Analysis of the 16S rRNA gene of micro-organism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order *Chlamydiales*: proposal of *Waddliaceae* fam. nov., *Waddlia chondrophila* gen. nov., sp. nov.

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The structural gene encoding the 16S rRNA of the new obligate intracellular organism presently designated WSU 86-1044^T was sequenced and analysed to establish its phylogenetic relationships. The 16S rDNA sequence was most closely related to those of chlamydial species, having 84.7–85.3% sequence similarity, while it had 72.4–73.2% similarity with rickettsia-like organisms. When the sequences of the four species of chlamydiae (*Chlamydophila psittaci*, *Chlamydia trachomatis*, *Chlamydophila pneumoniae* and *Chlamydophila pecorum*) were compared, they had > 93% sequence similarity indicating that WSU 86-1044^T was not close enough to be in the same family as current *Chlamydiaceae* members. However, based on the 84.7–85.3% 16S rDNA sequence similarity of WSU 86-1044^T and other previously described characteristics, WSU 86-1044^T belongs to a novel family within the order *Chlamydiales*; hence, the proposal of *Waddliaceae* fam. nov., *Waddlia chondrophila* gen. nov., sp. nov.

Keywords: chlamydia, phylogeny, 16S rRNA, 16S rDNA sequence, abortion

INTRODUCTION

The organism WSU 86-1044^T, was originally isolated from tissues of a first-trimester aborted bovine foetus at the Washington Animal Disease Diagnostic Laboratory (Dilbeck *et al.*, 1990). A cytopathic effect was observed within 2–3 d after the initial inoculation of bovine turbinate (BT) cell cultures with pooled spleen and liver homogenates. The organism was serially passaged numerous times and consistently induced cytopathic effect. It replicated rapidly to high levels, reaching peak titres exceeding 10⁸ 50% tissue-culture-infective doses (TCID₅₀) per ml within 3 d (Dilbeck *et al.*, 1990). Replication was inhibited by tetracycline, but not by penicillin or gentamicin (Dilbeck *et al.*, 1990). Light microscopy revealed organisms within

cytoplasmic inclusions that ranged in size from 0.2 to 0.4 µm (Dilbeck *et al.*, 1990). Electron microscopy confirmed that the organisms multiplied within a cytoplasmic vacuole with a developmental life cycle resembling that of ehrlichiae and chlamydiae (Dilbeck *et al.*, 1990; Kocan *et al.*, 1990). The organisms occurred in two forms; a reticulated form found within a cytoplasmic vacuole, and a dense infective form that was released from the cells.

Presently, WSU 86-1044^T is characterized as an obligate intracellular organism, which replicates within cytoplasmic vacuoles, exhibiting structural characteristics compatible with those of rickettsiae and chlamydiae (Dilbeck *et al.*, 1990; Kocan *et al.*, 1990). Serological identification of WSU 86-1044^T was not successful as the organism did not react with monoclonal or polyclonal antisera to a variety of *Rickettsia*, *Coxiella*, *Wolbachia*, *Anaplasma* or *Chlamydia* spp. (Dilbeck *et al.*, 1990). However, it did react weakly with antisera to *Cowdria ruminantium*. Thus, WSU 86-1044^T has not been taxonomically classified.

Abbreviations: BT, bovine turbinate; TCID₅₀, 50% tissue-culture-infective dose.

The GenBank accession number for the 16S rRNA gene sequence of WSU 86-1044^T is AF042496.

Progress in molecular biology using PCR and sequencing of DNA encoding 16S rRNA has enabled determination of the precise phylogenetic position of each bacterial species, particularly obligate intracellular bacteria which express few phenotypic characters (Hills *et al.*, 1996; Roux & Raoult, 1995). The primers are chosen from sequences that are highly conserved among the phylogenetic group referred to as the eubacteria (Wilson *et al.*, 1990; Woese, 1987), but which are not found in eukaryotes, archaea or mitochondria. It is therefore possible to amplify only bacterial 16S rDNA sequences even in the presence of nucleic acids from other types of organisms. Because the nucleotide sequences found in 16S rDNAs vary in an orderly fashion throughout the phylogenetic tree, they have been useful for the study of molecular evolution (Woese, 1987). Thus 16S rRNA or 16S rDNA sequencing is one of the most powerful and precise methods for determining the distant as well as close (intrageneric) genealogical relationships of bacteria (Hills *et al.*, 1996; Swofford *et al.*, 1996; Woese, 1987).

