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 1016 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 involved 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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	71	90
<i>C. pneumoniae</i>	ATAATGACCTT	CGGTGTGTAT
<i>C. psittaci</i>		
<i>C. trachomatis</i>	gc...tgt...caa..g.	
	171	200
<i>C. pneumoniae</i>	AATGTAGTGT AATTAGGCAT	CTAATATATA
<i>C. psittaci</i>	g..a. gt.....	a.c...
	a	
<i>C. trachomatis</i>	g.c...t..g.....	cg.gta.cg
	461	490
<i>C. pneumoniae</i>	GAGAGATTGG CTAATATCGA	ATCGATTGA
<i>C. psittaci</i>		
<i>C. trachomatis</i>	aggc..t....c..g	c.g.....
	1011	1035
<i>C. pneumoniae</i>	TTGACAACTG TAGAAATAGA	GCTTT
<i>C. psittaci</i>	cg.g. c.....gtc	t...
<i>C. trachomatis</i>	a....cg.g. c.....gtc	t...
	1451	1470
<i>C. pneumoniae</i>	CTCAACCTAT TTATAGGAGA	
<i>C. psittaci</i>	--gc.a.....	
<i>C. trachomatis</i>	--c gc.ag.....	

FIG. 1. Comparison of the unique 16S rRNA gene sequences of *C. pneumoniae*, *C. psittaci*, and *C. trachomatis*. The unique areas described in the text for *C. pneumoniae* (nucleotides 71 to 90, 176 to 182, 465 to 483, 1016 to 1032, and 1458 to 1465) are compared with the sequences of the other two species.

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 sulfa 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 planctomyces 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 planctomyces (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 phylogenetic, ancestral relationships of this unique group of microorganisms (14).

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