

# Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms

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**The current taxonomic classification of *Chlamydia* is based on limited phenotypic, morphologic and genetic criteria. This classification does not take into account recent analysis of the ribosomal operon or recently identified obligately intracellular organisms that have a chlamydia-like developmental cycle of replication. Neither does it provide a systematic rationale for identifying new strains. In this study, phylogenetic analyses of the 16S and 23S rRNA genes are presented with corroborating genetic and phenotypic information to show that the order *Chlamydiales* contains at least four distinct groups at the family level and that within the *Chlamydiaceae* are two distinct lineages which branch into nine separate clusters. In this report a reclassification of the order *Chlamydiales* and its current taxa is proposed. This proposal retains currently known strains with > 90% 16S rRNA identity in the family *Chlamydiaceae* and separates other chlamydia-like organisms that have 80–90% 16S rRNA relatedness to the *Chlamydiaceae* into new families. *Chlamydiae* that were previously described as '*Candidatus Parachlamydia acanthamoebae*' Amann, Springer, Schönhuber, Ludwig, Schmid, Müller and Michel 1997, become members of *Parachlamydiaceae* fam. nov., *Parachlamydia acanthamoebae* gen. nov., sp. nov. '*Simkania*' strain Z becomes the founding member of *Simkaniaceae* fam. nov., *Simkania negevensis* gen. nov., sp. nov. The fourth group, which includes strain WSU 86-1044, was left unnamed. The *Chlamydiaceae*, which currently has only the genus *Chlamydia*, is divided into two genera, *Chlamydia* and *Chlamydophila* gen. nov. Two new species, *Chlamydia muridarum* sp. nov. and *Chlamydia suis* sp. nov., join *Chlamydia trachomatis* in the emended genus *Chlamydia*. *Chlamydophila* gen. nov. assimilates the current species, *Chlamydia pecorum*, *Chlamydia pneumoniae* and *Chlamydia psittaci*, to form *Chlamydophila pecorum* comb. nov., *Chlamydophila pneumoniae* comb. nov. and *Chlamydophila psittaci* comb. nov. Three new *Chlamydophila* species are derived from *Chlamydia psittaci*: *Chlamydophila abortus* gen. nov., sp. nov., *Chlamydophila caviae* gen. nov., sp. nov. and *Chlamydophila felis* gen. nov., sp. nov. Emended descriptions for the**

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**Abbreviations:** CCon, *Chlamydia* consensus 16S rRNA sequence; EB, elementary body; LGV, lymphogranuloma venereum; MLE, maximum-likelihood estimate; MN, meningopneumonitis; MOMP, major outer-membrane protein; NJ, neighbour-joining; RB, reticulate body.

The GenBank accession numbers for the sequences used in this paper are given in Figs 1 and 2 and Tables 2 and 3.

**order *Chlamydiales* and for the family *Chlamydiaceae* are provided. These families, genera and species are readily distinguished by analysis of signature sequences in the 16S and 23S ribosomal genes.**

**Keywords:** *Chlamydiales*, taxonomy, ribosomal, human pathogens, animal pathogens

## INTRODUCTION

Members of the order *Chlamydiales* are obligately intracellular bacteria. They exhibit a two-stage developmental cycle of replication: the infectious bacterial form is endocytosed by eukaryotic cells and resides within a cytoplasmic inclusion, where it transforms into a vegetative form and replicates by binary fission (Moulder, 1991). As an inclusion fills with progeny, chlamydiae transform back into the metabolically inactive, infectious form and are released through host cell rupture or fusion of the inclusion/plasma membranes. Chlamydiae are disseminated by aerosol or by contact, requiring no alternate vector. Of the fifteen or more major groupings in the domain *Bacteria* (Stackebrandt *et al.*, 1997; Van de Peer *et al.*, 1994), *Chlamydiales* is the only lineage whose known members are exclusively intracellular parasites of members of the domain *Eucarya*.

The order *Chlamydiales* currently has one family, the *Chlamydiaceae*, containing one genus and four species (Herring, 1993). When the Approved Lists of Bacterial Names was published in 1980, the *Chlamydiaceae* had just two species (Skerman *et al.*, 1980). At that time, all bacteria with chlamydia-like chemical characteristics, morphology and developmental replication belonged to either *Chlamydia trachomatis* or *Chlamydia psittaci* in the order *Chlamydiales* (Page, 1966, 1968; Storz & Page, 1971). *Chlamydia trachomatis* strains were identified by their accumulation of glycogen in inclusions and their sensitivity to sulfadiazine. *Chlamydia psittaci* strains did not accumulate glycogen and were usually resistant to sulfadiazine. The establishment of this classification was a milestone in chlamydial taxonomy, as it renounced reliance on presumed host, on presumed tissue preference and on serology in grouping these organisms.

Before 1980, however, isolations had already been made that would not fit into these two species (Darougar *et al.*, 1980; Dwyer *et al.*, 1972; Forsey & Darougar, 1984). The development of DNA-based classification methods during the 1980s provided new techniques for differentiating chlamydial groups. DNA-DNA reassociation, in particular, was established as a tool for distinguishing species (< 70% homology) and genera (< 20% homology) (Amann *et al.*, 1995; Schleifer & Stackebrandt, 1983; Wayne *et al.*, 1987). According to these criteria, DNA-DNA reassociation studies of new chlamydial strains and of strains placed in the ATCC collection prior to 1971 supported eight groups on the level of genus or species (Table 1). These data contributed to the creation of two additional species, *Chlamydia pneumoniae*

(Grayston *et al.*, 1989) and *Chlamydia pecorum* (Fukushi & Hirai, 1992). In 1993, DNA sequence analysis identified an isolate from a ninth chlamydial group, *Chlamydia trachomatis*-like *Chlamydia* from swine (Kaltenboeck *et al.*, 1993). Since then, a number of closely related swine isolates and variants have been reported (Everett & Andersen, 1997; Kaltenböck *et al.*, 1997; Kaltenboeck & Storz, 1992; Rogers *et al.*, 1993, 1996; Rogers & Andersen, 1996; Schiller *et al.*, 1997). These strains cannot be designated as *Chlamydia trachomatis* using current criteria, as many of these strains are resistant to sulfadiazine (Andersen & Rogers, 1998). The nine clusters in the *Chlamydiaceae* are supported by analyses of phenotype, antigenicity, associated disease, host range, biological data and genomic endonuclease restriction (Everett & Andersen, 1997). Genetic data, including recent phylogenetic analyses using the ribosomal operon, are also consistent with the presence of nine groups in the *Chlamydiaceae* (Everett & Andersen, 1997; Kaltenboeck *et al.*, 1993; Pudjijatmoko *et al.*, 1997; Takahashi *et al.*, 1997).

Species within the *Chlamydiaceae* have 16S rRNA gene sequences that are > 90% identical (Pettersson *et al.*, 1997; Pudjijatmoko *et al.*, 1997; Takahashi *et al.*, 1997). All strains in the *Chlamydiaceae* are recognized by monoclonal antibodies (mAbs) that detect the LPS trisaccharide  $\alpha$ Kdo-(2  $\rightarrow$  8)- $\alpha$ Kdo-(2  $\rightarrow$  4)- $\alpha$ Kdo (Löbau *et al.*, 1995). Four additional groups of chlamydia-like organisms have been recently identified that have > 80% 16S rRNA gene sequence identity with chlamydiae: (i) the 'Simkania' strain Z (Kahane *et al.*, 1995, 1999); (ii) strains of 'Hall's coccus' (Birtles *et al.*, 1997) and the closely related 'Candidatus Parachlamydia acanthamoebae' Amann *et al.* 1997 (Amann *et al.*, 1997), all of which were isolated from amoebae; (iii) an agent identified as a cause of bovine abortion that was initially described as a rickettsia (Dilbeck *et al.*, 1990; Kocan *et al.*, 1990; Rurangirwa *et al.*, 1999); and (iv) a cluster of isolates from amoebae (T. Fritsche, personal communication; Gautom *et al.*, 1996). These bacteria meet the requirement for inclusion in *Chlamydiales* because they are obligate intracellular bacteria that have the chlamydia-like developmental cycle of replication. Inclusion of these novel chlamydia-like agents in *Chlamydiales* meets criteria recently established to define a bacterial class based exclusively on > 80% 16S rRNA gene sequence identity (Stackebrandt *et al.*, 1997). However, because the new groups at present include just a few species, a decision regarding whether *Chlamydiales* should become a class or remain an order can be deferred until more information on these and other new chlamydial

**Table 1.** *Chlamydia* spp. percentage similarity using DNA–DNA reassociation (*Chlamydia suis* not tested)

The total number of strains (*n*) that were used in these studies is shown for each species in the row heading, but many of the comparisons were performed with only a subset of these strains.

	8	7	6	5	4	3	2	1
1 <i>Chlamydia trachomatis</i> ( <i>n</i> = 8)	≤ 5*	6–11*, 18–24†	≤ 5–6*, 5–15†	7–12‡, 11§, ≤ 5*, 15–33†	4–32†	≤ 5–7*	65§, 20–52†	100‡§, 97–100*, 92–100†
2 <i>Chlamydia muridarum</i> ( <i>n</i> = 1)	–	1–10†	2–6†	11§, 3–20†	1–4†	–	100†	
3 <i>Chlamydophila pneumoniae</i> ( <i>n</i> = 4)	≤ 5–8*	6–8*	6–10*	≤ 5–7*	10¶	94–100*, 94–96¶		
4 <i>Chlamydophila pecorum</i> ( <i>n</i> = 3)	–	9–12†	1†	1–19†	88–100†			
5 <i>Chlamydophila psittaci</i> ( <i>n</i> = 6)	20–35*	21–37*, 31–33†	63–85*, 27–85†	100‡§, 93–100*, 73–100†				
6 <i>Chlamydophila abortus</i> ( <i>n</i> = 2)	27–38*	24–37*, 14–33†	100*†					
7 <i>Chlamydophila felis</i> ( <i>n</i> = 2)	30–33*	100*†						
8 <i>Chlamydophila caviae</i> ( <i>n</i> = 1)	100*							

\* Cox *et al.* (1988).

† Fukushi & Hirai (1989). Note also that interspecies analyses were carried out with one *Chlamydophila pecorum* strain, and that *Chlamydophila pecorum* intraspecies analysis used three.

‡ Kingsbury & Weiss (1968).

§ Weiss *et al.* (1970).

¶ Fukushi & Hirai (1992).

groups has become available. It seems likely that culture-free PCR techniques and DNA sequencing will soon expand our understanding of diversity within *Chlamydiales* and this will help resolve questions about higher level classification.

A reclassification of chlamydiae based on 16S rRNA sequence similarity clusters, on analyses of full-length 16S and 23S rDNAs, and on phenotypic and ecologic differentiation (Palys *et al.*, 1997) solves a number of current problems in chlamydial taxonomy. It provides a consistent method for identifying present taxa and a logical basis for defining new taxa using criteria that are comparable to those being established for other major bacterial lineages. It provides appropriate groupings for pathogens with distinctive underlying genetic relationships and diverse, well-established patterns of virulence. It further provides a usable system for organizing chlamydial data in the international sequence databases.

The purpose of this report is to update the description of taxa in the order *Chlamydiales*, presenting criteria that distinguish *Parachlamydiaceae* fam. nov., *Simkaniaceae* fam. nov. and *Chlamydiaceae* and designating appropriate genera and species within the *Chlamydiaceae*. To accomplish this, phylogenetic trees were constructed using 16S and 23S rRNA sequences from a number of strains within the *Chlamydiaceae* and closely related strains. To aid in the identification of new strains, 'signature sequence' segments for PCR amplification and sequencing were identified in the 16S and 23S rRNA genes. Information was compiled for the descriptions of species, chlamydial genome sizes and the presence or the absence of extrachromosomal plasmids. We offer the following proposal for revision of groupings within the order *Chlamydiales*, with two new families within the *Chlamydiales* and establishing a new genus and five new species in the *Chlamydiaceae*.

In this paper, the new names or combinations have only been used in the text following their first mention in the descriptions. However, the new names and combinations have been used throughout the tables and figures.

## METHODS

**Bacterial strains.** *Chlamydia* strains that were used for 23S rRNA gene analysis included *Chlamydia pneumoniae* TW-183<sup>T</sup>, *Chlamydia pecorum* IPA, *Chlamydia psittaci* NJ1, 6BC<sup>T</sup>, GPIC, FP Baker, *Chlamydia trachomatis* A/Har-13<sup>T</sup>, L2/434/BU, MoPn, SFPD and the *Chlamydia trachomatis*-like swine strain R22 and were obtained from previously described sources (Everett & Andersen, 1997). 'Simkania' Z was obtained from preparations of HeLa and Vero cells, and was identical to 'Simkania' Z1, which was a subculture of 'Simkania' Z (Everett & Andersen, 1997; Kahane *et al.*, 1993). Strain WSU 86-1044 was obtained from Fred Rurangirwa at the Washington Animal Disease Diagnostic Laboratory (WADDL) in Pullman, WA, USA. Chlamydial strains used for PFGE, extrachromosomal plasmid analysis, and testing of primers for signature sequence analysis were obtained from previously described sources (Everett & Andersen, 1997). *Escherichia coli* strain JM109 used in PFGE was obtained from Francis E. Nano (currently at University of Victoria, Victoria, BC, Canada).

**Sequence and phylogenetic analysis.** Oligonucleotide primer synthesis and DNA sequencing were performed by the Iowa State University DNA Sequencing and Synthesis Facility, Ames, IA, USA. Both the coding and the complementary strands were sequenced. The Sequencher data analysis software (Gene Codes) was used to assemble the 23S rRNA gene sequences. Sequence analysis programs described in the *Program Manual for the Wisconsin Package* (Genetics Computer Group, 1994) were used for DNA and RNA analyses. These programs were Reformat, SeqEd, Assemble, PILEUP, LineUp, Distances, FoldRNA and SQUIGGLES.

For phylogenetic analyses of the complete 16S rRNA genes, sequence data were retrieved from GenBank and extended to full-length as necessary using data from a recent study

(Everett & Andersen, 1997). The 16S rDNA from strain SFPD was resequenced; the 16S rRNA and 16S–23S intergenic spacer sequence for strain WSU 86-1044 were provided by Fred Rurangirwa (Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA). The 23S rRNA sequences from strain WSU 86-1044 and 'Simkania' strain Z were obtained directly from PCR products. Full-length 23S rRNA sequence data for phylogenetic analysis were obtained from PCR-amplified segments that supplemented data previously obtained (Everett & Andersen, 1997). Two PCR primers were used for these amplifications: primer 6F matching the coding strand of the *Chlamydia* 23S rRNA (5' GGATG-AGTTGTGAATAGG 3') and a complementary primer that PCR amplifies the 5S gene of *Brucella abortus* (5' AGTTCGGGATGGGATCGGGTG 3'). Additional primers were used to sequence both the PCR products and the cloned PCR-product templates. PCR conditions for all amplifications using AmpliTaq DNA polymerase (Perkin Elmer) were 30 s at 94 °C, 15 s annealing, 30 s at 72 °C in the GeneAmp PCR System 9600 thermal cycler (Perkin Elmer). Annealing temperatures were specific for each primer set. The 3' end of each ~3000 bp 23S rRNA gene was determined by FoldRNA analysis of sequence data for the intergenic spacer that had been spliced, by computer, to sequence data for a 200 base segment of 23S rRNA extending into the 5S gene. The 5' end of the 23S rRNA genes in 6BC<sup>T</sup>, NJ1 and R22 had previously been identified by primer extension analysis (Everett & Andersen, 1997).

