98). Strains WC and M56 evidently have not reappeared since the original outbreaks, while MN strains have been isolated repeatedly from birds. The *C. psittaci* abortion group is endemic in ruminants. Some strains have been observed to cause sporadic abortion in women who are sheep handlers (40). Abortion strains are highly invasive in placental tissue (63) and belong to a serovar that has been obtained from sheep, cattle, and goats worldwide (3, 18, 82). In the past, there was some confusion in classifying chlamydiae because preparation A22/M of ovine abortion strain A22 was evidently contaminated with an avian strain. Sequence analysis has shown that A22/M *omp1* (accession number X12647) is identical to duck strain Avn352 *omp1* (accession number L04980) and to MN strain VR 122 *omp1* (20). A22/M *omp1* is not identical to ovine A22 *omp1* (39).

Neither *C. pneumoniae* nor *C. pecorum* is limited to a specific host. *C. pneumoniae* strains have been isolated from hu-

mans, horses, and koalas (31, 46, 80). They are associated with respiratory disease, severe systemic infection, coronary artery disease, and ocular disease (32, 46). Human *C. pneumoniae* isolates are serologically nearly indistinguishable (46), and human *omp1* differs from both horse *omp1* and koala *omp1*. *C. pecorum* strains have been isolated from ruminants (cattle, goats, and sheep), koalas, and swine, so they, too, are not host specific (8, 18, 26, 32). *C. pecorum* is uniquely noninvasive in a mouse model of virulence (65). These strains are pathogenically and serologically diverse, having a disease range that includes abortion, conjunctivitis, encephalomyelitis, enteritis, pneumonia, and polyarthritis (8, 44).

The results obtained in this study are consistent with chlamydial groups determined by other methods. DNA-DNA reassociation studies of chlamydiae have indicated that the eight groups that have been studied are all less than 70% homologous and therefore qualify as separate species (1, 17, 25, 44, 71, 76). The *C. psittaci* avian and abortion groups were the most closely related groups as determined by the intergenic spacer and domain I analysis and also by the DNA-DNA homology analysis. The four current species were the most distantly related taxa as determined by the intergenic spacer and domain I analysis and also by DNA-DNA homology analysis. The genomic endonuclease restriction technique is also used to distinguish species (85). This technique has identified the nine *Chlamydia* groups: *C. trachomatis* human group (61), a mouse group (25, 61), a swine group (20), an avian group (2, 6, 33, 48), an abortion group (40, 47, 64), a feline group (25, 33, 40, 48), a guinea pig inclusion conjunctivitis group (14), *C. pecorum* (8, 25, 84), and *C. pneumoniae* (14, 33). The distinct separation of these nine groups is supported by the results of phylogenetic analyses of *omp1* (15, 22, 44, 80, 97).

The intergenic spacer and/or domain I may be used as a basis for phylogenetic analysis because each of these regions is subject to systematic selective pressure and functional conservation. For a classification system to be dependable, only gene sequences that are under comparable selective pressures (i.e., sequences that perform identical functions) may be compared (95). Intergenic spacers function in nucleolytic processing of rRNA genes by formation of complementary stem-loops with the 16S promoter region and with the segment downstream of the 23S gene (74). In this study, chlamydial intergenic spacers were found to be highly conserved with well-defined segments of species- or group-specific sequence and no tRNA genes (Fig. 2). A conserved segment of the *C. trachomatis* MoPn intergenic spacer complemented the 16S promoter sequence, and nucleolytic processing site P2 (19) was located in the complementary stem (Fig. 3). This suggests that the site of RNase III processing for the 16S rRNA is located between variable segments β and γ of the intergenic spacer. Conservation and complementarity in the intergenic spacers indicate that chlamydiae use these regions in ribosomal processing by a typically eubacterial mechanism and that they are under comparable selective pressures. The sole function of the chlamydial intergenic spacer is to facilitate processing of the 16S genes and, presumably, the 23S genes. Thus, the intergenic spacer is an appropriate basis for chlamydial classification. Domain I rRNA also meets the criteria for conservation of function as a basis for phylogenetic analysis. The secondary structure of 23S rRNA is comprised of six domains with long-range double helices (1, 36), and the function of domain I in the *E. coli* ribosome has been characterized (13). Homology between *Chlamydia* and *E. coli* domain I sequences indicates that the secondary structures are very similar and that they are therefore likely to have comparable functions (data not shown).

Thus, a new taxonomy for the genus *Chlamydia* in which the

C. trachomatis and non-*C. trachomatis* lineages would be separate genera and the nine groups would be species is fully consistent with established taxonomic criteria. Rapid identification and classification could be done by using the intergenic spacer and/or domain I, as the resolving power of these segments is at least equivalent to the resolving power of DNA-DNA homology. Primers #16SF2 and #23R might be used for a preliminary taxonomic assessment (Fig. 1). These primers amplify an approximately 600-bp PCR product that includes the intergenic spacer and 200 bp of domain I. This product is easily characterized by gel electrophoresis, RFLP analysis, or sequence analysis and is small enough to be efficiently amplified from clinical field specimens or partially intact DNA. In contrast, genus and species determinations based on the 16S gene and on *omp1* would have limitations, as the 16S gene lacks diversity and *omp1* has an excess of diversity (Table 4). The chlamydial 16S genes are well-suited for distinguishing *Chlamydia* spp. and chlamydia-like organisms at the family level. Serological data and *omp1* sequences, which are highly variable within each of the nine groups, are ideal for strain identification.

Evolutionary relationships among representative chlamydial isolates from mammals and birds were examined in this study. However, chlamydia-like organisms have now also been identified in the single-celled organism *Acanthamoeba* sp. (1a, 29). Analyses of domain I segments and 16S genes have indicated that the divergence of chlamydia-like organisms antedated the divergence of the *C. trachomatis* and non-*C. trachomatis* lineages. It is possible that the genus *Chlamydia* evolved from an ancestor that infected only single-celled organisms and that, as mammals and birds evolved, chlamydiae evolved to fit new niches. Some chlamydial groups, such as the *C. psittaci* avian group, have retained a promiscuous capacity for virulence. The relative inabilities of most other groups to cause endemic disease outside the observed host ranges may be due to adaptation associated with chronic persistence (59) and lack of opportunity. The presence of chlamydia-like organisms in the genus *Acanthamoeba* and the high degree of adaptation of chlamydial groups to specific hosts indicate that present methods of isolation need to be expanded to encompass more diverse hosts. Techniques for identifying unculturable strains can also greatly broaden our knowledge of the host range of chlamydiae (1, 10).

Our results indicate that *Chlamydia* spp. can be identified by analyzing short DNA segments in the ribosomal operon that contain numerous group-conserved sequence differences. With the information gained, problematic classification issues can be clarified and comprehensive epidemiologic studies can be conducted. The availability of ribosomal data makes possible ordered analysis of relationships among members of the *C. trachomatis* and non-*C. trachomatis* branches of chlamydiae. Ribosomal information developed in this study should facilitate detection and identification of the increasing number of chlamydiae and chlamydia-like organisms.

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23S genes of *C. psittaci* 6BC^T, *C. trachomatis* L2/434/BU, and *C. pneumoniae* TWAR.

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