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.

Chlamydiaceae 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...
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 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 five 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 Chlamydiae 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; Kaltenbock 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 Chlamydiales 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 Chlamydiales (Everett & Andersen, 1997; Kaltenboeck et al., 1993; Pudjiatmoko et al., 1997; Takahashi et al., 1997).

Species within the Chlamydiales have 16S rRNA gene sequences that are >90% identical (Pettersson et al., 1997; Pudjiatmoko et al., 1997; Takahashi et al., 1997). All strains in the Chlamydiales are recognized by monoclonal antibodies (mAbs) that detect the LPS trisaccharide αKdo(2→8)-αKdo(2→4)-αKdo (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
The purpose of this report is to update the description national sequence databases. Genetic relationships and diverse, well-established groupings for pathogens with distinctive underlying major bacterial lineages. It provides appropriate a consistent method for identifying present taxa and a 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., Simkania 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 Chlamydiaceae 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, Chlamydia pecorum IPA, Chlamydia psittaci NJ1, 6BC, GPIC, FP Baker, Chlamydia trachomatis A/Har-13, 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. Chlamydia1 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. Nono (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 Sequencer 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.
WSU 86-1044 and provided by Fred Rurangirwa (Department of Veterinary K. D. E. Everett, R. M. Bush and A. A. Andersen

AGTTGTGAATAGG 3') and a complementary primer (Everett

the cloned PCR-product templates. PCR conditions for all

that PCR amplifies the 5s gene of

sequence data for phylogenetic analysis were obtained from

directly from PCR products. Full-length 23s rRNA se-

Microbiology and Pathology, Washington State University,

AGTTCGGGATGGGATCGGGTG 3'). Additional

SFPD was resequenced; the 16s rRNA and 16s-23s

Annealing temperatures were specific for each primer set.

The 3' end of each

Elmer) were 30 s at 94 °C, 15 s annealing, 30 s at 72 °C in the

extension analysis (Everett

NJ1 and R22 had previously been identified by primer

into the 5s gene. The 5' end of the 23s rRNA genes in 6BCT,

sequence data for a 200 base segment of 23s rRNA extending

were trimmed to produce sequences of even length: 1558 nt

from strain Con. There were

were difficult to align, however, the results are robust

were narrowed to regions of sequence that were conserved and distinctive for each of

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-

CCTGGAGTAGGGCAT GAT CAT C 3'), which matched the 16s rRNA gene at position 40, and 16SIGR (5' TCAGTCCAGTTGTGGC 3'), which complemented the 16s rRNA gene at position 337 (E. coli and Chlamydia number). The rDNA template was PCR amplified with AmpliTaq DNA polyn

merase 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 Tth 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' GATGCGCTGGCATGAGGGGAGGAAGGA GA 3') and 23SIGR (5' TGTCCTCATCTGGAAAGGCCA 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' CCGGCCGTCACATCATGGGAT AGCGAGGAAGGA 3') and 23SIGR (5' TGTCCTCATCTGGAAAGG GCA 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, 23SIGR, IGSIGF (5' ATAAATAGACGT TTTAAGA 3') and 23R (5' TACTAAGTGTTTCATTGTC 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 Ca2+ and Mg2+ (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−1 (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 chromo-

some prior to electrophoresis. Chromosome from treated

untreated plugs, however, showed no reproducible differences in electrophoretic mobility. Subsequently, there-
Southern blot analysis. An oligonucleotide primer recognizing the origin of replication of all known chlamydioid plasmids was synthesized. This 102 base primer consisted of four direct repeats with a restriction site at each end (Integrated DNA Technologies): GCGGCCGCTTGCAG-CT(T/C)(T/A)GGTGGTAGACTTCGGGG. The primer was 5'-end-labelled with [32P]dATP (ICN; 10 pCi/μl) using T4 polynucleotide kinase (GibcoBRL Life Science) by capillary transfer in 10 mM NaOH at 4°C; neutralized in 1 M Tris•HCl, pH 7.5, and then electrophoresed in 1× TBE on 1% (w/v) agarose gels (10×65 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 μg ml−1). The blot was washed at 60°C in 1×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 ‘Simkmania’ 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 phylectic 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 GPC, was the single representative of its group. The order in which GPC diverged from the feline, abortion and C. psittaci clusters was unresolved. The parsimony and NJ trees showed GPC diverging between the feline cluster and C. psittaci, while the MLE tree made GPC 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
Table 2. Percentage differences among the 16S rRNA genes of Chlamydiales spp. type strains and some of their closest relatives

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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 ≤ 5% among themselves. The C. trachomatis-
Reclassification of order *Chlamydiaceae*

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 (±0.5%) or to the nearest decimal (±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 are 0.37% different and have identical percentage differences with all *Chlamydiaceae* strains as shown.