For phylogenetic analysis, full-length 16S rRNA and 23S rRNA data were aligned using CLUSTAL W (Thompson *et al.*, 1994). There were several short segments in the 23S rRNA which were difficult to align, however, the results are robust with respect to alternative alignment decisions and also to the removal of these segments. The 3' ends of both genes were trimmed to produce sequences of even length: 1558 nt positions were analysed for the 16S rRNA gene and 2956 nt positions were analysed for the 23S rRNA gene. Phylogenetic trees were generated using the maximum-parsimony and neighbour-joining routines of PAUP 4.0.0d63 (Swofford, 1993). PAUP's heuristic search parameters were set to trunk-bisection-resection branch swapping with no limit on the number of trees held per run, and with branches having a maximum length of zero collapsed to yield polytomies. Branching order reliability was evaluated by 1000 replications of bootstrap resampling using parsimony (Felsenstein, 1985). Trees were also constructed using the maximum-likelihood routine DNAML of PHYLIP 3.5c (Felsenstein, 1993). Parsimony and maximum-likelihood trees were both generated using 10 replicate runs in which the input sequences were read in random order. Saturation plots were constructed contrasting phyletic versus pairwise distances for each tree. Non-linearity of these plots would suggest that excessive homoplasy could be responsible for nodes being joined at random rather than due to similarity (Vuillaume *et al.*, 1997).

**Signature sequence identification.** 'Signature sequence' segments were identified by examining chlamydial 16S rRNA gene sequences and partial chlamydial 23S rRNA gene segments, most of which are currently in the GenBank database. Signature segments were narrowly defined regions of sequence that were conserved and distinctive for each of nine groups. The 16S signature segments were determined from the 59 discrete chlamydial 16S rRNA genes in GenBank plus the *Chlamydia pneumoniae* Koala sequence from strain Con. There were ≥ 5 representative strains

from six of the nine groups in the *Chlamydiaceae* and fewer strains in the other groups. The partial 23S rRNA sequences used to identify the 23S signature sequence included > 50 strains, with ≥ 5 representative strains from each of five of the nine groups in the *Chlamydiaceae* and fewer strains in the other groups. The 298 base 16S signature sequence could be PCR-amplified and sequenced using 51 °C annealing and primers which matched all chlamydiae: 16SIGF (5' CGG-CGTGGATGAGGCAT 3'), which matched the 16S rRNA gene at position 40, and 16SISR (5' TCAGTCCCAGTG-TTGGC 3'), which complemented the 16S rRNA gene at position 337 (*E. coli* and *Chlamydia* numbering). The rDNA template was PCR amplified with AmpliTaq DNA polymerase as described above. In some cases, the rRNA template was reverse transcribed and PCR was performed in a single reaction mix using the GeneAmp EZ *rTth* RNA PCR Kit (Perkin Elmer). PCR products were filtered with Microcon 100 microconcentrators (Amicon) prior to sequencing.

The 23S signature sequence, which was domain I of the 23S rRNA gene, was PCR-amplified and partially sequenced using either or both of two strategies. The first strategy was applied to reverse-transcribed rRNA or to rDNA template to amplify a 600 bp PCR product containing most of the signature sequence. This strategy used two conserved bacterial primers, U23F (5' GATGCCTTGGCATTGATAG-GCGATGAAGGA 3') and 23SISR (5' TGGCTCATCATGCAAAAGGCA 3') (annealing temperature 61 °C), which can also amplify non-chlamydial rRNAs. These primers were used in direct sequence analysis of PCR products that were filtered with Microcon 100 microconcentrators. For the *Chlamydiaceae* strains, a second strategy was also used to characterize the 23S signature sequence. A 1 kbp product was PCR-amplified from rDNA template using primers 16SF2 (5' CCGCCCGTCACATC-ATGG 3') and 23SISR (5' TGGCTCATCATGCAAAAGGCA 3'). This amplicon contained a complete 627 bp domain I segment, the ≥ 220 bp intergenic spacer, and 150 bp of the 16S rRNA gene; domain I was fully sequenced using primers 16SF2, 23SISR, IGSIGF (5' ATAATAAT-AGACGTTTAAGA 3') and 23R (5' TACTAAGATGTT-TCAGTTC 3').

**PFGE.** DNA plugs of 1% (w/v) low-melting-point agarose that contained Renografin-purified chlamydiae were prepared for PFGE. Pellets of chlamydiae and chlamydiae-infected cells were resuspended in 40% (w/v) Renografin (Solvay Animal Health) in PBS containing Ca<sup>2+</sup> and Mg<sup>2+</sup> (Sambrook *et al.*, 1989), incubated for 10 min at room temperature, and diluted in at least 2 vols PBS. Lysed host-cell DNA was sheared by repeated pipetting. Chlamydiae were then pelleted at 10000 r.p.m., resuspended in a few microlitres of PBS, mixed with 1% LMP agarose at 37 °C, and cooled in aliquots in a plug mould (Bio-Rad Laboratories). After refrigeration, the plugs were sequentially incubated in several buffers: 50 mM DTT, 30 mM Tris, 10 mM EDTA pH 9.0 at 37 °C overnight; 10 mM Tris, 10 mM EDTA pH 8.0 (TE) with DNase-free RNase (Boehringer Mannheim) at 37 °C for 5 h; TE containing 10 µg Proteinase K ml<sup>-1</sup> (Gibco BRL Life Technologies) at 50 °C overnight; and TE at 4 °C, with four or more changes of TE over a period of a week or more.

Initially, the plugs were subjected to 4 krad (40 gray) of gamma irradiation (Walker *et al.*, 1991) to linearize chromosome prior to electrophoresis. Chromosome from treated and untreated plugs, however, showed no reproducible differences in electrophoretic mobility. Subsequently, there-

fore, radiation treatment was removed from the protocol. The plugs were inserted into a 1% (w/v) agarose gel (12.5 × 12.5 × 1.2 cm) and electrophoresed at 150 V, 14 °C using the CHEF-DR Drive Module, Pulsewave 760, and power supply model 200/2.0 (Bio-Rad) with recirculating buffer containing 22.5 mM Tris/borate, 1 mM EDTA. A pulse time of 2 min was applied for 24 h, the buffer was replaced with fresh buffer, then a pulse time of 4 min was applied for 30 h (Bergthorsson & Ochman, 1995). Gels were stained with Sybr Green I nucleic acid stain (FMC BioProducts) (not shown) or with ethidium bromide. PFGE II size markers (Boehringer Mannheim) were used.

**Southern blot analysis.** An oligonucleotide primer recognizing the origin of replication of all known chlamydial plasmids was synthesized. This 102 base primer consisted of four direct repeats with a restriction site at each end (Integrated DNA Technologies): GCGGCCGC[TTGCAACT(T/C)T(T/A)GGTGGTAGACT]<sub>4</sub>CCCGGG. The primer was 5' end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP [ICN; 10  $\mu$ Ci  $\mu$ l<sup>-1</sup> (3.7 × 10<sup>5</sup> Bq  $\mu$ l<sup>-1</sup>), 4500 Ci mol<sup>-3</sup> (1.665 × 10<sup>14</sup> Bq mol<sup>-4</sup>)] using T4 polynucleotide kinase (GibcoBRL Life Technologies). The 25  $\mu$ l labelling reaction was stopped by the addition of 75  $\mu$ l H<sub>2</sub>O and 50  $\mu$ l 7.5 M ammonium acetate. Labelled primer was precipitated by adding 5  $\mu$ l 5 mg tRNA ml<sup>-1</sup> in H<sub>2</sub>O, then adding 450  $\mu$ l 95% ethanol, followed by centrifugation. Chlamydial DNA that was embedded in agarose plugs (above) was separated by standard electrophoresis in 1 × TBE on 1% (w/v) agarose gels (10 × 6.5 cm, or 10 × 14 cm). A DNA size standard, 1 kb DNA Ladder (GibcoBRL Life Technologies), was electrophoresed at the same time. After electrophoresis, the separated DNAs were denatured by soaking the gel for 45 min in several vols of 1.5 M NaCl, 0.5 M NaOH at 4 °C; neutralized in 1 M Tris pH 7.2, 1.5 M NaCl (Sambrook *et al.*, 1989); transferred to a Hybond-N+ nucleic acid transfer membrane (Amersham Life Science) by capillary transfer in 10 × SSC (0.15 M sodium citrate, 1.5 M NaCl pH 7.0), and baked onto the membrane at 80 °C, ambient pressure for 2 h. The blot was prehybridized and then hybridized with probe at 60 °C overnight in 6 × SSC containing 0.1% (w/v) SDS and sheared denatured calf thymus DNA (100  $\mu$ g ml<sup>-1</sup>). The blot was washed at 60 °C in 1.0 × SSC, 0.5% SDS and then washed repeatedly at 50 °C in 0.1 × SSC, 0.5% SDS prior to autoradiography.

## RESULTS

### Phylogenetic analysis of 16S rRNA revealed nine groups in two lineages in the *Chlamydiaceae*

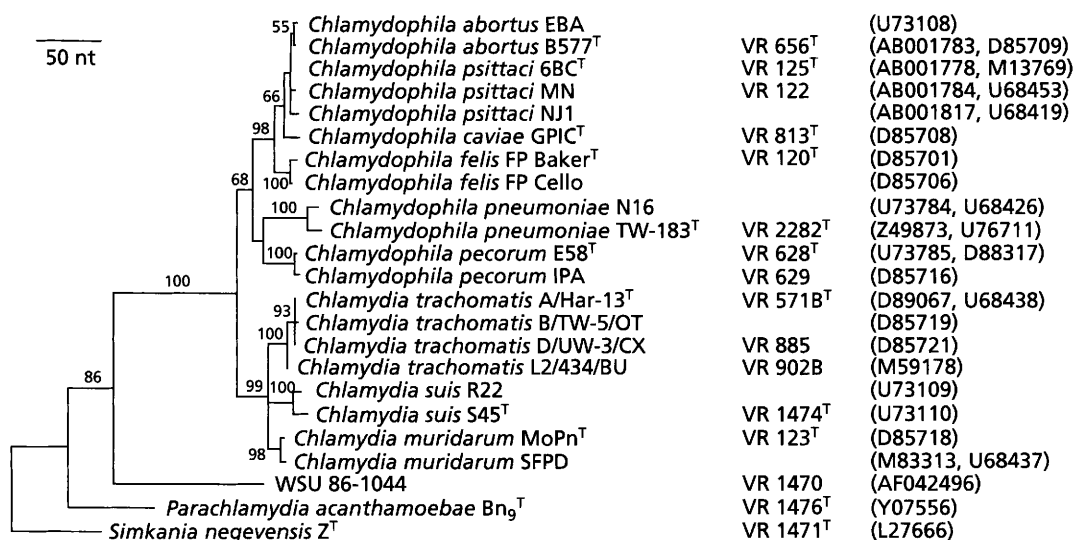
Ninety-three 16S rRNA sequences available from the *Chlamydiaceae*, 'Candidatus Parachlamydia' and 'Simkania' in GenBank, as of March 1998, were aligned with new data for SFPD and for WSU 86-1044. The alignment required few gaps, so no positions needed to be removed from the analysis. This large dataset contained a number of clusters of sequences that were so closely related as to be nearly identical. Phylogenetic trees were constructed using a subset of 23 of the 93 sequences, chosen so that all clusters were represented but so that nearly identical sequences were removed. Parsimony, maximum-likelihood (MLE) and neighbour-joining (NJ) algorithms were used to determine phylogenetic relationships among the 23 sequences.

Parsimony analysis found 28 different most-parsimonious trees, each requiring 693 nucleotide substitutions. Only 272 of these substitutions were within the *Chlamydiaceae* lineage. A consensus tree was constructed using a 50% majority rule. This tree had a consistency index of 0.75 (1.0 being the highest possible), indicating a lack of resolution at several nodes. Elimination of the outgroups resulted in 26 equally parsimonious trees and only increased the consistency index to 0.77, indicating that the distance of the outgroups was not responsible for there being more than one most parsimonious tree. The low consistency index was also not due to mutational saturation, because a plot of pairwise versus phyletic differences for all groups was linear. The MLE and NJ trees were similar to several of the parsimony trees, the differences being at nodes that were poorly resolved in the parsimony analysis. In particular, the NJ tree had a monophyletic clade containing feline, abortion, avian and *C. pecorum* clusters. This clade shared a common ancestor with *C. pneumoniae*, while the parsimony and MLE trees had *C. pecorum* paired with *C. pneumoniae*. The finding of more than one most parsimonious tree was due primarily to the presence of several nodes that were determined by a small number of nucleotide substitutions.

The MLE, NJ and parsimony trees were all in agreement with two observations: the same nine sequence clusters were always present in every tree and the *C. trachomatis*-like clusters were separated into a distinct, well-supported lineage. Eight of the nine specific clusters were the human strains of *C. trachomatis*, swine strains of *C. trachomatis*, *C. trachomatis* strains that infect Muridae species, *C. pneumoniae* strains, *C. pecorum* strains, feline strains, abortion strains and avian strains. The ninth, guinea pig strain GPIC, was the single representative of its group. The order in which GPIC diverged from the feline, abortion and *C. psittaci* clusters was unresolved. The parsimony and NJ trees showed GPIC diverging between the feline cluster and *C. psittaci*, while the MLE tree made GPIC a sister group with the abortion cluster.

Bootstrap analysis, in which 1000 replicate trees were produced using random subsamples of the nucleotide positions in the dataset, was used to assess statistical support for the individual branches (Fig. 1). In the non-*C. trachomatis*-like group, the B577 and EBA abortion strains were always grouped together, although they formed a monophyletic clade distinct from the avian cluster in only 55% of the bootstrap replicates. Thus, the abortion strains were closely related to each other but as a group had not yet diverged very far from the avian strains. A clade that contained feline, guinea pig, avian and abortion clusters was present in 98% of the trees. The bootstrap procedure provided 99% support for the monophyly of the *C. trachomatis*-like clusters, but little support for any particular order of divergence among the human, swine and Muridae clusters within *C. trachomatis*.

Sixty-eight per cent of the bootstrap replicates showed



**Fig. 1.** Phylogeny of the order *Chlamydiales*, constructed using full-length 16S rRNA genes. Branch lengths are measured in nucleotide substitutions. Numbers show the percentage of times each branch was found in 1000 bootstrap replicates. Branches without bootstrap values occurred in less than 50% of the trees. Values in parentheses are the GenBank sequence accession numbers.

**Table 2.** Percentage differences among the 16S rRNA genes of *Chlamydiales* spp. type strains and some of their closest relatives

*Chlamydia* type strains are MoPn<sup>T</sup>, A/Har-13<sup>T</sup> and S45<sup>T</sup>. *Chlamydia* type strains are B577<sup>T</sup>, 6BC<sup>T</sup>, GPIC<sup>T</sup>, FP Baker<sup>T</sup>, E58<sup>T</sup> and TW-183<sup>T</sup>. A consensus sequence (Ccon) was derived from these genes to identify a reference strain that contained the highest proportion of conserved 16S sequence motifs for the family *Chlamydiaceae*. Ccon is heavily weighted toward the *Chlamydiaceae* lineage because plurality for the consensus was 6, and all nine *Chlamydiaceae* type strains were included in the identification of Ccon. Percentages are to the nearest whole integer ( $\pm 0.5\%$ ) or to the nearest decimal ( $\pm 0.05\%$ ) (Jukes & Cantor, 1969).

