

Phylogenetic Position of *Taylorella equigenitalis* Determined by Analysis of Amplified 16S Ribosomal DNA Sequences

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The 16S ribosomal DNA sequence of *Taylorella equigenitalis* (formerly *Haemophilus equigenitalis*), the causative organism of contagious equine metritis, was determined. A phylogenetic analysis of this sequence revealed a phylogenetic position of *T. equigenitalis* in the β subclass of the class *Proteobacteria* apart from the position of *Haemophilus influenzae*, which belongs to the γ subclass of *Proteobacteria*. A close phylogenetic relationship among *T. equigenitalis*, *Alcaligenes xylosoxidans*, and *Bordetella bronchiseptica* was detected; *Spirillum volutans* and *Chromobacterium fluviatile* (*Iodobacter fluviatile*) were in the same group but slightly removed. This relationship is surprising in view of the considerable differences in the G+C contents of the genomes of these bacteria.

Contagious equine metritis is a sexually transmitted bacterial disease of horses that is caused by *Taylorella equigenitalis*. The disease was reported for the first time by Crowhurst in 1977 (2), and the causative agent was described by Taylor et al. in 1978 (18). Since then, *T. equigenitalis* has been detected in many countries and in different breeds of horses (19). The initial name of this microaerophilic, gram-negative coccobacillus that causes contagious equine metritis was *Haemophilus equigenitalis* (18). It was classified in the family *Pasteurellaceae* as a species incertae sedis belonging to the genus *Haemophilus*.

The taxonomic position of *T. equigenitalis* was reinvestigated in 1983, and it was compared with phenotypically similar organisms, such as *Haemophilus*, *Moraxella*, and *Legionella* species, by Sugimoto et al. On the basis of DNA base composition and DNA-DNA hybridization data, these authors proposed that *Haemophilus equigenitalis* should be transferred to a new genus, *Taylorella*, as *T. equigenitalis* (17).

To further clarify the phylogenetic position of *T. equigenitalis*, we determined the sequence of its 16S ribosomal DNA (rDNA).

We cultured type strain NCTC 11184 and strains N480/82 and N610/88 as described previously (1). These *T. equigenitalis* strains have been characterized by field inversion gel electrophoresis; two belong to field inversion gel electrophoresis group A, while strain N610/88 is a member of group C (1).

Genomic DNA was prepared as described by Garcia et al. (5). The 16S rDNA was amplified in a total volume of 100 μ l by using modified terminal primers fd1 (5'-gggctgcAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-cggatccAAGGAGG GTGATCCAGCCNCA-3') (20) and *Taq* DNA polymerase (Promega, Madison, Wis.). Primer fd1 contained a 5' *Pst*I restriction site (lowercase nucleotides). Amplified 16S rDNA that was about 1,500 bp long was isolated from an agarose gel by using a GeneClean kit (Bio101, La Jolla, Calif.) and was made blunt by incubation with the Klenow fragment of DNA polymerase I and 1.67 mM each of the four deoxy-nucleoside triphosphates for 30 min at 37°C (21). The frag-

ments were then cut with *Pst*I. The DNA was phenol extracted, ethanol precipitated, and ligated between the *Eco*RV and *Pst*I sites of pBluescript II KS (Stratagene, La Jolla, Calif.) by using T4 DNA ligase. Plasmid DNAs of clones were purified with a Qiagen plasmid kit (Qiagen, Inc., Chatsworth, Calif.) in accordance with the manufacturer's instructions.

The nucleotide sequences of both strands of the cloned 16S rDNA were determined by using the dideoxy chain termination method (14) and an Autoread laser fluorescent DNA sequencer (Pharmacia). The sequencing reactions were carried out by using an Autoread sequencing kit (Pharmacia) in accordance with the manufacturer's manual. The primers used for sequencing 16S rDNA, forward and reverse, were fluorescein isothiocyanate labeled and have been described previously (20).

The sequences of the *T. equigenitalis* 16S rDNAs of type strain NCTC 11184 and strains N480/82 and N610/88 were determined. They were identical. The sequence is available from the EMBL, GenBank, and DDBJ nucleotide sequence data bases.

The newly obtained sequence was compared with entries present in the EMBL and GenBank data bases. Close relationships with 16S rDNA sequences of *Bordetella bronchiseptica* S-1, *Alcaligenes xylosoxidans* ATCC 15173^T (T = type strain), *Chromobacterium fluviatile* (*Iodobacter fluviatile* [8, 9]) ATCC 33051, *Legionella pneumophila* ATCC 33152^T, *Pseudomonas aeruginosa* ATCC 25330, *Escherichia coli*, and *Spirillum volutans* ATCC 19554^T were detected. Two hypervariable regions which distinguish the three closest relatives most effectively are shown in Fig. 1. These regions are located at the 5' end of the 16S rDNA at positions 65 to 97 and 412 to 478 (*E. coli* numbering).