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**Fig. 2.** Phylogeny of 14 members of the order *Chlamydiaceae*, 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 ≤ 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 *Chlamydiaeae*.

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-
Fig. 3. 16S rRNA signature sequences for twelve species in the order Chlamydiales. The amplification/sequencing primers, 16SIGF (5' CGGCGTGGATGAGGCAT 3') and 16SIGR (5' TCAGTCCCAGTGTTGGC 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.

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’ (Aman 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 ≤ 5% among themselves; the *C. trachomatis*-like 23S rRNA gene sequences varied ≥ 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 ≥ 4 sequences were available for each. Variation within the 16S signature consensus for any single species was ≤ 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 ≥ 5 sequences were available for each. Variation within the domain I signature consensus for any single species was always ≤ 3%, but usually ≤ 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 1–1.1 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 Birkeland & 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− only if a genomic...
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**Consensus**

**Purrence**

**Simkov**

**WSU86-1044**

**Fig. 4.** For legend see facing page.

**International Journal of Systematic Bacteriology**
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' GATGCTTTGGCATTAGGCGATGAAGGA 3') (bases 31–60) and 23SIGR (5' TGGCTCATCATGCAAAGGCA 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.

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*Forty University of Massachusetts Medical Center *Chlamydia trachomatis* isolates from sexually transmitted disease (STD) or pelvic inflammatory disease (PID) 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.

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*TW-183T 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 Chlamydiales, 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 Chlamydiales is a well-characterized family and all strains have > 90% rRNA identity. Among 16S rRNA sequences in the Chlamydiales, 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 Chlamydiales, 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 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 Chlamydiales 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 Chlamydiales. 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 Chlamydiales and in determining relationships within the Chlamydiales 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 Chlamydiales (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 (Aumann 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 Chlamydiales 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 1.23 Mbp 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 (Birkeland & Stephens, 1992) and to genome sizes of 1.038680 bp (GENSET, 1997) and 1.042519 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, multicyclic 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 6BC²; 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- and plasmid+ 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 Chlamydiaceae, although this has not been widely published. Some strains that are known to be resistant include C. pecorum IPA and E58β, 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 Chlamydiaceae.

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 Chlamydiaceae spp. Currently, the order Chlamydiaceae includes the families Chlamydiaceae and Simkaniaeeae, 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 Chlamydiaceae 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 Chlamydiaceae 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 39α 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-13β, the original type strain. A new name, Chlamydophila, 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 Fukushima & 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 hydrophobic 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 chemoically 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, 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/Har13T. 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 1–1 Mb, 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 NPTI, TLNPTI, LNPtA or LNPTIA, 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/Har13T, 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; 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 bovar 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 bovar 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 bovar 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 bovar from the trachoma bovar. 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 bovar, including serovars A, B, Ba, D and E, are also phylogenically close than they are to L2 *kdtA*. The *groEL* sequence of L2 is phylogenetically almost as different as from the serovar A *groEL* as it is from *Chlamydia muridarum* MoPn. It would be inappropriate, therefore, to attempt to further separate the LGV bovar and the trachoma bovar using molecular criteria at the present time. The reference strain for the LGV bovar is L2/434/BU (ATCC VR 902B). The reference strain for the trachoma bovar 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 extrachromosomal plasmid, pMoPn. *Chlamydia muridarum* MoPn binds mAbs recognizing *Chlamydia trachomatis* MOMP vs4 core epitope (T)LNPTIA (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 MoPn₁ (= ATCC VR 571B⁰).

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

*Chlamydia suis* (su’is. L. fem. n. sus 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 extrachromosomal 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 TLPNTIDG(A/K/T)G(D/K/N/T), TWINPIAGGS or TLPNTISGKGQ. These epitopes are identical or nearly identical to the *Chlamydia MOMP* core epitopes NPTI, TLPNTI, LNPTIA or LNPTI, which are recognized by *Chlamydia trachomatis* vs4 mAbs; they are also identical or nearly identical to IAGAG and IAGAG epitopes, which are recognized by *Chlamydia trachomatis* B-serogroup mAbs (Batteiger *et al.*, 1996). *Chlamydia suis* is associated with con-
Description of the genus Chlamydophila gen. nov.

Chlamydophila (Chlamy.do'phi.la. Gr. fem. n. chlamys, chlamydis a cloak; L. fem. n. phila dear, beloved; M.L. fem. n. Chlamy.dophila 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 Chlamyphonella pecorum, Chlamyphonella pneumoniae and Chlamyphonella psittaci. The complete 16S rRNA and 23S rRNA genes of Chlamydophila species are ≥ 95% identical. Chlamyphonella species are readily identified and distinguished from other species by ribosomal intergenic spacer (Everett & Bush, 1996). The type strain for Chlamyphonella psittaci is E58T (= ATCC VR 628T).