	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2
1 Ccon	1.9	2.0	2.0	2.4	3.2	4.2	3.1	3.7	4.2	13.0	16.2	16.3	30.9	31.2	33.3	33.4
2 <i>Planctomycetes staley</i>	36	35	36	36	35	35	35	35	34	33	37	35	37	35	15	0
3 <i>Pirellula marina</i>	36	35	36	36	35	35	35	35	34	37	35	39	33	0		
4 <i>Verrucomicrobium VeGlc2</i>	33	33	33	33	33	33	33	33	31	33	33	37	0			
5 Chloroplast <i>Chlorella ellipsoidea</i>	32	32	32	33	32	32	31	31	34	35	35	0				
6 <i>Simkania</i>	18	18	18	18	18	18	18	18	13	18	0					
7 WSU 86-1044	18	18	18	18	18	18	18	18	14	0						
8 <i>Parachlamydia</i>	15	15	14	15	14	14	14	15	14	0						
9 S45 <sup>T</sup>	6	6	6	6	5	7	2	3	0							
10 A/Har-13 <sup>T</sup>	5	5	5	5	5	6	2	0								
11 MoPn <sup>T</sup>	4	4	4	5	5	6	0									
12 TW-183 <sup>T</sup>	4	4	5	5	4	0										
13 E58 <sup>T</sup>	4	4	4	4	0											
14 FP Baker <sup>T</sup>	2	2	2	0												
15 GPIC <sup>T</sup>	1	1	0													
16 6BC <sup>T</sup>	0.2	0														
17 B577 <sup>T</sup>	0															
Accession no.	D85709	M13769	D85708	D85701	D88317	L06108	D85718	D89067	U73110	Y07556	AF042496	L27666	X12742	X99390	X62912	M34126

the *Chlamydiaceae* splitting at its base into two monophyletic clades: the *C. trachomatis*-like group and a clade containing the rest of the *Chlamydiaceae* clusters. This pattern represents the most probable structure for the root of the *Chlamydiaceae* because the next most likely split, between *C. pneumoniae* and all other *Chlamydiaceae*, had only 31% bootstrap support. The clade containing just *C. pecorum* and *C. pneumoniae* in the Fig. 1 bootstrap tree, which also appeared in the MLE tree, had only 44% support. It

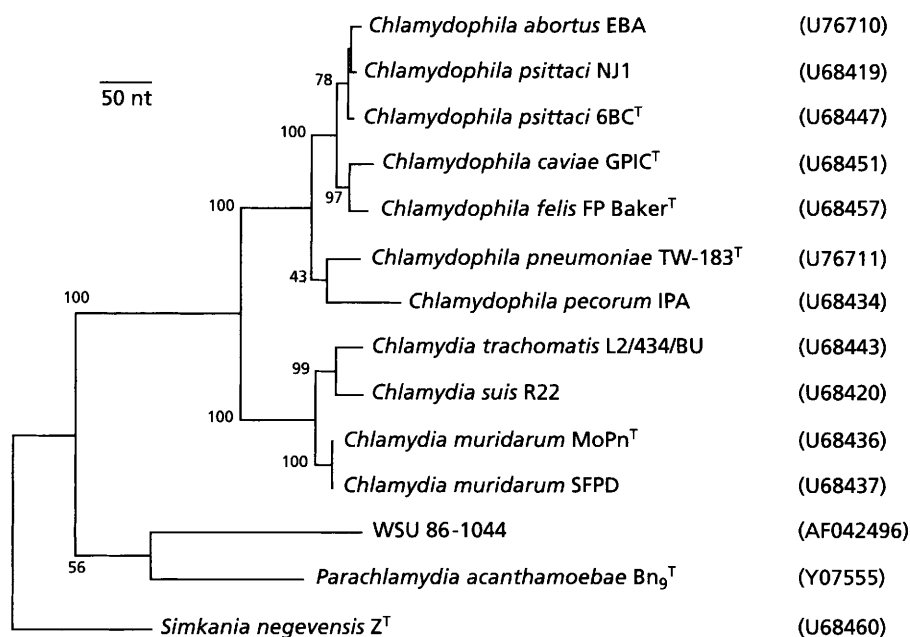
was not conclusive whether *C. pecorum*, *C. pneumoniae* or their common ancestor, diverged first from the *C. trachomatis*-like group. The order of divergence at the base of the *Chlamydiaceae* tree was most likely the *C. trachomatis*-like clusters first, then *C. pneumoniae* and *C. pecorum* groups followed by the feline, guinea pig, avian and abortion groups.

The non-*C. trachomatis*-like 16S rRNA gene sequences varied  $\leq 5\%$  among themselves. The *C. trachomatis*-

**Table 3.** Percentage differences among 23S rRNA genes of *Chlamydiales* spp. strains and some of their closest relatives

Percentages are to the nearest whole integer ( $\pm 0.5\%$ ) or to the nearest decimal ( $\pm 0.01\%$ ) (Jukes & Cantor, 1969). MoPn and SFPD are 0.03% different and have identical percentage differences with all strains as shown. NJ1 and 6BC<sup>T</sup> are 0.37% different and have identical percentage differences with all *Chlamydiaceae* strains as shown.

	13	12	11	10	9	8	7	6	5	4	3	2	1
1 Chloroplast <i>Chlorella ellipsoidea</i>	48	48	47	48	48	47	48	48	49	48	48	49	0
2 <i>Simkania</i>	18	18	19	19	20	19	18	18	19	15	18	0	
3 WSU 86-1044	19	19	19	19	19	19	18	19	18	15	0		
4 <i>Parachlamydia</i>	17	17	18	18	19	18	17	18	18	0			
5 R22	8	8	8	8	9	8	2	2	0				
6 L2/434/BU	8	8	8	8	9	8	2	0					
7 MoPn <sup>T</sup>	7	7	7	8	8	7	0						
8 TW-183 <sup>T</sup>	3	3	3	3	4	0							
9 IPA	4	4	5	5	0								
10 FP Baker <sup>T</sup>	2	2	2	0									
11 GPIC <sup>T</sup>	2	2	0										
12 6BC <sup>T</sup>	0.7	0											
13 EBA	0												
Accession no.	U76710	U68447	U68451	U68457	U68434	U76711	U68436	U68443	U68420	Y07555	AF042496	U68460	M36158



**Fig. 2.** Phylogeny of 14 members of the order *Chlamydiales*, constructed using full-length 23S rRNA genes. Branch lengths are measured in nucleotide substitutions. Numbers show the percentage of times each branch was found in 1000 bootstrap replicates. Branches without bootstrap values occurred in less than 50% of the trees. Values in parentheses are the GenBank sequence accession numbers.

like gene sequences varied  $\leq 3\%$  among themselves (Table 2). The non-*C. trachomatis*-like gene sequences were 4–7% different from the *C. trachomatis*-like genes. The 4–7% differences were comparable to 16S rRNA differences that separate a number of bacterial species and genera, as determined by BLAST searching of the GenBank database, and approached neither the near-zero difference limit for species discussed by Palys *et al.* (1997) nor the 80% identity limit proposed for the class *Actinobacteria* by Stackebrandt *et al.* (1997). The separation of these nine groups was also consistent with the phyletic separation of chlamydiae based on

this gene (Fig. 1) and with biochemical and phenotypic differences between the groups. Thus, genetic differences in the 16S rRNA gene supported the proposed revision of species and genera in the *Chlamydiaceae*.

#### 16S reference sequence determined for the *Chlamydiaceae*

To identify a 16S rRNA sequence that could be used to specifically and easily recognize members of the *Chlamydiaceae*, a consensus sequence (Ccon) of rep-

	40				90				139	
<i>Chlamydia muridarum</i>	-----	-----	-----	--Cn--TG--	-----	-----	-----	-----	-----	-----
<i>Chlamydia trachomatis</i>	-----	-----	-----	-GC--TGT-	-----	-----	-----	-----	-----	-----
<i>Chlamydia suis</i>	-----	-----	-----	--CnG-TTT.	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila felis</i>	-----	-----	-----	-C--GA-	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila abortus</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila psittaci</i>	-----	-----	-----	-t-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila caviae</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila pecorum</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila pneumoniae</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<b>Consensus</b>	CGGCGTGGAT	GAGGCATGCA	AGtCgAACGG	AataaaT--ct	tCggttatTa	TTTAGTGGCg	GAAGGGTTAG	TAaTaCATAG	ATAAAttGtC	cTtAACTTGG
<i>Parachlamydia</i>	-----	-----	-----	-A--CGG....	-----	-----	-----	-----	-----	-----
<i>Simkania</i>	-----	-----	-----	-G-----	-----	-----	-----	-----	-----	-----
WSU86-1044	-----	-----	-----	-A--A--G-GTGTCT--	-----	-----	-----	-----	-----	-----
	140				190				239	
<i>Chlamydia muridarum</i>	-----	-----	-----	-G-----	-----	-----	-----	-----	-----	-----
<i>Chlamydia trachomatis</i>	-----	-----	-----	-G-----	-----	-----	-----	-----	-----	-----
<i>Chlamydia suis</i>	-----	-----	-----	-G-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila felis</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila abortus</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila psittaci</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila caviae</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila pecorum</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<i>Chlamydomphila pneumoniae</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
<b>Consensus</b>	GaATAACGGT	TGGAAACGac	CGCTAATACC	GAATGtgTg	-tgtTTaGgc	ATCtaaa--a	taTTAAAGAA	GgGGATCTTc	---GGACCTT	TcGGTTaagg
<i>Parachlamydia</i>	-----	-----	-----	-G-----	-----	-----	-----	-----	-----	-----
<i>Simkania</i>	-----	-----	-----	-G-----	-----	-----	-----	-----	-----	-----
WSU86-1044	-----	-----	-----	-G-TA-----	-----	-----	-----	-----	-----	-----
	240				290				337	
<i>Chlamydia muridarum</i>	-----	-----	-----	-T-----	-----	-----	-----	-----	-----	< 1%
<i>Chlamydia trachomatis</i>	-----	-----	-----	-T-----	-----	-----	-----	-----	-----	< 1%
<i>Chlamydia suis</i>	-----	-----	-----	-G-----	-----	-----	-----	-----	-----	< 3%
<i>Chlamydomphila felis</i>	-----	-----	-----	A-----	-----	-----	-----	-----	-----	0%
<i>Chlamydomphila abortus</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	0%
<i>Chlamydomphila psittaci</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	< 2%
<i>Chlamydomphila caviae</i>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-
<i>Chlamydomphila pecorum</i>	-----	-----	-----	-A-----	-----	-----	-----	-----	-----	< 2%
<i>Chlamydomphila pneumoniae</i>	-----	-----	-----	A-----	-----	-----	-----	-----	-----	< 2%
<b>Consensus</b>	gagAGTCTaT	GgGATATCAG	CTtGTTGGTg	GGGTAA--GGC	CTACCAAGGc	taTGACGTCT	AGGCGGATTG	AGAGATTGac	CGCCAACACT	GGGACTGA
<i>Parachlamydia</i>	-----	-----	-----	AGAG--C--	-----	-----	-----	-----	-----	-----
<i>Simkania</i>	-----	-----	-----	AGAG-CTC--	-----	-----	-----	-----	-----	-----
WSU86-1044	-----	-----	-----	--G-C-C-C	-----	-----	-----	-----	-----	-----

**Fig. 3.** 16S rRNA signature sequences for twelve species in the order *Chlamydiales*. The amplification/sequencing primers, 16SIGF (5' CGGCGTGGATGAGGCAT 3') and 16SIGR (5' TCAGTCCCAGTGTGGC 3') (51 °C annealing temperature), match or complement the first and last 17 bases in this sequence, respectively. The *Chlamydiaceae* species lines: all strains in the species are identical (upper case), are identical and match the consensus line (-), or most but not all strains use the indicated nucleotide (lower case); n = no consensus for that species; (.) = alignment gap. Consensus line: all known *Chlamydiaceae* are identical (upper case), at least one strain of the *Chlamydiaceae* is not identical to the consensus nucleotide (lower case), or fewer than six species have the same base at this position (-). The number of strains used to compile the consensus for each species is appended to the end of the first lines of sequence. Base 40 is the position 40 in the 16S rRNA gene of *Chlamydiales* and also *E. coli*. The percentage variation within each species signature is appended to the ends of the last lines of sequence. Dots were inserted into the sequences to improve alignment.

representative 16S rRNA genes from diverse *Chlamydia* strains and from closely related bacteria was identified. The Ccon sequence represented a plurality  $\geq 6$  identities at each position and matched the most conserved sequence data among these bacteria. A percentage difference analysis of Ccon with these groups was also carried out (Table 2). This comparison indicated that the 16S rRNA sequence from *C. psittaci* B577 best matched a consensus of conserved *Chlamydia* sequences.

**Phylogenetic analysis of 23S rRNA revealed two major lineages within the *Chlamydiaceae***

Thirteen complete *Chlamydiales* 23S rRNA gene sequences, including 11 *Chlamydia* strains, 'Simkania' Z and WSU 86-1044, were sequenced and compared to the previously reported 23S rRNA sequence of 'Candidatus Parachlamydia' (Amann *et al.*, 1997). The

*Chlamydia* sequences differed from the outgroup sequences by 15–20% (Table 3). Parsimony analysis produced a single most parsimonious tree with 1380 nucleotide substitutions and a consistency index of 0.76 (Fig. 2). The MLE tree was identical to the parsimony tree and *C. pecorum* and *C. pneumoniae* were paired together. The NJ tree differed from this tree only in that it showed *C. pecorum* strain IPA to be more closely related to the outgroups than was *C. pneumoniae* TW-183.

The *Chlamydiaceae* 23S rRNA tree split at its base into two monophyletic lineages with 100% bootstrap support (Fig. 2). One lineage included the *C. trachomatis*-like strains and the other included the non-*C. trachomatis*-like strains. Among the *C. trachomatis*-like strains, the swine and human isolates separated from the Muridae isolates with almost 100% bootstrap support. The base of the non-*C. trachomatis*-



like lineage was not well-defined: either *C. pneumoniae* or a common ancestor of *C. pneumoniae* and of *C. pecorum* could have been most closely related to the *C. trachomatis*-like clade. A monophyletic lineage containing the *C. psittaci* abortion/avian/feline/guinea pig strains formed the rest of the non-*C. trachomatis*-like clade with 100% bootstrap support. A saturation plot of pairwise versus phyletic differences did not show evidence of mutational saturation; thus the low consistency index was due not to excessive homoplasy but to a lack of information needed to resolve some specific nodes (e.g. the *C. pneumoniae* + *C. pecorum* node and the WSU 86-1044 + 'Candidatus Parachlamydia' node).

The non-*C. trachomatis*-like 23S rRNA gene sequences varied  $\leq 5\%$  among themselves; the *C. trachomatis*-like 23S rRNA gene sequences varied  $\leq 2\%$  among themselves (Table 3). The non-*C. trachomatis*-like gene sequences were 7–9% different from the *C. trachomatis*-like genes. The 7–9% gene sequence differences were comparable to 23S rRNA differences that separate a number of bacterial genera, as determined by BLAST searching of the GenBank database. They were also consistent with the phyletic separation of groups in the *Chlamydiaceae* (Figs 1 and 2) and with biochemical and phenotypic differences between the groups. Thus, genetic differences in the 23S rRNA gene supported the proposed revision of species and genera in the *Chlamydiaceae*.