In an effort to classify the agent WSU 86-1044^T, the DNA encoding the 16S rRNA was PCR-amplified and sequenced. Comparison of the 16S rDNA sequence with other 16S rDNA sequences in GenBank indicated that WSU 86-1044^T belonged to the order *Chlamydiales*. However, the similarity was not sufficient to allow its classification in any of the families within the order.

In a recent paper by Everett *et al.* (1999) on a revised classification scheme for the order *Chlamydiales*, *Chlamydia pneumoniae*, *Chlamydia pecorum* and *Chlamydia psittaci* were reclassified to *Chlamydophila* gen. nov. as *Chlamydophila pneumoniae* comb. nov., *Chlamydophila pecorum* comb. nov. and *Chlamydophila psittaci* comb. nov., respectively. Their new names will be used throughout this paper.

METHODS

WSU 86-1044^T. The isolation of the organism from pooled foetal liver/lung homogenate has been described previously (Dilbeck *et al.*, 1990). The organism was cloned at the 8th passage by three serial limiting dilutions in 96-well plates of bovine turbinate (BT) cells (ATCC CRL-1390) (Dilbeck *et al.*, 1990). Cloned organisms at the 13th passage in BT cells with a titre of $1 \times 10^{6.2}$ TCID₅₀ were used in these studies.

DNA extraction. Genomic DNA was prepared from WSU 86-1044^T passaged in bovine turbinate cells. The supernatants were harvested when the cytopathic effect was advanced and stored at -70°C . For use, the fluid was thawed and clarified at 500 *g* for 10 min, and the organisms pelleted at 20000 *g* for 30 min. DNA was prepared from the sedimented WSU 86-1044^T organisms using Puregene DNA Isolation Kit (Gentra Systems).

PCR amplification. The DNA encoding the 16S rRNA was amplified *in vitro* via PCR (White, 1993) by using a primer set (forward 5' AGA GTT TGA TCC TGG 3' and reverse 5' TAC CTT GTT ACG ACT T 3') selected from the conserved sequences at the beginning and the end of the eubacterial kingdom 16S rDNA (Wilson *et al.*, 1990; Woese, 1987).

PCR amplification was done in 100 μl reaction mixture and *Taq* DNA polymerase from Gibco-BRL. Typically, a tube contained 10 μl 10 $\times$ PCR buffer made of 200 mM Tris/HCl (pH 8.4) and 500 mM KCl, 5 μl 50 mM MgCl₂, 2 μl 10 mM dinucleotide mix (Invitrogen), 0.5 μl 50 mM of the reverse and forward primers, 79.5 μl double-distilled water, 2 μl containing 0.25 μg target DNA and 0.5 μl (2.5 U) *Taq* DNA Polymerase. Before addition of the target DNA and *Taq* polymerase a wax gem (Perker-Elmer) was added to the tube and heated at 75 $^{\circ}\text{C}$ for 5 min and then allowed to cool to room temperature. Amplification was performed in a GeneAmp PCR System 9600 Thermal Cycler (Perkin-Elmer) in which the target DNA was denatured by incubation at 95 $^{\circ}\text{C}$ for 5 min followed by 35 cycles of denaturation (94 $^{\circ}\text{C}$ for 1 min), primer annealing (55 $^{\circ}\text{C}$ for 3 min) and primer extension (72 $^{\circ}\text{C}$ for 3 min). At the end of the cycling, the reaction mixture was held at 72 $^{\circ}\text{C}$ for 7 min and cooled to 4 $^{\circ}\text{C}$. The size of the PCR product was verified by agarose gel electrophoresis of 10 μl reaction mixture (Sambrook *et al.*, 1989).

