Phylogenetic Relationship of *Chlamydia pneumoniae* to *Chlamydia psittaci* and *Chlamydia trachomatis* as Determined by Analysis of 16S Ribosomal DNA Sequences

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The 16S ribosomal DNA sequence of *Chlamydia pneumoniae* was determined and compared with the corresponding gene sequences of *Chlamydia psittaci* and *Chlamydia trachomatis*. *C. pneumoniae* has been reported to exhibit little chromosomal DNA homology with the other chlamydial species, and its phylogenetic relationships within the genus *Chlamydia* have not been described. A polymerase chain reaction was employed to determine the 16S rRNA gene sequence of *C. pneumoniae*. Ten primers from the *C. psittaci* sequences were used to amplify a *C. pneumoniae* template in overlapping segments of the gene. Sequence data for 1,554 bases indicated that the levels of homology of *C. pneumoniae* with *C. psittaci* and *C. trachomatis* were 96.19 and 94.07%, respectively. These data support the results of previous biochemical and developmental studies indicating that *C. pneumoniae* is more closely related to *C. psittaci* than to *C. trachomatis*.

*Chlamydia pneumoniae* has been established as a cause of respiratory infections in humans and may account for up to 10% of cases of community-acquired pneumonia (1, 5, 7, 9, 13). Although *C. pneumoniae* is similar to *Chlamydia trachomatis* and *Chlamydia psittaci* in its developmental cycle, its morphology, its biochemical characteristics, its restriction endonuclease patterns, and the results of whole chromosomal DNA analysis have established that it is a distinct species (2, 3, 8, 10, 11). However, the phylogenetic relationship of this species is unknown because the 16S rRNA sequence of *C. pneumoniae* has not been reported previously. Examinations of *C. trachomatis* and *C. psittaci* 16S rRNA sequences have indicated a eubacterial origin for *Chlamydia* spp., yet these organisms represent a major, phylogenetically distinct group of bacteria (20, 21). We sequenced the 16S rRNA gene of *C. pneumoniae* in order to define the relationship of this species to *C. psittaci* and *C. trachomatis* and to discover unique sequences in *C. pneumoniae* which might be useful for such a determination.

*C. pneumoniae* TW183 and IOL207 were grown as previously described (6). Standard procedures were used for DNA extraction, polyacrylamide and agarose gel electrophoresis, and ethidium bromide staining (17). Elementary bodies were purified in a Percoll density gradient (6). Oligonucleotide primers were synthesized with a model 380 DNA synthesizer (Applied Biosciences, Foster City, Calif.). Because 16S rRNA sequences are highly conserved, most of the initial sequencing of the gene was performed by using primers from the previously published sequence of *C. psittaci*, using *C. pneumoniae* as the template (20). This approach has been used for unculturable organisms by other investigators who used eubacterial conserved 16S rRNA sequences (4, 12, 15, 16). A polymerase chain reaction was performed as previously described (6). Sequences of polymerase chain reaction products were determined with an Applied Biosystems model 373A automated DNA sequencer. Analysis of the sequences was performed with the GCG sequence software (Genetics Computer Group, Madison, Wis.).

After *C. pneumoniae*-specific gene sequences were determined, new primers specific for the *C. pneumoniae* gene were synthesized and used to create new overlapping gene segments for sequencing. All sequencing reactions were performed in both the sense and antisense orientations. By this means, the gene sequences of *C. pneumoniae* TW183, exclusive of 34 bases at the 5' end and 25 bases at the 3' end, were determined. For *C. pneumoniae* IOL207, DNA was also cloned by constructing genetic libraries in pBR322, pUC19, and M13 bacteriophage vectors. Recombinant plasmids and phage were identified by hybridization and were isolated by standard methods (17). These clones were then sequenced by the dideoxynucleotide chain termination method (18). A comparison of the *C. pneumoniae* sequence with the previously published sequences of *C. psittaci* and *C. trachomatis* indicated that there was substantial similarity. However, differences in these sequences were most evident at five areas (Fig. 1). The most diverse region of the *C. pneumoniae* gene was the region from nucleotide 3016 to nucleotide 1032, where *C. pneumoniae* differed in 8 bases from the 17 bases that are conserved in *C. psittaci* and *C. trachomatis*. A second unique area included nucleotides 1458 and 1459, where a thymidine and an adenine were present in *C. pneumoniae*, but deleted in *C. psittaci* and *C. trachomatis*. Following this area, the next six nucleotides (nucleotides 1460 to 1465) were somewhat conserved in *C. psittaci* and *C. trachomatis*, but consisted of five unique thymidine residues in *C. pneumoniae*. Another short area of diversity was also noted between nucleotides 176 and 182. Two additional areas where *C. pneumoniae* differed from *C. trachomatis* but not from *C. psittaci* were nucleotides 71 to 90 and 465 to 483. A direct comparison of the sequence of the 16S rRNA gene of *C. pneumoniae* (GenBank accession

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number L06108) with the C. psittaci 6BC sequence (GenBank accession number M13769) and the C. trachomatis L2 sequence (GenBank accession number M59178) indicated that the levels of homology were 96.19 and 94.07%, respectively. C. psittaci and C. trachomatis were 95.49% homologous (20). Our analysis of the 16S rRNA genes of the Chlamydia species therefore demonstrated that the 16S rRNA gene is remarkably conserved in the three species, indicating a common ancestral lineage. However, the differences in the 16S rRNA genes confirmed that C. pneumoniae is a unique species, different from either C. psittaci or C. trachomatis. The level of sequence homology between C. pneumoniae and C. psittaci was consistent with the biochemical similarity between the two organisms. Both of these bacteria are incapable of accumulating glycogen in inclusion bodies, whereas C. trachomatis can synthesize glycogen from ADP-glucose (10, 19). Both C. pneumoniae and C. psittaci are resistant to sulfida drugs, whereas C. trachomatis is susceptible (8).

The unique 16S rRNA sequences of C. pneumoniae have proven to be useful for the development of a diagnostic polymerase chain reaction (6). The C. pneumoniae sequence can be amplified by a polymerase chain reaction by using primers CPnA and CPnB, which correspond to the sequences of the diverse regions between nucleotides 1016 and 1032 and nucleotides 1460 and 1465. This primer pair has not amplified DNA from C. psittaci or C. trachomatis (6).

While Chlamydia species are remotely related to other eubacteria, Weisburg et al. have reported the levels of relatedness between Chlamydia species and plantomycetes on the basis of the degrees of sequence similarity (20). These authors defined eight characteristics of the higher-order structure of the 16S rRNA which suggested the putative relationship of Chlamydia species to plantomycetes (20). Each of these structural characteristics and unique base locations is conserved in C. pneumoniae, C. psittaci, and C. trachomatis. Whether the Chlamydia species share a common unknown ancestor or evolved from one parental species cannot be determined yet. Chlamydia-like organisms have been found in invertebrate hosts (clams, scallops, oysters, crabs, spiders) mostly on the basis of morphological evidence, and these bacteria need to be assessed to gain further understanding of the phylogenet, ancestral relationships of this unique group of microorganisms (14).

REFERENCES