#### Sequence signatures identified for the *Chlamydiaceae* groups

Previously it was shown by sequence comparison that the 16S–23S ribosomal intergenic spacer is a defined region of sequence that is functionally conserved and distinctive for each of the nine chlamydial groups (Everett & Andersen, 1997). A 'signature sequence' such as this can identify these groups for purposes of classification. Signature sequences were identified in the 16S and 23S rRNA genes of the *Chlamydiaceae* by aligning all of the ribosomal segments for chlamydial organisms that are in GenBank. The 16S signature sequence started at base 40 in the gene and was 298 bases long (Fig. 3). The homologous 'Simkania', 'Candidatus Parachlamydia' and WSU 86-1044 sequences were also examined (Fig. 3). Confidence in the 16S species signature was highest for human *C. trachomatis* strains, *C. pneumoniae* strains, *C. pecorum* strains, and *C. psittaci* feline, abortion and avian strains because  $\geq 4$  sequences were available for each. Variation within the 16S signature consensus for any single species was  $\leq 3\%$ . New data from 16S signature sequence analysis of *C. pneumoniae* N16 and *C. trachomatis* SFPD were used to update N16 and SFPD sequences in GenBank and in Fig. 3.

The 23S signature sequence, which was domain I of this gene, started at base 1 in the gene and was 627 bases long (Fig. 4). The homologous 'Simkania' and

'Candidatus Parachlamydia' sequences were also examined (Fig. 4). Confidence in the 23S species signature was highest for human *C. trachomatis* strains, swine *C. trachomatis*-like strains, *C. pneumoniae* strains, *C. pecorum* strains and *C. psittaci* abortion and avian strains, because  $\geq 5$  sequences were available for each. Variation within the domain I signature consensus for any single species was always  $\leq 3\%$ , but usually  $\leq 1\%$  (Fig. 4).

#### PFGE revealed conserved genome size in the *Chlamydiaceae*

PFGE of agarose-embedded chlamydial DNA was employed to determine genome sizes for six groups in *Chlamydia* and to compare these sizes with 'Simkania' and *E. coli*. The data indicated that uncut chlamydial genome migrates at an approximate size of 1–1.2 Mbp (Fig. 5). *C. trachomatis* strains and *C. trachomatis*-like swine strains had an apparent size of 1–1.1 Mbp and non-*C. trachomatis* strains had an apparent size of about 1.2 Mbp. This agreed with the size of the 1.045 Mbp *C. trachomatis* strain L2/434/BU genome identified by Birkelund & Stephens (1992). Additional study of these genomes using restriction endonucleases will be needed to confirm the actual genome sizes.

#### Extrachromosomal plasmids were strain- and group-specific

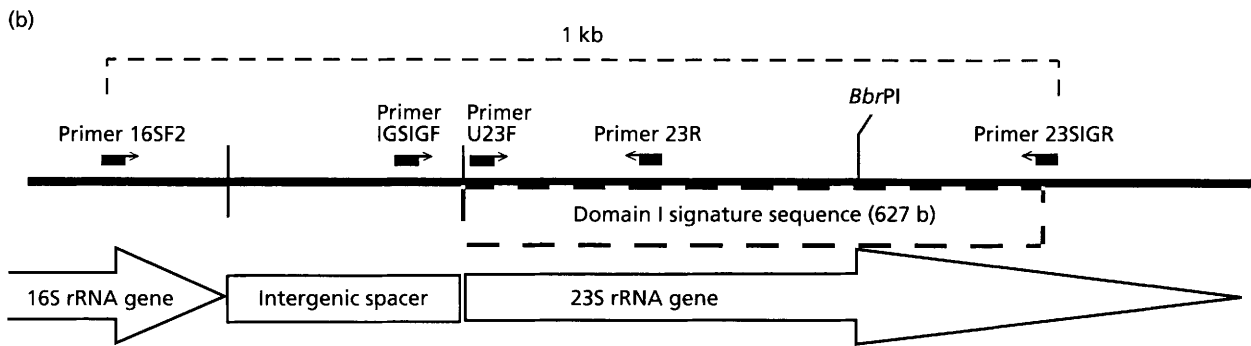
To compile information that could contribute to a more complete description of groups in the *Chlamydiaceae*, chlamydial DNA was examined for the presence of an extrachromosomal plasmid. DNAs that had been prepared in agarose plugs for PFGE were separated by standard electrophoresis on 1% gels, were examined by ethidium bromide staining, and were Southern blotted using a probe that matched the plasmid origin of replication. A sharp band that stained only weakly with ethidium bromide hybridized strongly to the probe. The mobility of this band was markedly slower than the mobility of a band of sheared DNA that stained strongly with ethidium bromide. There was no hybridization of the probe to the sheared DNA. These bands were larger than the 12 kbp DNA molecular size marker. A faint band with a mobility of 7.5 kbp was only rarely detected with the probe on Southern blot. Handling of the chromosomal/plasmid preparations in liquid form (rather than in agarose plugs) greatly increased the amount of 7.5 kbp plasmid detected by Southern blot and reduced the amount of detectable  $> 12$  kbp plasmid. The retarded mobility of the  $> 12$  kbp plasmid band was consistent with this band being an open circular form of the chlamydial plasmid (Levene & Zimm, 1987) or with its being a dimer of interlinked circular forms, as have been previously reported.

The presence of plasmids was determined or confirmed in 14 strains (Table 4). The absence of plasmid was discovered or confirmed for six strains (Table 4). Strains were designated plasmid<sup>-</sup> only if a genomic

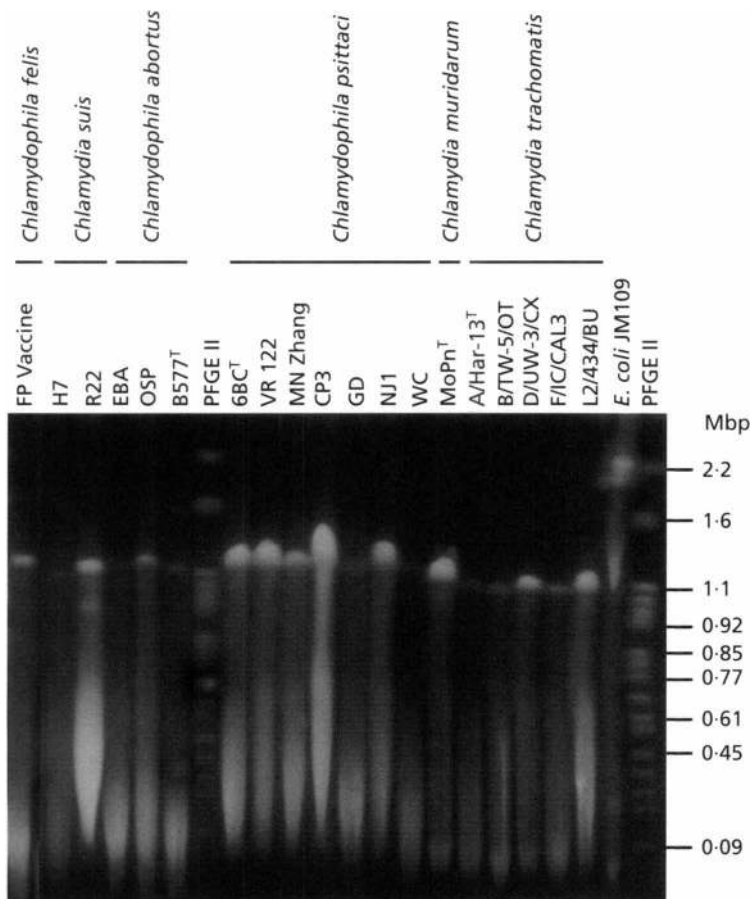
(a)

	1	50	110	2
<i>Chlamydia muridarum</i>	-----GA-----	-----	-----AA-----GAA-G-----AT-----A-A-----	2
<i>Chlamydia trachomatis</i>	-----A-----	-----A-----	-----GAA-----GAA-G-----GAT-----A-A-----	5
<i>Chlamydia suis</i>	-----GA-----	-----A-----	-----AA-----GAA-G-----GAT-----A-c-----	7
<i>Chlamydomytila felis</i>	-----G-----C-----	-----	-----G-----	3
<i>Chlamydomytila abortus</i>	-----T-----	-----	-----G-----	20
<i>Chlamydomytila psittaci</i>	-----	-----	-----G-----	15
<i>Chlamydomytila caviae</i>	-----G-----C-----	-----	-----G-----	1
<i>Chlamydomytila pecorum</i>	-----G-----	-----A-----	-----T-----	6
<i>Chlamydomytila pneumoniae</i>	-----G-----C-----g	-----	-----T-----G-----	7
<b>Consensus</b>	<b>TACAGACCAA GTTatTAAGA GcTATTGGTG GATGCGTTGg CATTGcaAGG CGAtGAAGGA tGCgttTACC TGCa-tAAtC TtCGGcGAGC TGGTataaAG CtatGACCCG</b>			
<i>Parachlamydia</i>	ATTT-GT--C-----G-----GC-----A-----CA-T-----		-----GAA-----G-----G-----A-----GC-----	1
<i>Simkania</i>	-TT-TT--C-----G-----C-----		-----CG-----GA-----G-----G-----ATAG-----AT-----A-----	1
WSU86-1044	-TT-TGTT--C-----GC-----G-----CA-----		-----CG-----GA-----C-----T-----A-AG-----G-A-----	1
	111	160	220	
<i>Chlamydia muridarum</i>	-----G-----A-----G-----A-----G-----A-----T--GC-----A-----			
<i>Chlamydia trachomatis</i>	-----A-----A-----G-----A-A-----GC-----T--GC-----C-----			
<i>Chlamydia suis</i>	-----A-----a-----G-----A-t-----G-----a-----C-----t-----C-----			
<i>Chlamydomytila felis</i>	-----C-----C-----T-----A-----A-----A-----			
<i>Chlamydomytila abortus</i>	-----C-----C-----A-T-----A-----T--G-C-----G-----G-----T-----			
<i>Chlamydomytila psittaci</i>	-----C-----C-----T-----A-----c-----A-C-----T-----g-G-----G-----			
<i>Chlamydomytila caviae</i>	-----A-----T-----T-----A-----A-----A-----T-----			
<i>Chlamydomytila pecorum</i>	-----T-----AA-----A-----T-----A-----T-----A-C-----C-----G-----G-----A-----A-c-----T-g-----			
<i>Chlamydomytila pneumoniae</i>	-----A-----C-----A-----C-----A-----t-----A-----t-----GAAaCa TAGgcaTata AaGcGAcACC tgcTGAACtG AAACATCTTA GTAAGcaGAG			
<b>Consensus</b>	<b>GAGGT-TCCG AATGGG-AA CCCGgTAGA-TAAAtcgtCt AccATT-tAt gcTGAaCa TAGgcaTata AaGcGAcACC tgcTGAACtG AAACATCTTA GTAAGcaGAG</b>			
<i>Parachlamydia</i>	-----A-A-T-----C-----AATG-GA C-----TC-C-----TT--CT-TA-----T-----TATAG-----T-----			
<i>Simkania</i>	A--A-T-----G-----A--G--G T--G--C--C-----T--CCTA-----C-----GT-GG-----A-T-----T--G-----A-----G-----			
WSU86-1044	-G-AT-----C-----AA-G-GA T--CACC-C-----TT--CT-TA-----C-----A-----GTATAG-----G-----G-----C-----G-----A-----G-T-----			
	221	270	330	
<i>Chlamydia muridarum</i>	-----G-----T-----	-----T-----GA-C--A--G . .-TC-----A-T-----GGG-T-AA-- --AAG-T-C		
<i>Chlamydia trachomatis</i>	-----G-----T-----	-----T-----GAGC-GA-- GgCTC-----A-T-----aGG-T-AA-- --AGg-C-C		
<i>Chlamydia suis</i>	-----G-----c-----T-----	-----T-----GAGCtGAAG- AGC-A-----T-----GGAaAA-- --AGG-C-C		
<i>Chlamydomytila felis</i>	-----TT-----	-----A-----CG-----		
<i>Chlamydomytila abortus</i>	-----T-----GA-----A-----C-----			
<i>Chlamydomytila psittaci</i>	-----T-----a-----g-----GA-----A-----aC-----			
<i>Chlamydomytila caviae</i>	-----T-----A-----GA-----A-----CGCC--GG . .-A-----A-----			
<i>Chlamydomytila pecorum</i>	-----T-----T-----C-----T-----T-----T-----A-----G-----A-----			
<i>Chlamydomytila pneumoniae</i>	-----T-----T-----A-----			
<b>Consensus</b>	<b>GAAAAGAAAT CAa. AGAGAT TCcCTgaGTA GCGCGAGCG AAAGGGGAA- AGcCaAAACC a-atTTTTaa --tgtGGGT TGTAGGgTcG ataacaTgG ATCTtaAgTt</b>			
<i>Parachlamydia</i>	-----tt-----ta-----c-----tg-----			
<i>Simkania</i>	-----tt-----gt-----gt-----g-----ga-aa--t . t-tc-----acta g-c-t-aa-c t-aagt-g-ac			
WSU86-1044	-----tt-----gt-----T-----G-----A-GACG-G TT-T-----AC-A-----G-C--A-A-----T-GGAG-T-G			
	331	380	440	
<i>Chlamydia muridarum</i>	C-----C--A-----G-G-T-----A-----GG- G-TCA--C GACC--A-C-----A-----c-----			
<i>Chlamydia trachomatis</i>	C-----C--A-----A-G-T-----GG- G-GAA--C GACC--A-C-----A-----c-----			
<i>Chlamydia suis</i>	C-----C--A-----G-G-T-----GG- G-GAA--C GACC--a-----a-----			
<i>Chlamydomytila felis</i>	-----C-----A-----C-----A-----G-----T-----			
<i>Chlamydomytila abortus</i>	-----C-----A-----T-----G-----			
<i>Chlamydomytila psittaci</i>	-----tA-----G-----T-----			
<i>Chlamydomytila caviae</i>	-----T-----A-----G-----T-----G-----			
<i>Chlamydomytila pecorum</i>	-----T-----a-----A-----A-----G-----A-----T-----T-----A-----a-----			
<i>Chlamydomytila pneumoniae</i>	-----T-----A-----a-----g-----A-----			
<b>Consensus</b>	<b>tTAGTtGAAActTCTGGAA AGttGAcGA tACAGGGTGA TaTCCCCTG aACGAAAaa cAa--AGACT cta-TCgAta CCTGAGTAG gCTAGACACG TGAAacCTAG</b>			
<i>Parachlamydia</i>	C-----C-----AG-----G-----C-----C-----G-----G-----ATGACCA-AC AAGGCA-GC-----CG-----CG-----CG-----			
<i>Simkania</i>	-----CAT-----A-----G-----G-----G-----A--C-----G TG-A--G-G-A-GCTTA-G GAGA-TAGC-----CG-----CG-----CG-----			
WSU86-1044	-----C-----C-----G-----G-T-----C-----G-----GC- AG-TCC--A GGGT.-GC-----CG-----CG-----CG-----			
	441	490	550	
<i>Chlamydia muridarum</i>	-----G-----	-----G-----	-----C-----	
<i>Chlamydia trachomatis</i>	-----C-----	-----G-----	-----C-----	
<i>Chlamydia suis</i>	-----G-----	-----G-----	-----C-----	
<i>Chlamydomytila felis</i>	-----T-----	-----	-----	
<i>Chlamydomytila abortus</i>	-----T-----	-----	-----	
<i>Chlamydomytila psittaci</i>	-----T-----	-----	-----a-----	
<i>Chlamydomytila caviae</i>	-----T-----A-----A-----		-----a-----A-----	
<i>Chlamydomytila pecorum</i>	-----T-----A-----A-----		-----a-----A-----	
<i>Chlamydomytila pneumoniae</i>	-----c-----		-----	
<b>Consensus</b>	<b>TcTGAATCTG GGGAGACCAC TCTCaAAGgC TAAATACTAg TCAATGACCT ATAGTGAACC AGTaCTGTga aGGAAAGGtG AAAAGAACCC TTGTTAAGGG AgtGAAATAG</b>			
<i>Parachlamydia</i>	-----A-----TG-----G-----G-----C-----G-----C-----G-----CGA-G-G-----			
<i>Simkania</i>	-----C-----G-----G-----C-----A-----A-----T-----C-----G-----G-----			
WSU86-1044	-----C-----TG-----G-----C-----G-----C-----G-----C-----G-----C-----G-----			
	551	600	650	
<i>Chlamydia muridarum</i>	-----C-----G-----G-----G-----T--CTT--AAC G-G--CGA-- --		-----	0%
<i>Chlamydia trachomatis</i>	-----C-----G-----A--G G-- --G-AA --G--C-A-- --		-----	< 0.7%
<i>Chlamydia suis</i>	-----a-----C-----G-----GtAa --g--T-A-- --		-----	< 3%
<i>Chlamydomytila felis</i>	-----A-----T AG-TTT--C AAA-CA-- --		-----	< 0.2%
<i>Chlamydomytila abortus</i>	-----A-T, T--C GAA--C-- --		-----	< 0.2%
<i>Chlamydomytila psittaci</i>	-----A-T, T--A GAA--C-- --		-----	< 1%
<i>Chlamydomytila caviae</i>	-----G-----G-T, T--C--C GAA--CC-- --		-----	-
<i>Chlamydomytila pecorum</i>	-----A-T, T--C --A--G-- --		-----	< 0.5%
<i>Chlamydomytila pneumoniae</i>	-----G-----A-T, T--C --A--A-- --		-----	< 2%
<b>Consensus</b>	<b>AACTGAAAC CAgTAGCTTA tAAGCGGTG aaGACcTata -cc--ott-g -gagt-atGG TTGACGGCT GCCTTTTGCA TGATGAGCCA GGGAGTTAAG</b>			
<i>Parachlamydia</i>	-----C-----C-----A-----G-----G-----T-----T-----TT AAG-AA-----C-----C-----CT			
<i>Simkania</i>	-----T-----C-----A-----G-----T-----T-----TT A-G-AA-----C-----T-----			
WSU86-1044	-----C-----C-----G-----G-----AG-- . AC-AA GTSC-A-----C-----T-----			

Fig. 4. For legend see facing page.