Cloning, sequencing and sequence analysis. The amplified fragment was ligated into the *EcoRI* site of vector pCR 2.1 (Invitrogen) and used to transform *Escherichia coli* (one-shot cell INV α F' Competent Cells; Invitrogen). Individual, transformed colonies were grown in Luria Broth, DNA was isolated and then digested with *EcoRI*. DNA fragments were separated in 1.5% agarose gel and stained with ethidium bromide to verify the size of the fragment ligated into the plasmid. Nucleotide sequencing of the recombinant inserts from selected colonies was performed by the Laboratory for Biotechnology and Bioanalysis, Washington State University, using the Perkin Elmer Applied Biosystems Prism Dye Terminator Kit and analysed on an ABI 373 DNA Sequencer. Cloning the amplified fragment into pCR 2.1 enabled sequencing of the whole amplicon, initially using M13 forward and reverse primers (Invitrogen) approximately 100 bases up- and downstream from the *EcoRI* cloning site, and subsequently with commercially synthesized specific primers (Life Technologies) selected as sequence information was obtained. Table 1 shows the primers used to sequence through the entire 16S rDNA amplicon of the WSU 86-1044^T. Sequence analysis programs REFORMAT, GAP, REVERSE, PILEUP, DISTANCES, NEIGHBOR and GROWTREE in the University of Wisconsin Genetics Computer Group (GCG), version 8 (1994), were used for DNA analyses (Devereux *et al.*, 1984). GenBank search for similarities was accomplished using BLAST and FASTA programs on-line (Pearson & Lipman, 1988). Comparison of the WSU 86-1044^T 16S rDNA sequence with published sequences of other organisms, most of which were obligate intracellular bacteria, was performed both by PILEUP and by DISTANCES analysis to calculate the Kimura two-parameter distances. Sequences of the following micro-organisms (GenBank accession no. and strain designation in parentheses) were compared: *Chlamydophila* (formerly *Chlamydia*) *psittaci* (M13769, strain 6BC^T), *Chlamydia trachomatis* (M59178, strain 434^T), *Chlamydophila* (formerly *Chlamydia*) *pneumoniae* (Z49873, strain TW-183^T), *Chlamydophila* (formerly *Chlamydia*) *pecorum* (U73782), *Simkania negevensis* (L27666, strain Z^T), *Parachlamydia acanthamoebae* (Y07556, strain Bn9^T), *Rickettsia rickettsii* (M21293, strain R), *Cowdria ruminantium* [X61659, strain Crystal Springs (Zimbabwe) isolate], *Anaplasma marginale* (M60313), *Ehrlichia phagocytophila* (M73220, strain OS^T) and *Escherichia coli* (M24996).

The neighbour-joining method and tree construction were

performed by using the NEIGHBOR and GROWTREE programs, respectively. All sequences were aligned using the PILEUP multiple-sequence alignment program.

RESULTS

PCR product

Conserved eubacterial rDNA primers amplified a major WSU 86-1044^T fragment slightly above 1.5 kb. When the plasmid with the insert was isolated and digested with *Eco*RI endonuclease, three fragments resulted comprising the vector pCR 2.1 (3.9 kb), one insert fragment slightly longer than 0.8 kb, and a second insert fragment of approximately 0.7 kb, indicating an internal *Eco*RI site in the amplicon. An *Eco*RI site at nucleotide positions 679–685 from the 5' of WSU 86-1044^T was confirmed by sequence analysis. An *Eco*RI site also was reported approximately 680 nucleotides from the 5' end of rDNA for *Chlamydo-phila* (formerly *Chlamydia*) *psittaci* (Weisburg *et al.*, 1986). The sequence of *Chlamydo-phila psittaci* rDNA obtained from GenBank has an *Eco*RI site between nucleotides 681 and 687.

Sequences

The sequence of the WSU 86-1044^T 16S rDNA consisted of 1526 nucleotides (400 A, 300 C, 453 G, 333 T). On-line BLAST and FASTA searches of GenBank for similar sequences revealed that WSU 86-1044^T rDNA was > 80 % similar to chlamydiae. The first 30 items of the search indicated similarity to the four known chlamydiae (*Chlamydo-phila psittaci*, *Chlamydia trachomatis*, *Chlamydo-phila pneumoniae* and *Chlamydo-phila pecorum*), small subunit of chlamydia rRNA, and to *Simkania negevensis* (Kahane *et al.*, 1995; Everett *et al.*, 1999). Similarity ranking by the Ribosomal Database Project (RDP) at the University of Illinois Urbana-Champaign Department of Microbiology indicated that the WSU 86-1044^T rDNA sequence was most similar to *Chlamydo-phila psittaci*.