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 (pCpEl). 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-183T (= ATCC VR 2282T).

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, with a specific serovar, and are genetically almost indistinguishable (Kuo et al., 1995; Pettersson et al., Glassick et al., 1996; M. Jackson et al., 1997) and swine (Anderson et al., 1996; Everett & Andersen, 1997; Kaltenboeck & Storz, 1992). In koalas, Chlamyphonella pecorum causes reproductive disease, infertility and urinary tract disease. In other animals, Chlamyphonella pecorum has been associated with abortion, conjunctivitis, encephalomyelitis, enteritis, pneumonia and polyarthritis (Anderson et al., 1996; Kaltenboeck et al., 1993). The type strain for Chlamyphonella pecorum is E58T (= ATCC VR 628T).
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-183T.

**Chlamydia pneumoniae, biovar Koala.** *Chlamydia 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, CellLabs-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 *Chlamydiaphila pneumoniae* is further differentiated from other biovars of *Chlamydia pneumoniae* by the sequence of variable segment 4 of *ompA* (Wardrop et al., 1999; eight isolates), the partial 60 kDa cysteine-rich gene (Glassick et al., 1996; two isolates), and the groESL intergenic region (Wardrop et al., 1999). Chlamydiae belonging to the Koala biovar of *Chlamydiaphila 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.

**Chlamydia pneumoniae, biovar Equine.** The Equine biovar of *Chlamydiaphila 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 *Chlamydiaphila pneumoniae* isolates (Storey et al., 1993) and > 30% different from other chlamydial species. The 16S rRNA of *Chlamydiaphila 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 Chlamydia 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 *Chlamydia psittaci*, with specific former *Chlamydia psittaci* strains N16, chlamydial pneumonia, N16, chlamydial pneumonia, psittaci, known as Cal-10, MP or MN (meningopneumonitis), were first isolated during an outbreak of pneumonitis (Page, 1967; Spalatin et al., 1975). The emended species, *Chlamydiaphila 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 Chlamydiophila abortus sp. nov., including abortion isolates previously belonging to Chlamydia psittaci

Chlamydiophila abortus (a.bor'tus. L. masc. gen. n. abortus of miscarriage, referring to the principal symptom of infection).

Chlamydiophila 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 Chlamydiophila 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 Chlamydiophila abortus. Chlamydiophila 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). Chlamydiophila 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 Chlamydiophila 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 Chlamydiophila abortus is B5777 (= ATCC VR 6567). B5777 has a slightly divergent rDNA sequence compared to other Chlamydiophila abortus strains.

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

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

Chlamydiophila felis is endemic among house cats worldwide, primarily causing conjunctivitis and rhinitis (Gaillard et al., 1984; TerWee et al., 1998). Chlamydiophila 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 Chlamydiophila 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 Chlamydiophila felis has been reported (Schachter et al., 1969). The type strain for Chlamydiophila felis is FP Baker7 (= ATCC VR 1207).

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

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

Chlamydiophila 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 Chlamydiophila 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 Chlamydiophila caviae infection is the conjunctiva, however, it is possible to infect the genital tract of guinea pigs with Chlamydiophila caviae and elicit a disease that is very similar to human genital infection. Chlamydiophila 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. Chlamydiophila caviae infects primarily the mucosal epithelium and is not invasive. The type strain for Chlamydiophila caviae is GPIC7 (= ATCC VR 8137).

Description of Parachlamydiaceae fam. nov.

Parachlamydiaceae (Par.a.chla.my.dia.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 zKdo-(2→8)-zKdo-(2→4)-zKdo of the Chlamydiaceae. The description of this family derives from the description of 'Candidatus Parachlamydia acanthamoebae' strain Bn5, strain Berg17, 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\textsuperscript{T}. The Parachlamydiaceae belong to the order Chlamydiales and form a sister taxon to the Chlamydiaceae because they have a chlamydial-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.


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* [a.can.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\textsuperscript{T} (= ATCC VR 1476\textsuperscript{T}), isolate Berg\textsubscript{1T} 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\textsubscript{658}: 5'-TCCGTTTTCTCCGC-CTAC 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 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\textsuperscript{T} (= ATCC VR 1471\textsuperscript{T}). *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\textsuperscript{T} 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 PbrP1 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 zKdo-(2→8)-zKdo-(2→4)-zKdo, a unique component of the LPS 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 PbrP1 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.

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REFERENCES


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