**Fig. 4.** 23S domain I rRNA signature sequences for eleven species in the order *Chlamydiales*. This segment could be PCR amplified using an annealing temperature of 61 °C and amplification/sequencing primers U23F (5' GATGCCTTGGCATTGATAGGCGATGAAGGA 3') (bases 31–60) and 23SIGR (5' TGGCTCATCATGCAAAAGGCA 3') (extending 13 bases past base 627). (a) *Chlamydiaceae* species lines: all strains in the species are identical (upper case), are identical and match the consensus line (-), or most but not all strains use the indicated nucleotide (lower case); (.) = alignment gap. Consensus line: all known *Chlamydiaceae* are identical (upper case), at least one strain of the *Chlamydiaceae* is not identical to the consensus nucleotide (lower case), or fewer than six species have the same base at this position (-). The number of strains used to compile the consensus for each species is appended to the end of the first lines of sequence. Base 1 is the first nucleotide of the 23S rRNA gene, based on primer extension analysis (Everett & Andersen, 1997). The percentage variation within each species signature is appended to the ends of the last lines of sequence. Dots were inserted into the sequences to improve alignment. (b) Map of the domain I signature sequence in the 23S rRNA gene. Primers 16SF2, U23F and 23SIGR recognize all members of the *Chlamydiales*. Primers IGSIGF and 23R are specific for the *Chlamydiaceae*.



**Fig. 5.** PFGE of chlamydial chromosomes. Linearized chromosomes migrated at 1.0–1.2 Mbp. *E. coli* JM109 chromosome was included as a control.

**Table 4.** Presence or absence of extrachromosomal plasmid in selected strains of *Chlamydia* and *Chlamydophila* spp.

Compiled from Farencena *et al.* (1997); Girjes *et al.* (1988); Grayston *et al.* (1989); Joseph *et al.* (1986); Lusher *et al.* (1989); Peterson *et al.* (1990); Thomas *et al.* (1997); and Timms *et al.* (1988). Strains in bold were identified or confirmed in this study.

Species	Present	Absent
<i>Chlamydia trachomatis</i>	<b>A/Har-13<sup>T</sup>, D/UW-3/CX, B/TW-5/OT, F/IC/CAL3, L1/440/LN, L2/434/BU</b>	L2 (pCT <sup>-</sup> ); Ba (pCT <sup>-</sup> ); GTS-3, GTS-10, GTS-17, GTS-18, GTS-21, GTS-23, GTS-31, GTS-36, GTS-39*
<i>Chlamydia suis</i>	<b>S45<sup>T</sup>, R22, R24, R27, H7</b>	—
<i>Chlamydia muridarum</i>	<b>MoPn<sup>T</sup></b>	—
<i>Chlamydophila caviae</i>	<b>GPIC<sup>T</sup></b>	—
<i>Chlamydophila felis</i>	Cello, Pring	<b>FP Baker<sup>T</sup>, FP Vaccine</b>
<i>Chlamydophila psittaci</i>	<b>6BC<sup>T</sup>, A24, M56</b> , MN Zhang, N352, RA1/56 gull, 360 duck	MN VR-122
<i>Chlamydophila abortus</i> †	—	<b>B577<sup>T</sup>, EBA, OSP, A22, S26,</b>
<i>Chlamydophila pneumoniae</i>	N16	<b>TW-183<sup>T</sup>, Koala I, IOL-207</b>
<i>Chlamydophila pecorum</i>	<b>E58<sup>T</sup></b> , Koala II	—

\* Forty University of Massachusetts Medical Center *Chlamydia trachomatis* isolates from sexually transmitted disease (STD) or pelvic inflammatory disease (ID) patients were grown and characterized; nine were plasmid minus (An & Olive, 1994; An *et al.*, 1992).

† A. Rodolakis, personal communication.

**Table 5.** Taxa in the order *Chlamydiales*

Source information is provided where possible. Biovar reference strains may be obtained from ATCC or from the original sources.

Taxon	Type/reference strain	
	Name	ATCC no.
<b>Family I: <i>Chlamydiaceae</i></b>		
<i>Chlamydia muridarum</i> sp. nov.	MoPn <sup>T</sup>	VR 123 <sup>T</sup>
<i>Chlamydia suis</i> sp. nov.	S45 <sup>T</sup>	VR 1474 <sup>T</sup>
<i>Chlamydia trachomatis</i>	A/Har-13 <sup>T</sup>	VR 571B <sup>T</sup>
Biovar trachoma	C/PK-2	
Biovar LGV	L2/434/BU	VR 902B
<i>Chlamydophila abortus</i> sp. nov.	B577 <sup>T</sup>	VR 656 <sup>T</sup>
<i>Chlamydophila caviae</i> sp. nov.	GPIC <sup>T</sup>	VR 813 <sup>T</sup>
<i>Chlamydophila felis</i> sp. nov.	FP Baker <sup>T</sup>	VR 120 <sup>T</sup>
<i>Chlamydophila pecorum</i> comb. nov.	E58 <sup>T</sup>	VR 628 <sup>T</sup>
<i>Chlamydophila pneumoniae</i> comb. nov.	TW-183 <sup>T*</sup>	VR 2282 <sup>T</sup>
Biovar TWAR	TW-183	VR 2282
Biovar Koala	LPCon	
Biovar Equine	N16	
<i>Chlamydophila psittaci</i> comb. nov.	6BC <sup>T</sup>	VR 125 <sup>T</sup>
<b>Family II: <i>Simkaniaceae</i> fam. nov.</b>		
<i>Simkania negevensis</i> sp. nov.	Z <sup>T</sup>	VR 1471 <sup>T</sup>
<b>Family III: <i>Parachlamydiaceae</i> fam. nov.</b>		
<i>Parachlamydia acanthamoebae</i> sp. nov.	Bn <sub>9</sub> <sup>T</sup>	VR 1476 <sup>T</sup>
<b>Family IV:</b>	WSU 86-1044	VR 1470

\* TW-183<sup>T</sup> is also available through the Washington Research Foundation, 1107 NE 45th St, Suite 205, Seattle, WA 98105, USA (Tel: +1 206 633 3569; Fax: +1 206 633 2981; WWW URL: <http://www.halcyon.com/lori/wrf.htm>).

band was detected by PFGE and no plasmids were detected by Southern blot analysis.

## DISCUSSION

### Identification of coherent groupings in *Chlamydiales*: ribosomal evidence

We propose a reclassification of groups in the order *Chlamydiales* based on 16S rRNA sequence similarity clusters, on concurrence between analyses of full-length 16S and 23S rDNA data for all known groups, and on supporting evidence showing these groups to be phenotypically and ecologically differentiated (Palys *et al.*, 1997). Some of these groups are well-characterized (see descriptions, below), while others have only recently been identified and phenotypic studies are under way. Ecological differentiation of groups is evident by the often host-related variations in virulence, morphology, propagation and chronic maintenance. According to this proposal, members of the order *Chlamydiales* are obligately intracellular bacteria that have the chlamydia-like developmental cycle of replication and that have > 80% rDNA sequence identity with chlamydial 16S rRNA genes and/or 23S rRNA genes (Figs 1 and 2, Tables 2 and 3). This order includes strains belonging to the *Chlamydiaceae*, strains closely related to '*Candidatus Parachlamydia acanthamoebae*', '*Simkania*' strain Z and strain WSU 86-1044. This proposal separates these groups at the family level. Actual rRNA gene differences between these groups were 13–20%, and between other bacterial lineages this range of differences can typically separate classes, orders or families. The *Chlamydiaceae* is a well-characterized family and all strains have > 90% rRNA identity. Among 16S rRNA sequences in the *Chlamydiaceae*, strain B577 has diverged less than all others from 'Ccon', a chlamydial consensus sequence (Table 2). Therefore, to be identified as a member of the *Chlamydiaceae*, the 16S rRNA gene of a new chlamydia-like isolate should have > 90% identity with the 16S rRNA gene of B577 (accession no. D85709).

Analysis of the rooted, 1558 nt 16S rRNA dataset supported two specific conclusions. The first major conclusion was that nine sequence clusters were present in the *Chlamydiaceae*. These clusters were present in all phylogenetic trees and were independent of the algorithms used to construct the trees. These groupings agreed with many phenotypic and ecological differences among chlamydial groups, including antigenicity, associated disease, host range and genomic endonuclease restriction (Everett & Andersen, 1997). They were also in agreement with observations by Herring (1993) that these groupings would provide an 'outline for a new taxonomy of chlamydial species'. Genetic studies of the *Chlamydiaceae* have shown patterns of relatedness that were consistent with these clusters (Carter *et al.*, 1991; Everett & Andersen, 1997; Fitch *et al.*, 1993; Glassick *et al.*, 1996; Kaltenboeck *et*

*al.*, 1993; Löbau *et al.*, 1995; Pettersson *et al.*, 1997; Pudjiatmoko *et al.*, 1997; Storey *et al.*, 1993; Takahashi *et al.*, 1997; Zhang *et al.*, 1993). The 16S rRNA tree failed to show strong genetic divergence between the EBA/B577 abortion cluster and the avian cluster, from which the abortion group is evolving. However, the abortion cluster was well-supported by DNA–DNA reassociation (Table 1), by the intriguing absence of plasmids from all known isolates (Table 4) and by virulence studies (Jorgensen, 1997; Page, 1967; Rodolakis *et al.*, 1989).

The second major conclusion derived from the 16S rRNA analysis was that the *C. trachomatis*-like group diverged before other groups in the *Chlamydiaceae*. There was 99% support for the monophyly of the *C. trachomatis*-like group and 68% support for the early divergence of this group (Fig. 1). This conclusion was possible because of the unambiguous rooting provided by the '*Candidatus Parachlamydia*', '*Simkania*' and WSU 86-1044 genes. Problems in rooting the *Chlamydiaceae* and in determining relationships within the *Chlamydiaceae* have been ongoing since 1986, when ribosomal data were first obtained for chlamydiae (Weisburg *et al.*, 1986). Most recently, Pettersson *et al.* (1997) used eight ribosomal sequences from '*Simkania*', '*Candidatus Parachlamydia*' and more distantly related bacteria to root a set of nine chlamydial sequences. However, because of difficulty in aligning the chlamydial sequences to the distant outgroups, 22% of the 16S rRNA sequence positions contained gaps and on this basis were removed from their analysis. Their resulting phylogeny had little support in our analyses, which used complete gene sequences.

Analysis of the 23S rRNA showed 100% support for the early divergence of *C. trachomatis*-like strains in the *Chlamydiaceae* (Fig. 2). The 23S rRNA phylogeny was in complete agreement with the most probable 16S rRNA phylogeny. Separation of lineages was greatest and bootstrap support was strongest, using the 23S rRNA data.

### Evolution of coherent clusters in *Chlamydiales*

Recent discoveries of chlamydiae in amoebae (Amann *et al.*, 1997; Birtles *et al.*, 1997; Gautom *et al.*, 1996) suggest the possibility that chlamydial parasites radiated from single-celled eukaryotes into multicelled hosts. This radiation would have been accompanied by intense selective pressure in animal hosts. This selective pressure may have influenced the evolution of all of the proposed groupings in *Chlamydiales*, because members of all of the families have been associated with infection of vertebrates (Birtles *et al.*, 1997; Dilbeck *et al.*, 1990; Kahane *et al.*, 1999; Moulder, 1991). Intense selective pressure is consistent with the large differences seen between the rRNAs of the proposed families. Between some *Chlamydiaceae* groups, however, branching order was affected by relatively small numbers of nucleotide substitutions in

the rRNA, although there were large DNA–DNA reassociation differences (Table 1). (In Figs 1 and 2 such branches had the weakest bootstrap support or the branching orders were shown as unresolved.) This suggests that intense selective pressures did not always involve the rRNA. An unusually low rate of rDNA change could very likely be due to the inability of the replicating chlamydiae to survive the extracellular environment or to infect new host cells. Propagation of changes in rRNA sequence and in ribosome function can take place in chlamydiae only if mutants remain in step with the replication cycle of the total population in the inclusion; other mutants may not survive host cell lysis or may not be able to compete successfully for new host cells in chronically infected hosts.

#### Evidence for the early divergence of the *C. trachomatis*-like group

The strong ribosomal evidence for early divergence of the monophyletic *C. trachomatis*-like group in the *Chlamydiaceae* was supported by other data: genome size (Fig. 5), glycogen production only by the *C. trachomatis*-like strains (Moulder, 1991; Rogers *et al.*, 1996), *ompA* sequence analysis (Kaltenboeck *et al.*, 1993), and conservation of epitopes deduced from the *ompA* sequences of *C. trachomatis*-like strains (see descriptions below). Genome size among the *C. trachomatis*-like strains was smaller than among the non-*C. trachomatis*-like strains (Fig. 5). These sizes were consistent with recent determinations of genome size for specific isolates: the 1.2 Mbp size seen in Fig. 5 for non-*C. trachomatis*-like strains was close to the 1230025 bp genome size of *C. pneumoniae* (GENSET, 1997). The 1.1 Mbp size for *C. trachomatis* L2/434/BU and D/UW-3/CX in Fig. 5 corresponded to a mapped size of 1.045 Mbp (Birkelund & Stephens, 1992) and to genome sizes of 1038680 bp (GENSET, 1997) and 1042519 bp (Stephens *et al.*, 1998), respectively. Glycogen production by bacteria has been shown to be correlated with the management of nutrient deprivation (Sakamoto & Bryant, 1998). Thus, it is possible that a gene which was normally essential during nutrient deprivation was lost by genetic deletion in the formation of the *C. trachomatis*-like lineage.