The 16S rDNA sequence of *T. equigenitalis* was aligned with the sequences of the 16S rDNAs of these bacteria. Aligned 16S rRNA sequences were obtained from the Ribosomal Database Project (10). All bacterial sequences were truncated from position 27 (*E. coli* numbering). Positions at which all of the 16S rRNA sequences had a gap were eliminated. This alignment was used for a sequence signature analysis (22) and a phylogenetic analysis.

With additional sequence masking we eliminated positions at which the sequences had gaps. In this way, only those

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T. equigenitalis 65 AACGGCAGCA TAAGGAGAGC TTGCTTTTCT TGA 97
A. xylosoxidans ..... C-----GA. ..CG-.C--- -.G
B. bronchiseptica G..... C-----G.. ..CG-GCCT- -.G
    
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T. equigenitalis 412 TTGAAGGCCTT GGGTTGTAA AGCACITTTG * TCAGGGAAGA AAAGTTTGT * GTTAATACCA TGGACTGCTG ACGGTACCTGA * 478
A. xylosoxidans .-..... C..... G...A.... ..C.TCG..G ..... C.CGGCA... .....C
B. bronchiseptica .-..... C..... G...A.... ..C..CAC.G .C.....T.C ..TG.AA... .....C
    
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FIG. 1. Two most variable regions of the 16S rDNA genes of *T. equigenitalis*, *A. xylosoxidans*, and *B. bronchiseptica*. The first region is located from position 65 to position 97; the second region is located from position 412 to position 478 (*E. coli* numbering). The dots in the alignments indicate identical sequences; the dashes indicate deletions. Asterisks indicate signature sequences.

positions that were present in all of the sequences were used for the alignment. This sequence masking affected only the lengths of the branches, not the branching order (data not shown).

For the phylogenetic analysis we performed a distance matrix and parsimony analysis, as described in the Felsenstein-Phylip package, version 3.3 (3). The distance matrix FITCH (4) and NJTree (13) algorithms were used for treeing algorithms that assume no evolutionary clock. The KITCH program (4) uses a treeing algorithm that assumes an evolutionary clock.

The evolutionary distance tree generated by the distance matrix analysis and the FITCH program is shown in Fig. 2. *T. equigenitalis* forms a cluster with *A. xylosoxidans*, *B. bronchiseptica*, *S. volutans*, and *C. fluviatile*. The evolutionary distance trees generated by the other programs and by

the parsimony analysis program DNAPars were not significantly different from the tree shown in Fig. 2 (data not shown). The levels of sequence identity among the members of this phylogenetic cluster varied between 87.6 and 97.6% (Table 1).

The cluster belongs to the β subclass of the class *Proteobacteria* (16). The position of *T. equigenitalis* in the β subclass is supported by the results of an analysis of the helices between positions 180 and 220. The first helix, between position 184 and 193, contains 3 bp. The second helix, between positions 198 and 219, contains 9 bp, a feature typical of the β and γ subclasses of *Proteobacteria* (22). Finally, a sequence signature analysis showed that the 16S rRNA of *T. equigenitalis* contains the signature sequences of the β subdivision of the purple bacteria in 63 of 66 positions.

The phylogenetic position of *T. equigenitalis* apart from

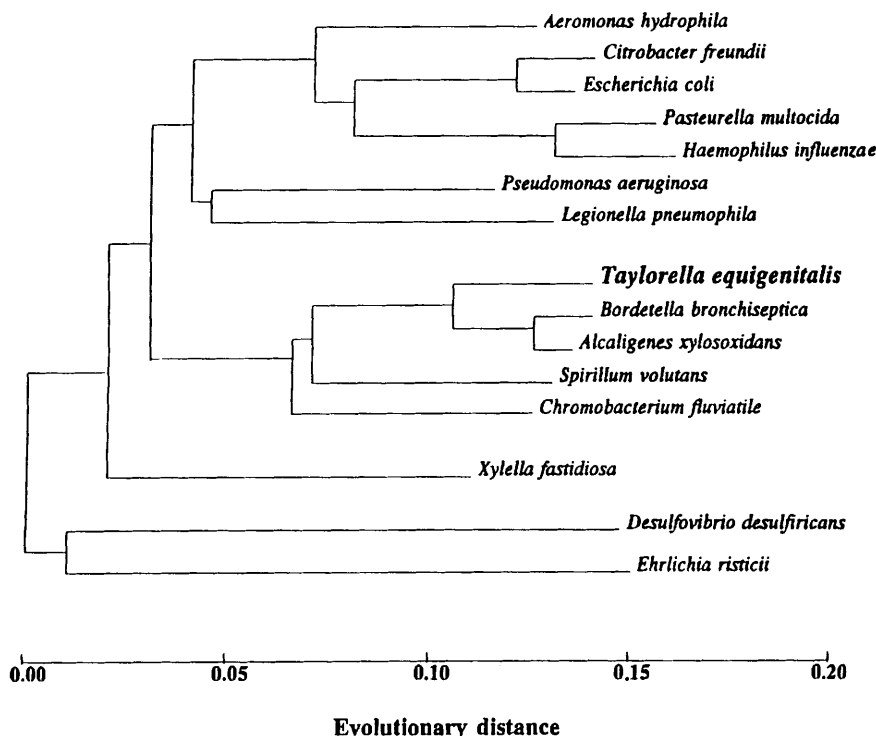


FIG. 2. Evolutionary distance tree generated by distance matrix analysis without sequence masking by using the FITCH program. The evolutionary distances (represented by the lengths of the horizontal lines) are shown in Table 1.