Phylogenetic analyses

A new classification of the order Chlamydiales has been proposed in which the currently known strains with > 90 % 16S rRNA similarity form the Chlamydiaceae family. The chlamydia-like organisms which have 80–90 % 16S rRNA similarity form two new families: Simkaniaceae and Parachlamydiaceae (Everett *et al.*, 1999). We compared 16S rDNA sequences of some members of the Chlamydiaceae, Simkaniaceae and Parachlamydiaceae (Everett *et al.*, 1999), WSU 86-1044^T, and some intracellular parasites from the Rickettsiaceae to assess their percentage sequence similarity. WSU 86-1044^T exhibited the greatest similarity to members of the order Chlamydiales (> 84.5 %). Three rickettsia species, which included *Cowdria ruminantium*, had values ranging from 72 to 72.9 % while one *Ehrlichia* species had sequence similarities of 72.1 %. The reported anti-

genic cross-reactivity between WSU 86-1044^T and *Chlamydia ruminantium* (Dilbeck *et al.*, 1990) was not reflected in significant rDNA sequence identity.

DISCUSSION

Analysis of 16S rDNA of WSU 86-1044^T indicated that the abortion agent was closely related to the members of the order Chlamydiales, having 84.5–85.3 % sequence similarity. The relatively high degree of similarity of WSU 86-1044^T 16S rDNA to 16S rDNA from the members of the order Chlamydiales provided justification for its inclusion in this group/order. However, when the rDNA sequences of four members from the family Chlamydiaceae were aligned they had sequence similarities of 93.8–96.7 %. Therefore the lower level of sequence similarity between WSU 86-1044^T and the members of the Chlamydiaceae family did not justify inclusion of WSU 86-1044^T into this family (Kahane *et al.*, 1995; Everett & Andersen, 1997; Everett *et al.*, 1999; Takahashi *et al.*, 1997).

It has been shown that WSU 86-1044^T is morphologically similar to rickettsiae and chlamydiae but is biochemically and antigenically distinct (Dilbeck *et al.*, 1990; Kocan *et al.*, 1990). Two developmental forms (dense bodies and reticulate bodies) of WSU 86-1044^T organisms were observed in intracytoplasmic vacuoles by electron microscopy. WSU 86-1044^T was completely resistant to penicillin *in vitro*, whereas all known

Fig. 1. Dendrogram (based on the distances calculated by PILEUP analysis) showing relationship between WSU 86-1044^T, chlamydiae and rickettsiae. Bar indicates 10 nucleotide substitutions.

Table 1. Primers for sequencing the rDNA amplicon of WSU 86-1044^T

Primer	Sequence (position/source)
Forward	
F1 (M13)	5' GTTTTCCAGTCACGACGTTGTA (Invitrogen)
F2	5' GCTCACCAGGCTAAGACGTC (277–298)
F3	5' CTAGCTTTGACCTGACGCTGAT (752–774)
F4	5' GAATCTGCAACTCGGCTCCATG (1323–1345)
Reverse	
R1 (M13)	5' TTGTGAGCGGATAACAATTTC (Invitrogen)
R1	5' CATCCTAAATGCTGGCAAC (392–373)
R2	5' CACCGCTACATGTGGAATTCC (843–822)
R4	5' GATCCTCTCTAGCACCATATCC (1358–1336)

chlamydia are sensitive (Moulder, 1991). Furthermore, WSU 86-1044^T did not react with either polyclonal or monoclonal antibodies used to type chlamydia, although there was some reactivity with antibodies to *Chlamydia ruminantium* (Dilbeck *et al.*, 1990).

Evolutionary distance values calculated using DISTANCES were used to construct the phylogenetic tree shown in Fig. 1. The tree shows the distinctions between chlamydiae and rickettsiae. Although WSU 86-1044^T, *Simkania negevensis* and *Parachlamydia acanthamoebae* formed a cluster, the three are as far apart from one another (84.6–87.2% sequence similarity) as they are from the chlamydiae.