#### Evidence for ecologically coherent clusters

At least two of the proposed families have evolved specific ecological niches: the *Chlamydiaceae* are primarily vertebrate pathogens and strains in the 'Candidatus Parachlamydia' group appear to reside primarily in amoebae. Ecologically coherent clusters can be identified by DNA–DNA reassociation (Palys *et al.*, 1997). DNA–DNA reassociation analysis of the *Chlamydiaceae* showed groupings that were congruent with the nine groups identified using 16S and 23S rRNA data (Table 1). The range of DNA–DNA reassociation similarity among the groups in the *Chlamydiaceae* was 1–100%. Within *C. pecorum* and

*C. pneumoniae* the ranges were 88–100%. In the current *C. psittaci*, however, the range was much broader (20–85%) because it contained four of the groups separated by ribosomal clustering. The two *C. trachomatis*-like groups that have been characterized were 20–65% similar by DNA–DNA reassociation. Both the *C. psittaci* and the *C. trachomatis* ranges were sufficiently broad that they could each encompass new species: the value established for the discrimination of species is 70% and for the discrimination of genera is about 20% (Amann *et al.*, 1995; Schleifer & Stackebrandt, 1983; Wayne *et al.*, 1987). Therefore, DNA–DNA reassociation provided good evidence that the ribosomal clusters were ecologically distinct groups.

DNA–DNA reassociation is a difficult technique to apply to the measurement of relatedness among intracellular bacteria, however. Values are affected not only by genetic distance but also by the presence of diverse host DNAs, multicopy extrachromosomal DNAs (Brenner & Falkow, 1971), strain integrity, and methodology. Some of these influences are evident in the available DNA reassociation data for chlamydiae. For example, *C. psittaci* B577 has been reported to have similarities of 34 and 85% versus *C. psittaci* 6BC<sup>T</sup>; B577 has reported similarities of 27 and 46% with *C. psittaci* Cal-10 MN (Fukushi & Hirai, 1989) (Table 1). Such wide variances in chlamydial data indicate that DNA–DNA reassociation is not the method of choice for conclusively determining relatedness among chlamydial populations.

Other evidence also contributes to the suggestion that the *Chlamydiaceae* is made up of a number of ecologically distinct populations. An ecologically distinct population can typically occur when one group outgrows others in a particular niche. It has been observed that most chlamydial isolations produce single serovars, rather than mixed populations and that most *Chlamydia* groups have specific host or disease associations. Thus, apparently, long-term adaptation between chlamydiae and animal species has contributed to the evolution of ecologically distinct populations. The apparent host ranges are fairly narrow. For example, one group of *C. trachomatis*-like chlamydiae has been isolated only from swine, some *C. trachomatis*-like strains and the TWAR serovar of *C. pneumoniae* are found exclusively in humans. *C. pecorum* has been isolated only from mammals. Some *C. psittaci* strains are primarily isolated from birds; one group primarily infects cats. Another group is associated with colonization of the placenta, abortion and weak neonates. Disease and host associations have been used for years to help restrict the probable identity of chlamydial agents. However, the condition of the host or the presence of other bacteria or viruses can also play a role in the severity of chlamydial disease and may lead to inappropriate conclusions about the virulence of isolates. In addition, apparently host-specific strains can be made to cause disease in experimentally infected animals (Page, 1967) and

*Chlamydia* strains that infect birds have caused sporadic disease in mammals and tortoises. Thus, although host and disease associations are not definitive markers for purposes of classification, they are consistent with the ecological separation of these populations.

#### Variable traits in *Chlamydiaceae*

Some data assembled in this study contributed to descriptions of groups but proved to be inappropriate for primary group separations. Specifically, these data included plasmid<sup>-</sup> and plasmid<sup>+</sup> genotypes, which could be found in most groups (Table 4) and resistance to sulfadiazine, which interferes with folate synthesis. Sulfadiazine resistance appears to characterize many of the lineages in *Chlamydiales*, although this has not been widely published. Some strains that are known to be resistant include *C. pecorum* IPA and E58<sup>T</sup>, *C. pneumoniae* AR-39, *C. psittaci* FP Baker, GPIC, VS1, CP3, MN, B577 (A. A. Andersen, unpublished; B. Kaltenböck, unpublished), *C. trachomatis*-like swine strains (Andersen & Rogers, 1998) and 'Simkania'. *C. trachomatis*-like isolates from humans and Muridae are sensitive to sulfadiazine, however (Fox *et al.*, 1993; Moulder *et al.*, 1984). Furthermore, both resistance and sensitivity in apparently isogenic *C. psittaci* 6BC strains has been reported (Lin & Moulder, 1966). Fan *et al.* (1992) determined that sulfadiazine-resistant and sulfadiazine-sensitive chlamydiae synthesize folates but vary in their ability to transport host folates. Fan *et al.* (1992) concluded that chlamydial synthesis and transport of folate is in a transitional stage of evolution and probably varies from one strain of *Chlamydia* to the next. For these reasons, it is inappropriate to use sulfadiazine resistance as a phyletic marker for chlamydiae.

The observations described in this report are consistent with the presence of two genera and nine species in the *Chlamydiaceae*, as distinguished by genetic, phenotypic and ecologic differences. We found that ribosomal sequence data could be used to easily assign isolates to these groups and to also distinguish families within the *Chlamydiales*.

#### Emended description of the order *Chlamydiales*

We emend the description of the order *Chlamydiales* to include obligately intracellular bacteria that have a chlamydia-like developmental cycle of replication, Gram-negative or Gram-positive infectious EBs (elementary bodies), > 80% 16S rRNA gene sequence identity, and/or > 80% 23S rRNA gene sequence identity with *Chlamydiales* spp. Currently, the order *Chlamydiales* includes the families *Chlamydiaceae* and *Simkaniaceae*, which have Gram-negative EBs, and *Parachlamydiaceae*, which has variable Gram staining of EBs. The EB form has an inner and an outer membrane and a variable periplasmic space. EBs are round, highly electron-dense, 0.2–0.6 µm in diameter, and metabolically inactive. The chlamydial cycle of replication is initiated when EBs are endocytosed by

eukaryotic cells. During this stage, EBs reside within cytoplasmic inclusions where they differentiate by enlarging to 0.6–1.5 µm, becoming less electron-dense RBs (reticulate bodies), and undergoing binary fission. Inclusions are unique vacuoles that do not undergo acidification or lysosomal fusion. Inclusions that have been studied in detail do not correspond to canonical endocytic vesicles, being essentially dissociated from the endocytic pathway and having some similarities with recycling endosomes (Hackstadt *et al.*, 1997; van Ooij *et al.*, 1997; Taraska *et al.*, 1996). Over a period of days, RBs condense into EBs and chlamydiae are released by host cell rupture or by fusion of the inclusion membrane with the host cell plasma membrane. No alternate host vector is required. Many species of *Chlamydiales* coexist in an asymptomatic state within specific hosts, and it is widely believed that these hosts provide a natural reservoir for these species. The type genus for *Chlamydiales* is *Chlamydia*.

#### Emended description of the family *Chlamydiaceae*

The family *Chlamydiaceae* was previously described by Rake (1957). In accordance with rRNA analyses and corroborating sequence data, the emended family *Chlamydiaceae* is divided into two genera. Rules 39a and 39b of the International Code of Nomenclature of Bacteria state that when a genus is subdivided, the original generic name must be retained by the genus containing the original type species (Lapage *et al.*, 1992). Therefore, the genus name *Chlamydia* must remain with *Chlamydia trachomatis*, which contains A/Har-13<sup>T</sup>, the original type strain. A new name, *Chlamydothila*, has been coined for the genus containing non-*Chlamydia trachomatis*-like species and strains.

The family *Chlamydiaceae* includes current members of the order *Chlamydiales* that have < 10% overall 16S rRNA gene diversity, < 10% overall 23S rRNA gene diversity, a genome size of 1.0–1.24 Mbp, either one or two ribosomal operons in each genome, no tRNA in the 16S–23S intergenic spacer, little or no muramic acid, and the antigenic LPS trisaccharide αKdo-(2 → 8)-αKdo-(2 → 4)-αKdo. The G + C content for the *Chlamydiaceae* spp. is about 40%: 42.7 ± 1.4 mol% according to Moulder *et al.* (1984) and 40.5 ± 1.5 mol% according to Cox *et al.* (1988), using thermal denaturation methods; 39.5 ± 0.3 mol% according to Fukushi & Hirai (1992) using an HPLC method. Among the nearly 100 16S rRNA genes that have been analysed for the *Chlamydiaceae*, the gene from strain B577 is most similar to a consensus sequence for the family (Table 3). Therefore, to be identified as a member of the *Chlamydiaceae*, the 16S rRNA gene of a new chlamydia-like isolate should have > 90% identity with the 16S rRNA gene of B577 (accession no. D85709). The *Chlamydiaceae* are Gram-negative. Host-cell ATP is known to be required by some strains of *C. psittaci* and *C. trachomatis*, and may possibly be a requirement for all members of this family. The extracellular osmotic stability of the

*Chlamydiaceae* EBs is maintained by a complex of disulfide-cross-linked envelope proteins. These include a major outer-membrane protein (MOMP; translation product of *ompA*) with a molecular mass of 40 kDa, a hydrophilic cysteine-rich 60 kDa protein, and a low-molecular-mass cysteine-rich bacterial lipoprotein. When chlamydiae infect cells, the disulfide cross-links within and among these envelope proteins become chemically reduced, allowing transformation of EBs into RBs. Penicillin binding studies and genetic analyses indicate that at least some of the genes that are required for the synthesis of peptidoglycan are present in the chlamydial genome. However, chlamydiae do not contain muramic acid, indicating that chlamydial cell walls do not contain significant amounts of peptidoglycan. The bright flickering appearance, varying in intensity and duration, that is seen in chlamydiae-filled inclusions using visible light microscopy, suggests some degree of bacterial motility for strains of *C. trachomatis*, *C. trachomatis*-like swine strains, *C. psittaci* strains FP Baker, 6BC<sup>T</sup>, VS1, CP3 and B577, and *C. pecorum* strain IPA (A. A. Andersen, unpublished data). Many, but not all, members of the *Chlamydiaceae* possess an extrachromosomal plasmid of about 7.5 kbp (Table 4). These plasmids exhibit 60–80% interspecies conservation of sequence (Thomas *et al.*, 1997), and shared features suggest that these plasmids have a monophyletic origin. These shared features include the conserved organization of eight open reading frames and an iteron in the origin of replication, which may regulate plasmid copy number (Nordström, 1990; Thomas *et al.*, 1997). The iteron consists of four highly conserved tandem repeats. Plasmid copy number in some strains may also be regulated by antisense RNA (Nordström, 1990; Thomas *et al.*, 1997).

The family *Chlamydiaceae* is distinguished from *Simkaniaceae* by 16S rRNA sequence differences (18.6±0.1%), 23S rRNA sequence differences (18.6±0.3%), and by the absence of detectable *Chlamydiaceae*-specific Kdo trisaccharide antigen in 'Simkania' using a family-specific mAb (Kahane *et al.*, 1999). The *Chlamydiaceae* spp. are distinguished from 'Candidatus Parachlamydia' by 16S rRNA sequence differences (14.9±0.7%), 23S rRNA sequence differences (17.7±0.5%), and by the variable responses of 'Candidatus Parachlamydia' EBs to Gram staining (Amann *et al.*, 1997; R. J. Birtles, personal communication). Members of the *Chlamydiaceae* can be readily identified and differentiated by signature sequence analysis (Figs 3 and 4). The type genus for the family *Chlamydiaceae* is *Chlamydia*.

#### Emended description of the genus *Chlamydia*, including specific human, hamster, mouse and swine isolates

The genus *Chlamydia* previously included all known chlamydiae (Moulder, 1984). As emended, *Chlamydia* is one of two genera in the *Chlamydiaceae* and includes

*Chlamydia trachomatis*, *Chlamydia muridarum* gen. nov., sp. nov. and *Chlamydia suis* gen. nov., sp. nov. The previous species, *Chlamydia pecorum*, *Chlamydia pneumoniae* and *Chlamydia psittaci*, have been transferred to the genus *Chlamydophila*. The complete 16S and 23S rRNA gene sequences of *Chlamydia* species that have thus far been examined are each ≥97% identical. To be included in this genus, 16S or 23S rRNA sequences of new isolates should be ≥95% identical to the type strain, A/Har13<sup>T</sup>. *Chlamydia* species are readily identified and distinguished from other species by signature sequences (Figs 3 and 4) or by any of several other genes. Glycogen is produced by *Chlamydia* species and has been characterized by Garrett (1975). Glycogen is easily detected in the human *Chlamydia trachomatis* serovars and is detectable to varying degrees in *Chlamydia muridarum* and *Chlamydia suis*. The *Chlamydia* genomes characterized thus far are 1.0–1.1 Mbp, somewhat smaller than *Chlamydophila* genomes (Fig. 5). *Chlamydia* genomes examined thus far have two identical ribosomal operons (Engel & Ganem, 1987; Everett & Andersen, 1997; Fukushi & Hirai, 1993; Scieux *et al.*, 1992). The majority of strains in this genus have extrachromosomal plasmids, but not all (Table 4). *Chlamydia* strains have varying inclusion morphology and varying sensitivity to sulfadiazine. The currently known strains in *Chlamydia* have an epitope in MOMP that matches the core epitopes NP1, TLNP1, LNPTIA or LNPTI, recognized by vs4 *Chlamydia trachomatis* mAbs (see descriptions below) (Batteiger *et al.*, 1996). The type species for the genus *Chlamydia* is *Chlamydia trachomatis*.

#### Emended description of *Chlamydia trachomatis*

*Chlamydia trachomatis*, as previously described (Moulder *et al.*, 1984), comprised two human biovars and a murine biovar. As emended, only strains from the human biovars of *Chlamydia trachomatis* are being retained in *Chlamydia trachomatis*. Therefore, according to Rule 40b of the International Code of Nomenclature of Bacteria, the original *Chlamydia trachomatis* epithet is retained by the group of human strains which contain A/Har13<sup>T</sup>, the original type strain (Lapage *et al.*, 1992). As emended, *Chlamydia trachomatis* strains have a high degree of sequence conservation. The *Chlamydia trachomatis* 16S rRNA genes that have been sequenced to date differ by <0.65%. *Chlamydia* species are readily identified and distinguished from other species by ribosomal signature sequences (Figs 3 and 4) or by inspection of the 16S–23S intergenic spacer (Everett & Andersen, 1997). Any of several other gene sequences may also be used to identify *Chlamydia trachomatis*. In particular, the gene for the MOMP, *ompA* (*omp1*), is widely used to distinguish *Chlamydia trachomatis* strains. Many, but not all, *Chlamydia trachomatis* strains have the extrachromosomal plasmid, pCT (Table 4). Glycogen accumulation by *Chlamydia trachomatis* is readily detected with iodine staining of inclusions. *Chlamydia*



*trachomatis* strains are generally sensitive to sulfadiazine and tetracyclines. Most strains of *Chlamydia trachomatis* are recognized by mAbs to vs4 core epitopes NPTI, TLNPTI, LNPTIA and LNPTI in MOMP (Batteiger *et al.*, 1996; Peterson *et al.*, 1991). *Chlamydia trachomatis* causes trachoma, sexually transmitted disease, some forms of arthritis, and neonatal inclusion conjunctivitis and pneumonia. The type strain for *Chlamydia trachomatis* is A/Har-13<sup>T</sup> (= ATCC VR 571B<sup>T</sup>).