TABLE 1. Levels of sequence identity and evolutionary distances among rRNA and rDNA sequences of *T. equigenitalis* and other eubacteria

Organism	% Sequence identity or evolutionary distance (10 ³) ^a														
	<i>Escherichia coli</i>	<i>Taylorella equigenitalis</i>	<i>Alcaligenes xylosoxidans</i>	<i>Bordetella bronchiseptica</i>	<i>Spirillum volutans</i>	<i>Chromobacterium fluviatile</i>	<i>Xylella fastidiosa</i>	<i>Pseudomonas aeruginosa</i>	<i>Legionella pneumophila</i>	<i>Citrobacter freundii</i>	<i>Aeromonas hydrophila</i>	<i>Pasteurella multocida</i>	<i>Haemophilus influenzae</i>	<i>Ehrlichia risticii</i>	<i>Desulfivibrio desulfuricans</i>
<i>Escherichia coli</i>															
<i>Taylorella equigenitalis</i>	21.8														
<i>Alcaligenes xylosoxidans</i>	21.3	6.4													
<i>Bordetella bronchiseptica</i>	21.4	6.6	2.4												
<i>Spirillum volutans</i>	20.9	13.0	12.4	12.6											
<i>Chromobacterium fluviatile</i>	20.3	13.4	12.8	12.9	12.4										
<i>Xylella fastidiosa</i>	20.5	21.0	20.5	20.6	20.1	19.5									
<i>Pseudomonas aeruginosa</i>	17.1	19.2	18.6	18.8	18.3	17.6	17.8								
<i>Legionella pneumophila</i>	19.0	21.0	20.4	20.6	20.1	19.4	19.6	15.4							
<i>Citrobacter freundii</i>	3.2	22.1	21.6	21.7	21.2	20.6	20.8	17.4	19.2						
<i>Aeromonas hydrophila</i>	12.3	20.4	19.9	20.0	19.5	18.9	19.1	15.7	17.5	12.6					
<i>Pasteurella multocida</i>	13.7	24.2	23.6	23.8	23.3	22.6	22.8	19.4	21.3	14.0	14.6				
<i>Haemophilus influenzae</i>	14.0	24.4	23.9	24.0	23.5	22.9	23.1	19.7	21.5	14.3	14.9	5.67			
<i>Ehrlichia risticii</i>	28.5	29.0	28.5	28.7	28.1	27.5	26.0	25.8	27.6	28.8	30.8	31.1	73.7		
<i>Desulfivibrio desulfuricans</i>	28.2	28.8	28.2	28.4	27.9	27.2	25.7	25.5	27.4	28.5	30.5	30.8	31.1	76.9	

^a The values on the upper right are the levels of sequence identity, and the values on the lower left are the evolutionary distances.

the position of *H. influenzae*, which belongs to the γ subclass of *Proteobacteria*, supports the exclusion of *T. equigenitalis* from the genus *Haemophilus* and the creation of the genus *Taylorella*.

The relationship of *T. equigenitalis* to *B. bronchiseptica*, and *A. xylosoxidans* subsp. *denitrificans*, and other species belonging to rRNA superfamily III was determined by Rossau et al. (12). Despite the low G+C content of its genome, these authors included *T. equigenitalis* in rRNA superfamily III on the basis of DNA-rRNA hybridization data.

The variation in the G+C contents of the genomes of the five bacteria in the cluster is remarkable; *T. equigenitalis* and *S. volutans* have G+C contents of about 37 mol% (7, 18), *C. fluviatile* has a G+C content of about 51 mol% (15), and *A. xylosoxidans* and *B. bronchiseptica* have G+C contents of about 69 mol% (6, 11). Although G+C content is not a phylogenetic marker per se, such extreme variation is usually not found within a bacterial subdivision. Clearly, some additional parameters are needed to identify further subdivisions within the β subclass of *Proteobacteria*.

The 16S rRNA gene sequences of *Taylorella* strains belonging to field inversion gel electrophoresis groups A and C are identical. This implies that the 16S rRNA gene sequences are not useful for epidemiological studies. On the other hand, the 16S rRNA gene may be useful as a target for a diagnostic polymerase chain reaction.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers for the 16S rDNAs of *T. equigenitalis*, *B. bronchiseptica* S-1, *A. xylosoxidans* ATCC 15173^T, *C. fluviatile* ATCC 33051, *L. pneumophila* ATCC 33152^T, *P. aeruginosa* ATCC 25330, *E. coli*, and *S. volutans* ATCC 19554^T are X68645, X57026, M22509, M22511, M59157, M34133, J01695, and M34131, respectively.

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