The genetic data described herein, and the morphological similarity (Kocan *et al.*, 1990), are consistent with a close relationship between WSU 86-1044^T and the chlamydiae. However, since rDNA sequence similarity of > 90% has been suggested as a criterion for classifying chlamydia-like organisms within the same family of the order *Chlamydiales* (Everett *et al.*, 1999), WSU 86-1044^T having < 90% similarity does not fit into any of the present families in the order *Chlamydiales*. Therefore, we propose that WSU 86-1044^T be classified as *Waddliaceae* fam. nov., *Waddlia chondrophila* gen. nov., sp. nov.

Description of *Waddliaceae* fam. nov.

Waddliaceae (Wadd'li.a'ce.ae M.L. fem. n. *Waddlia* the type genus of the family; -aceae ending to denote a family; M.L. fem. pl. *Waddliaceae* the *Waddlia* family).

Waddliaceae currently includes the type genus, *Waddlia*. The description of this family is identical at present to that of 'micro-organism WSU 86-1044' (Dilbeck *et al.*, 1990; Kocan *et al.*, 1990) which are obligate intracellular organisms resistant to penicillin. They grow well in BT producing multiple cytoplasmic vacuoles and Gram-negative, periodic acid-Schiff negative and non-acid-fast inclusions. The inclusions contain coccoid organisms ranging from 0.2 to 0.5 µm in size. The BT infectivity is abolished by tetracycline and/or chloroform treatment. The organism

multiplies by binary fission and has two developmental forms: the dense form which is infective, and the reticulated form, usually associated with mitochondria, which undergoes binary fission. These organisms do not react with antisera used for typing chlamydiae or rickettsiae. The 16S rDNA of the *Waddliaceae* strains are > 90% similar to ribosomal genes in WSU 86-1044^T. The family *Waddliaceae* belongs to the order *Chlamydiales* and is a sister taxon of the *Chlamydiaceae* because the ribosomal genes are 80–90% similar to ribosomal genes in the *Chlamydiaceae*. Phylogenetic analyses of the *Waddliaceae* 16S rDNA sequence is presented here. At present this family comprises a single genus, the type genus *Waddlia*.

Description of *Waddlia* gen. nov.

Waddlia [Wadd'li.a. N.L. fem. n. *Waddlia* arbitrary name derived from the abbreviation WADDL (Washington Animal Disease Diagnostic-Laboratory)].

Members of the genus *Waddlia* have 16S rDNA that is > 90% similar to that of the type species, *Waddlia chondrophila* strain WSU 86-1044^T.

Description of *Waddlia chondrophila* sp. nov.

Waddlia chondrophila (chon.dro'phi.la. Gr. n. *chondros* clump; Gr. adj. *philos*, -a friendly to; M.L. *chondrophila* liking clumps, in reference to the association of the organism with cellular mitochondria).

The species *Waddlia chondrophila* currently includes only the type strain, WSU 86-1044^T (= ATCC VR 1470^T). *Waddlia chondrophila* was isolated from the tissues of a first-trimester aborted bovine foetus. The description of this species is identical to that of 'micro-organism WSU 86-1044' (Dilbeck *et al.*, 1990; Kocan *et al.*, 1990). The full length of *Waddlia chondrophila* 16S rDNA sequence is 15.0 ± 0.25% different from the 16S rDNA of *Chlamydiaceae* spp., fitting into the 80–90% identity range that makes this organism a member of the order *Chlamydiales*, but not a member of the family *Chlamydiaceae*. The *Waddlia*

chondrophila 16S rDNA sequence is 15.8% different from that of *Simkania negevensis* strain Z^T, while it is 12.8% different from that of *Parachlamydia acanthamoebae* strain Bn₉^T, which excludes it from *Simkaniaceae* and *Parachlamydiaceae* families of the order *Chlamydiales* (Everett *et al.*, 1999). For a newly identified strain to be described as a member of *Waddliaceae*, a nearly full-length rDNA of the new strain may only differ from *Waddlia chondrophila* 16S rDNA by < 10%.

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