The 18 serovars of *Chlamydia trachomatis* are clustered into two biovars: trachoma and lymphogranuloma venereum (LGV) (Batteiger, 1996; Moulder *et al.*, 1984). These serovars are recognized by specific mAbs and can be distinguished by *ompA* sequence differences. Worldwide, each serovar has only a minor amount of diversity, according to *ompA* sequence analysis. The trachoma biovar currently has 14 serovars designated by the letters A through K, plus Ba, Da and Ia. Infection is limited primarily to squamocolumnar cells of mucous membranes. Serovars A–C are primarily associated with endemic trachoma and serovars D–K with sexually transmitted infection. The LGV biovar consists of four serovars, L1, L2, L2a and L3. LGV serovars are sexually transmitted and can invade lymphatic tissue. There is a clear separation of these biovars based on pathogenicity and on growth in cell culture and in laboratory animals. However, there is not a clear molecular pattern of separation of the LGV biovar from the trachoma biovar. Antigenic analyses of MOMP and sequence analyses of the *ompA* gene that expresses MOMP suggest that L1 and L2 are closely related to the B- and E- trachoma serovars and that L3 is related to the A-, C- and H-trachoma serovars (Fitch *et al.*, 1993). Analyses of the extrachromosomal plasmid and of the gene for the 60 kDa cysteine-rich protein, however, indicate that the LGV serovars are similar and that they differ from serovar B. The 16S rRNA sequences of serovars A, B and D are closer to each other than they are to the serovar L2 sequence. The *kdtA* sequences in the trachoma biovar, including serovars A, B, Ba, D and E, are also phylogenetically closer than they are to L2 *kdtA*. The *groEL* sequence of L2 is phylogenetically almost as different from the serovar A *groEL* as it is from *Chlamydia muridarum* MoPn *groEL*. It would be inappropriate, therefore, to attempt to further separate the LGV biovar and the trachoma biovar using molecular criteria at the present time. The reference strain for the LGV biovar is L2/434/BU (ATCC VR 902B). The reference strain for the trachoma biovar is C/PK-2.

**Description of *Chlamydia muridarum* sp. nov., including hamster and mouse isolates previously belonging to *Chlamydia trachomatis***

*Chlamydia muridarum* [mu.ri.da'rum. M.L. pl. gen. fem. n. *muridarum* of the Muridae (i.e. the mouse/hamster family)].

Two strains of *Chlamydia muridarum*, MoPn and SFPD (Fox *et al.*, 1993; Nigg, 1942; Stills *et al.*, 1991), have been isolated from mice and hamsters. The recently reconstructed family Muridae includes both mice and hamsters (Alderton, 1996). The *Chlamydia muridarum* 16S rRNA genes that have been sequenced to date differ by < 0.13% (using updated SFPD sequence data), and their 16S–23S intergenic spacers and 23S domain I segments are identical. Analyses of other gene sequences can also identify this species. Glycogen production by both strains has been demonstrated, although it is difficult to detect in MoPn (Moulder *et al.*, 1984). MoPn has an extra-chromosomal plasmid, pMoPn. *Chlamydia muridarum* MoPn binds mAbs recognizing *Chlamydia trachomatis* MOMP vs4 core epitope (T)LNPT(IA) (Peterson *et al.*, 1991). DNA sequence analysis indicates that these mAbs should recognize SFPD and that *Chlamydia trachomatis* B-serogroup mAbs specific for the vs4 epitope IAGAG should recognize SFPD (Batteiger *et al.*, 1996). MoPn was isolated in 1942 from the lungs of asymptomatic albino Swiss mice and was subsequently shown to be capable of producing disease in mice. SFPD was obtained from a hamster, concurrent with a causative agent of proliferative ileitis. MoPn has been shown to be sensitive to sulfadiazine. The type strain for *Chlamydia muridarum* is MoPn<sup>T</sup> (= ATCC VR 123<sup>T</sup>).

**Description of *Chlamydia suis* sp. nov.**

*Chlamydia suis* (su'is. L. fem. n. *suis* pig; L. fem. gen. n. *suis* of the pig, because the only currently known natural host is *Sus scrofa*).

*Chlamydia suis* has recently been characterized and has only been isolated from swine. A high incidence of *Chlamydia suis* is being found in enteric porcine specimens, indicating that it may be endemic (Schiller *et al.*, 1997; Szeredi *et al.*, 1996; Zahn *et al.*, 1995). The *Chlamydia suis* 16S rRNA genes that have been sequenced to date differ by < 1.1%. Glycogen has been detected in *Chlamydia suis* inclusions in infected swine tissues and in cell culture. Some strains have enhanced resistance to sulfadiazine and tetracycline (Andersen & Rogers, 1998). Several strains of *Chlamydia suis* are known to have an extra-chromosomal plasmid, pCS (Table 4). DNA sequence analysis of *ompA* indicates that *Chlamydia suis* strains are somewhat more diverse than are other chlamydial species. The deduced *ompA* gene products of various *Chlamydia suis* strains contain vs4 epitopes TLNPTI-AG(A/K/T)G(D/K/N/T), TWNPTIAGAGS or TLNPTISGKGQ. These epitopes are identical or nearly identical to the *Chlamydia* MOMP core epitopes NPTI, TLNPTI, LNPTIA or LNPTI, which are recognized by *Chlamydia trachomatis* vs4 mAbs; they are also identical or nearly identical to TIAGAGD and IAGAG epitopes, which are recognized by *Chlamydia trachomatis* B-serogroup mAbs (Batteiger *et al.*, 1996). *Chlamydia suis* is associated with con-

conjunctivitis, enteritis and pneumonia (Rogers & Andersen, 1996; Rogers *et al.*, 1996). The type strain for *Chlamydia suis* is S45<sup>T</sup> (= ATCC VR 1474<sup>T</sup>).

#### Description of the genus *Chlamydophila* gen. nov.

*Chlamydophila* (Chla.my.do'phil.a. Gr. fem. n. *chlamys*, *chlamydis* a cloak; L. fem. n. *phila* dear, beloved; M.L. fem. n. *Chlamydophila* dear to the cloak).

*Chlamydophila* is one of two genera in the *Chlamydiaceae* and is composed of the current species *Chlamydia pecorum*, *Chlamydia pneumoniae* and *Chlamydia psittaci*. These taxa, therefore, are renamed *Chlamydophila pecorum*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci*. The complete 16S rRNA and 23S rRNA genes of *Chlamydophila* species are  $\geq$  95% identical. *Chlamydophila* species are readily identified and distinguished from other species by ribosomal signature sequences (Figs 3 and 4), or by inspection of the ribosomal intergenic spacer (Everett & Andersen, 1997). Any of several other genes may also be used to identify *Chlamydophila* species on the basis of DNA homology. The *Chlamydophila* genome is approximately 1.2 Mbp. *Chlamydophila* species do not produce detectable quantities of glycogen. The *Chlamydophila* genome is slightly larger than the *Chlamydia* genome and contains a single ribosomal operon (Fig. 5) (Fukushi & Hirai, 1993; Scieux *et al.*, 1992). *Chlamydophila* strains have varying morphology and varying resistance to sulfadiazine. Extrachromosomal plasmid is present in many *Chlamydophila* species (Table 4). The type species for the genus *Chlamydophila* is *Chlamydophila psittaci*.

#### Emended description of *Chlamydophila pecorum* (Fukushi and Hirai 1992) comb. nov.

The proposal to establish two genera in the family *Chlamydiaceae* necessitates transfer of the species *Chlamydia pecorum*, which has been described previously (Fukushi & Hirai, 1992), to the species *Chlamydophila pecorum* comb. nov. The emended *Chlamydophila pecorum* strains have a high degree of ribosomal sequence conservation; *Chlamydophila pecorum* 16S rRNA genes that have been sequenced to date differ by < 0.6%. Analyses of signature sequences (Figs 3 and 4) or of several other gene sequences can help identify this species. Two strains have been shown to have extrachromosomal plasmids (Table 4). *Chlamydophila pecorum* strains are generally non-invasive in a mouse model of virulence (Rodolakis *et al.*, 1989; Rodolakis & Souriau, 1992; A. Rodolakis, personal communication), but are serologically and pathogenically diverse. *Chlamydophila pecorum* strains characterized thus far have been limited to mammals, but not to a specific host family: *Chlamydophila pecorum* has been isolated from ruminants (cattle, sheep and goats) (Denamur *et al.*, 1991; Fukushi & Hirai, 1992), a marsupial (koala) (Girjes *et al.*, 1993;

Glassick *et al.*, 1996; M. Jackson *et al.*, 1997) and swine (Anderson *et al.*, 1996; Everett & Andersen, 1997; Kaltenboeck & Storz, 1992). In koalas, *Chlamydophila pecorum* causes reproductive disease, infertility and urinary tract disease. In other animals, *Chlamydophila pecorum* has been associated with abortion, conjunctivitis, encephalomyelitis, enteritis, pneumonia and polyarthritides (Anderson *et al.*, 1996; Kaltenboeck *et al.*, 1993). The type strain for *Chlamydophila pecorum* is E58<sup>T</sup> (= ATCC VR 628<sup>T</sup>).

#### Description of *Chlamydophila pneumoniae* (Grayston, Kuo, Campbell and Wang 1989) comb. nov.

The proposal to establish two genera in the family *Chlamydiaceae* necessitates transfer of the species *Chlamydia pneumoniae* (Grayston *et al.*, 1989) to the species *Chlamydophila pneumoniae* comb. nov. *Chlamydophila pneumoniae* is considered to be primarily a respiratory pathogen. The emended species, *Chlamydophila pneumoniae*, has been isolated from humans worldwide (Kuo *et al.*, 1995). In addition, a number of isolates obtained from koalas and a horse have genetic and antigenic characteristics very similar to the human isolates, which warrants including all of these in *Chlamydophila pneumoniae* (Glassick *et al.*, 1996; Storey *et al.*, 1993). DNA sequence analyses of *ompA* indicate that the amino acid sequence of MOMP in all *Chlamydophila pneumoniae* strains varies < 6%, while other species are > 30% different from *Chlamydophila pneumoniae*. The coherence of the *Chlamydophila pneumoniae* taxon has also been demonstrated by DNA sequence analysis of rRNA, *kdtA* (KDO-transferase), and the 60 kDa cysteine-rich gene. Partial analysis of any of these genes or analysis of signature sequences (Figs 3 and 4) can be used to identify this species. Only the equine strain, N16, has an extrachromosomal plasmid (pCpnE1). Three biovars, TWAR, Koala and Equine, are proposed within *Chlamydophila pneumoniae*, based on genetic and biological differences. The type strain for *Chlamydophila pneumoniae* is TW-183<sup>T</sup> (= ATCC VR 2282<sup>T</sup>).

***Chlamydophila pneumoniae*, biovar TWAR.** Previously, strain TWAR was synonymous with the designation of *Chlamydia pneumoniae* (Kuo *et al.*, 1995). The description of biovar TWAR is identical to the description of the prototypical TWAR serovar (Grayston *et al.*, 1989), except that biovar TWAR EBs may have either of two distinct ultrastructural morphologies: the prototypical pear-shaped EB (Grayston *et al.*, 1989) or the classic coccoid morphology of the *Chlamydiaceae* spp. (Carter *et al.*, 1991; Miyashita *et al.*, 1993; Popov *et al.*, 1991). The name TWAR was formed by merging the first two letters of isolates TW-183 and AR-39 (Grayston *et al.*, 1989). TWAR strains have been obtained only from humans, form a distinct serovar, and are genetically almost indistinguishable (Kuo *et al.*, 1995; Pettersson *et al.*,

1997). TWAR is primarily a pathogen of the respiratory tract, predominantly causing acute or chronic bronchitis and pneumonia. TWAR has also been associated with acute and chronic respiratory diseases such as otitis media, obstructive pulmonary disease and pulmonary exacerbation of cystic fibrosis. It has also been associated with Alzheimer's, atherosclerosis, asthma, erythema nodosum, reactive airway disease, Reiter's syndrome and sarcoidosis (Balin *et al.*, 1998; Braun *et al.*, 1997; Ellis, 1997; L. A. Jackson *et al.*, 1997; Kuo *et al.*, 1995). The reference strain for the TWAR biovar is TW-183<sup>T</sup>.

***Chlamydophila pneumoniae*, biovar Koala.** *Chlamydophila pneumoniae* isolates obtained from the marsupial *Phascolarctos cinereus* have distinctive features that merit separation of the Koala biovar from the TWAR biovar. All isolates to date have only been obtained from *Phascolarctos cinereus*. The MOMP in the Koala biovar isolate designated 'Type I' is 2.2% different from the protein homologue in TWAR, 5.5% different from Equine MOMP, and > 30% different from MOMP in other chlamydial species. The KDO-transferase of Koala biovar Type I and TWAR differ by < 8% (Girjes *et al.*, 1997), but are > 40% different from the *kdtA* product in other chlamydial species. The TWAR mAb, CeLLabs-28E, does not recognize the Type I isolate (Girjes *et al.*, 1994). Ultrastructural analyses of Type I EBs show the classic coccoid morphology of the *Chlamydiaceae* spp. (Girjes *et al.*, 1993). The Koala biovar of *Chlamydophila pneumoniae* is further differentiated from other biovars of *Chlamydophila pneumoniae* by the sequence of variable segment 4 of *ompA* (Wardrop *et al.*, 1999; eight isolates), the partial 60 kDa cysteine-rich gene (Glas-sick *et al.*, 1996; two isolates), and the *groESL* intergenic region (Wardrop *et al.*, 1999). Chlamydiae belonging to the Koala biovar of *Chlamydophila pneumoniae* have been identified in all five Australian free-ranging koala populations that have been tested, including a colony that was suffering from respiratory disease. Isolates are commonly obtained from ocular and urogenital sites, where they do not appear to be highly pathogenic. The reference strain for the Koala biovar is LPCon.

***Chlamydophila pneumoniae*, biovar Equine.** The Equine biovar of *Chlamydophila pneumoniae* currently includes only strain N16, which was isolated from the respiratory tract of a horse (Wills *et al.*, 1990). The MOMP of N16 is 5.5% different from that in other *Chlamydophila pneumoniae* isolates (Storey *et al.*, 1993) and > 30% different from other chlamydial species. The 16S rRNA of *Chlamydophila pneumoniae* strain N16 differs 1.1% from TWAR 16S rRNA and > 4% from the 16S rRNA of other chlamydial species. N16 and TWAR 16S-23S intergenic spacer differ by 0.9%, and they are > 11.3% different from other chlamydial species. The 23S rRNA Domain I of N16 and TWAR differ by 1.3%, and are > 5.6% different from other chlamydial species. Two mAbs have been identified that are specific for the TWAR biovar, but that do not

recognize N16 (Storey *et al.*, 1993). Ultrastructural analyses of N16 EBs show the classic coccoid morphology of the *Chlamydiaceae* spp. (Wills *et al.*, 1990). Inoculation of horses with N16 leads to asymptomatic infections (Mair & Wills, 1992). The reference strain for the Equine biovar is N16.

#### **Description of *Chlamydophila psittaci* (Page 1968) comb. nov.**

*Chlamydia psittaci*, as previously described (Moulder *et al.*, 1984), has been a taxon with considerable heterogeneity, having four groups that are phenotypically, pathogenically and genetically distinct. Only avian-like strains are being retained in *Chlamydophila psittaci*, with specific former *Chlamydia psittaci* abortion, feline and guinea pig strains being transferred into three new species. According to Rule 40b of the International Code of Nomenclature of Bacteria, the original epithet for *Chlamydia psittaci* is retained by *Chlamydophila psittaci* because it contains avian strain 6BC<sup>T</sup>, the original type strain (Lapage *et al.*, 1992). As emended, *Chlamydophila psittaci* 16S rRNA genes differ by < 0.8%. Analyses of other gene sequences can also help identify this species. Isolates are similar in virulence and grow readily in cell culture. All should be considered to be readily transmissible to humans. Several *Chlamydophila psittaci* strains have an extrachromosomal plasmid (Table 4). Many *Chlamydophila psittaci* strains are susceptible to bacteriophage Chp1 (Storey *et al.*, 1992). The type strain for *Chlamydophila psittaci* is 6BC<sup>T</sup> (= ATCC VR 125<sup>T</sup>).

The emended species, *Chlamydophila psittaci*, has eight known serovars that are readily distinguished using serovar-specific mAbs (Andersen, 1991, 1997; Vanrompay *et al.*, 1993). These specific serovars can also be identified by *ompA* sequence differences. Some serovars have a capability for infecting more than one type of host (Andersen *et al.*, 1997; Centers for Disease Control and Prevention, 1997; Francis & Magill, 1938; Page, 1967; Spalatin *et al.*, 1966; Vanrompay *et al.*, 1994). Serovar A is endemic among psittacine birds and causes sporadic zoonotic disease in humans. Serovar A infection of birds is often systemic and can be inapparent, severe, acute or chronic with intermittent shedding. Serovar B is endemic among pigeons, has been isolated from turkeys, and has also been identified as the cause of abortion in a dairy herd. Serovar C isolates GD, CT1 and Par1 were obtained from duck, turkey and partridge, respectively, so a specific avian host family has not been identified. Serovar D strains have been isolated from turkeys, a seagull, a budgerigar and humans. Serovars C and D are occupational hazards for slaughterhouse workers and for people in contact with birds. Serovar E isolates, known as Cal-10, MP or MN (meningopneumonitis), were first isolated during an outbreak of pneumonitis in humans in the late 1920s and early 1930s. Subsequently, MN isolates have been obtained from a

variety of avian hosts worldwide, including ducks, pigeons, ostriches and rheas (Andersen *et al.*, 1998). These isolations often come from ill or dying birds, and a specific reservoir for serovar E has not been identified. A single serovar F isolate, VS225, was obtained from a parakeet. The M56 serovar was isolated during an outbreak in muskrats and hares. The WC serovar was isolated during an outbreak of enteritis in cattle. Other monoclonal serotyping systems have also been developed (Fukushi *et al.*, 1987; Takahashi *et al.*, 1988, 1997), but a comparative study of these methods has not been done.

**Description of *Chlamydomphila abortus* sp. nov., including abortion isolates previously belonging to *Chlamydia psittaci***

*Chlamydomphila abortus* (a.bo'rus. L. masc. gen. n. *abortus* of miscarriage, referring to the principal symptom of infection).

*Chlamydomphila abortus* strains are endemic among ruminants and efficiently colonize the placenta (Rodolakis *et al.*, 1989). They have a distinctive serotype and nearly 100% conservation of ribosomal and *ompA* sequences. The *Chlamydomphila abortus* 16S rRNA genes that have been sequenced to date differ by < 0.07%. Analyses of signature sequences (Figs 3 and 4) or other gene sequences readily identify this species. An extrachromosomal plasmid has not been identified in any strain of *Chlamydomphila abortus*. *Chlamydomphila abortus* has been primarily associated with cases of abortion and weak neonates. Typical isolates include strains B577, EBA, OSP, S26/3 and A22 and have been obtained from cases of abortion in sheep, cattle and goats worldwide (Andersen, 1991; Denamur *et al.*, 1991; Storz *et al.*, 1960). *Chlamydomphila abortus* isolates have also been characterized by serology and sequence analysis from cases of abortion in a horse, a rabbit, guinea pigs and mice (J. Storz, K. D. E. Everett, A. A. Andersen, unpublished data) and by PCR from a pig (Kaltenböck *et al.*, 1997). Sporadic zoonotic abortion due to *Chlamydomphila abortus* has been confirmed by genetic analysis of isolates from women who work with sheep (Herring *et al.*, 1987; Jorgensen, 1997). The type strain for *Chlamydomphila abortus* is B577<sup>T</sup> (= ATCC VR 656<sup>T</sup>). B577<sup>T</sup> has a slightly divergent rDNA sequence compared to other *Chlamydomphila abortus* strains.

**Description of *Chlamydomphila felis* sp. nov., including feline isolates previously belonging to *Chlamydia psittaci***

*Chlamydomphila felis* (fe'lis. L. gen. n. *felis* of the cat, because the domestic cat is the primary host of the strains identified to date).

*Chlamydomphila felis* is endemic among house cats worldwide, primarily causing conjunctivitis and rhinitis (Gaillard *et al.*, 1984; TerWee *et al.*, 1998). *Chlamydomphila felis* strains are characterized by a high

degree of rRNA and *ompA* conservation and have a distinctive serotype (Andersen, 1991; Pudjiatmoko *et al.*, 1997; Sayada *et al.*, 1994). The *Chlamydomphila felis* 16S rRNA genes that have been sequenced to date differ by < 0.6%. Strains FP Pring and FP Cello have an extrachromosomal plasmid, while the FP Baker strain does not. FP Cello produces lethal disease in mice while the FP Baker does not (May *et al.*, 1996). An attenuated FP Baker strain is used as a live vaccine for cats. Zoonotic infection of humans with *Chlamydomphila felis* has been reported (Schachter *et al.*, 1969). The type strain for *Chlamydomphila felis* is FP Baker<sup>T</sup> (= ATCC VR 120<sup>T</sup>).

**Description of *Chlamydomphila caviae* sp. nov., including guinea pig inclusion conjunctivitis isolates previously belonging to *Chlamydia psittaci***

*Chlamydomphila caviae* [ca'vi.ae. L. gen. fem. n. *caviae* of the guinea pig (*Cavia cobaya*) because the type strain was isolated from *Cavia cobaya*].

*Chlamydomphila caviae* was first recovered from the conjunctiva of guinea pigs and the description of this species is identical to that of the isolate GPIC (Gordon *et al.*, 1966; Murray, 1964; Prain & Pearce, 1989). There are five known *Chlamydomphila caviae* isolates, and the *ompA* (*omp1*) sequences of these isolates are identical (Zhao *et al.*, 1993). Analyses of signature sequences (Figs 3 and 4) or other gene sequences, including 16S rRNA, can be used to distinguish this species. GPIC contains an extrachromosomal plasmid, pCpGP1 (Lusher *et al.*, 1989). The natural site of *Chlamydomphila caviae* infection is the conjunctiva, however, it is possible to infect the genital tract of guinea pigs with *Chlamydomphila caviae* and elicit a disease that is very similar to human genital infection. *Chlamydomphila caviae* is markedly specific for guinea pigs, as attempts to infect mice, hamsters, rabbits and gerbils have been unsuccessful, except for one experimentally infected gerbil. *Chlamydomphila caviae* infects primarily the mucosal epithelium and is not invasive. The type strain for *Chlamydomphila caviae* is GPIC<sup>T</sup> (= ATCC VR 813<sup>T</sup>).

**Description of *Parachlamydiaceae* fam. nov.**

*Parachlamydiaceae* (Par.a.chla.my.di.a'ce.ae. M.L. fem. n. *Parachlamydia* type genus of the family; -aceae ending to denote a family; M.L. fem. pl. n. *Parachlamydiaceae* the *Parachlamydia* family).

The *Parachlamydiaceae* naturally infect amoebae and can be grown in cultured Vero cells. The *Parachlamydiaceae* are not recognized by monoclonal antibodies specific for the antigenic LPS trisaccharide  $\alpha$ Kdo-(2 → 8)- $\alpha$ Kdo-(2 → 4)- $\alpha$ Kdo of the *Chlamydiaceae*. The description of this family derives from the description of '*Candidatus* Parachlamydia acanthamoebae' strain Bn<sub>9</sub><sup>T</sup>, strain Berg<sub>17</sub>, and several unnamed isolates (Amann *et al.*, 1997; Birtles *et al.*, 1997; Michel *et al.*, 1994). The *Parachlamydiaceae*

comprise strains with rRNAs that are > 90% identical to the ribosomal genes of strain Bn<sub>9</sub><sup>T</sup>. The *Parachlamydiaceae* belong to the order *Chlamydiales* and form a sister taxon to the *Chlamydiaceae* because they have a chlamydia-like cycle of replication and their ribosomal genes are 80–90% identical to ribosomal genes in the *Chlamydiaceae*. Phylogenetic analysis of a 16S rRNA from the *Parachlamydiaceae* gene has been published previously (Amann *et al.*, 1997; Pettersson *et al.*, 1997). At present, this family comprises but a single genus, the type genus *Parachlamydia*.

**Description of *Parachlamydia* (Amann, Springer, Schönhuber, Ludwig, Schmid, Müller and Michel 1997) gen. nov. 'Candidatus Parachlamydia' Amann, Springer, Schönhuber, Ludwig, Schmid, Müller and Michel 1997**

*Parachlamydia* (Par.a.chla.my'di.a. Gr. prep. *para* alike, alongside of; M.L. fem. n. *Chlamydia* taxonomic name of a bacterial genus).

Currently the description of *Parachlamydia* corresponds to the description of the family *Parachlamydiaceae*. *Parachlamydia* have variable Gram staining characteristics and are mesophilic. The 16S and/or 23S rRNA genes of known members of *Parachlamydia* are > 95% identical to those of the type species, *Parachlamydia acanthamoebae*. New members of this genus should have > 95% rRNA sequence identity with *Parachlamydia acanthamoebae*.

**Description of *Parachlamydia acanthamoebae* sp. nov. 'Candidatus Parachlamydia acanthamoebae' Amann, Springer, Schönhuber, Ludwig, Schmid, Müller and Michel 1997**

*Parachlamydia acanthamoebae* [acan.tha.moe'bae. M.L. gen. sing. n. of *Acanthamoeba* taxonomic name of a genus of *Acanthamoebidae*; of (living in) members of the genus *Acanthamoeba*].

*Parachlamydia acanthamoebae* sp. nov. currently includes the type strain Bn<sub>9</sub><sup>T</sup> (= ATCC VR 1476<sup>T</sup>), isolate Berg<sub>17</sub> and several unnamed isolates (Amann *et al.*, 1997; Birtles *et al.*, 1997; Michel *et al.*, 1994). The 16S rRNA and rDNA of *Parachlamydia acanthamoebae* can be specifically detected with oligonucleotide probe Bn<sub>9</sub>658: 5' TCCGTTTTCTCCGCCTAC 3'. Trophozoites of *Acanthamoeba* hosting these strains were isolated from humans in an outbreak of humidifier fever in Vermont USA ('Hall's coccus') and also from asymptomatic women in Germany. In Nova Scotia, four patients whose sera recognized Hall's coccus did not show serological cross-reaction with antigens from the *Chlamydiaceae* (Birtles *et al.*, 1997).

**Description of *Simkaniaceae* fam. nov.**

*Simkaniaceae* (Sim.ka.ni.a'ce.ae. M.L. fem. n. *Simkania* type genus of the family; -*aceae* ending to

denote a family; M.L. fem. pl. n. *Simkaniaceae* the *Simkania* family).

The family *Simkaniaceae* currently comprises but a single genus, the type genus *Simkania*. The description of this family corresponds to that of the 'micro-organism Z' (Kahane *et al.*, 1993, 1995, 1999). The *Simkaniaceae* are not recognized by mAbs specific for the antigenic LPS trisaccharide  $\alpha$ Kdo-(2 → 8)- $\alpha$ Kdo-(2 → 4)- $\alpha$ Kdo of the *Chlamydiaceae*. *Simkaniaceae* belongs to the order *Chlamydiales* and is a sister taxon of the *Chlamydiaceae* because it has a chlamydia-like cycle of replication and the ribosomal genes are 80–90% identical to ribosomal genes in the *Chlamydiaceae*. Phylogenetic analyses of the *Simkaniaceae* 16S rRNA gene have been published previously (Amann *et al.*, 1997; Kahane *et al.*, 1995; Pettersson *et al.*, 1997). New strains belonging to the *Simkaniaceae* should have ribosomal genes that are > 90% identical to the ribosomal genes of strain Z<sup>T</sup>. The type genus for *Simkaniaceae* is *Simkania*.

**Description of *Simkania* gen. nov.**

*Simkania* (Sim.ka'ni.a. M.L. fem. n. *Simkania* arbitrary name formed from the personal name Simona Kahane).

Currently the description of *Simkania* corresponds to the description of the family *Simkaniaceae* and includes a single isolate, strain Z<sup>T</sup>. The natural host of *Simkania* is not known, but serological evidence and PCR indicate that it is widespread among humans. New members of the genus *Simkania* should have 16S or 23S rRNA genes that are > 95% identical to those of *Simkania negevensis*, the type species.

**Description of *Simkania negevensis* sp. nov.**

*Simkania negevensis* (ne.ge.ven'sis. M.L. adj. *negevensis* of or pertaining to the Negev, a desert in southern Israel).

The species *Simkania negevensis* currently includes only the type strain, *Simkania negevensis* strain Z<sup>T</sup> (= ATCC VR 1471<sup>T</sup>). *Simkania negevensis* was discovered as a bacterial contaminant in cell cultures. The description of this species is derived from that of the 'micro-organism Z' (Kahane *et al.*, 1993, 1995, 1999). Strain Z<sup>T</sup> does not contain an extrachromosomal plasmid and has an unusually slow developmental cycle in cultured Vero cells (up to 14 d), compared to other chlamydiae. Unlike other chlamydial rRNA genes that have been characterized, *Simkania negevensis* has a group I intron in 23S rRNA position 1931 (*E. coli* numbering) and this intron is not spliced out of the rRNA. The intron is closely related to group I introns in the 23S rRNA of chloroplasts and mitochondria in algae and amoebae.

### Rapid identification and classification of new chlamydial strains

Identification of strains can be readily and accurately performed to determine whether a specimen belongs to one of the nine groups in the *Chlamydiaceae*, to *Simkaniaceae*, to *Parachlamydiaceae*, or is similar to strain WSU 86-1044. Three simple PCR methods have been described (Everett *et al.*, 1999). Another simple method is to PCR-amplify and directly sequence the 16S and/or 23S signature sequences from chromosomal template and to compare these data with Figs 3 or 4. When GenBank entries have been updated to reflect the new groups, identification of signature sequences will also be possible by using a BLAST search of that database. Because it is essential to have good sequence data to make these determinations, template preparations for PCR may need to be free of contamination with other bacterial templates or, alternatively, sequence data can be obtained from cloned gene segments. If other bacteria are present in the chlamydial isolate, hot-start PCR methods may partially alleviate the frequency of mismatch amplification of contaminating template. If cloning is necessary, a conserved *PbrPI* restriction site has been identified in the 23S sequence to facilitate the selection of 23S signature sequence clones (Fig. 4b). Sequence data not matching a species in Fig. 3 or 4 will represent either a novel isolate or a non-chlamydial organism. Novel isolates require more extensive phenotypic characterization and/or sequence analysis before they can be validly identified.

DNA sequence analysis of the ribosomal intergenic spacer or of non-ribosomal genes can be used to corroborate signature sequence identification of the *Chlamydiaceae*. Members of strains belonging to the family *Chlamydiaceae* are also identified using mAbs that recognize the trisaccharide antigen  $\alpha$ Kdo-(2  $\rightarrow$  8)- $\alpha$ Kdo-(2  $\rightarrow$  4)- $\alpha$ Kdo, a unique component of the LPS and previously referred to as a genus-specific marker (Löbau *et al.*, 1995).

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