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Assignment of the Agent of Tyzzer's Disease to *Clostridium piliforme* comb. nov. on the Basis of 16S rRNA Sequence Analysis

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The small-subunit rRNA (16S rRNA) sequence of Tyzzer's bacillus (also known as "*Bacillus piliformis*") was elucidated by using the polymerase chain reaction followed by reverse transcriptase sequencing. By using maximum-likelihood analysis, a phylogenetic tree was constructed from this and other 16S rRNA sequences available from the first release of the Ribosomal Database Project (G. J. Olsen, R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W.-M. Kuan, T. J. Macke, Y. Xing, and C. R. Woese, Nucleic Acids Res. 20:2199–2200, 1992). Tyzzer's bacillus grouped with a specific set of anaerobic bacteria, most of which are *Clostridium* spp. The closest identified relatives are *Clostridium coccoides*, *Clostridium oroticum*, *Clostridium clostridiiforme*, *Clostridium symbiosum*, and *Streptococcus hansenii*. *Clostridium aminovalericum* and "*Acetitomaculum ruminis*" are also solidly allied with this ensemble. We propose that Tyzzer's bacillus be reclassified as *Clostridium piliforme* on the basis of its 16S rRNA sequence.

In 1917 Ernest Tyzzer described an infectious disease of laboratory mice. Affected animals had diarrhea for up to 3 days, followed by inappetence, weight loss, and death approximately 6 days after the first signs appeared. At necropsy, gross internal pathology was confined to a focal necrotic hepatitis. The more chronic the condition, the more numerous were the lesions. Pleomorphic, subterminally sporulating, rod-shaped bacteria, often in roughly parallel bundles, were seen in the otherwise healthy liver cells surrounding the areas of necrosis. Similar bundles of bacteria were seen in the cecal and proximal colonic epithelia (25).

Tyzzer's bacillus remains to be cultivated in an acellular medium, although it can be grown in cultured mammalian cells (16, 22). Consequently, published reports on the bacterium have largely been restricted to in vivo studies in which susceptible laboratory animals were used and to the reporting of clinical cases of the disease. Mammals that have been diagnosed as having Tyzzer's disease include mice, rats, guinea pigs, gerbils, hamsters, muskrats, rabbits, cottontails, raccoons, coyotes, dogs, grey foxes, cats, snow leopards, horses, and rhesus monkeys (reviewed by Borriello and Carman [2]). Although there are no reports of Tyzzer's disease in humans, Fries (12) has reported elevated serum antibody levels in 166 of 287 (58%) antenatal women. Some titers exceeded 1/1,000 (12).

Tyzzer's bacillus has been called "*Bacillus piliformis*" (25), although it has been suggested that it may more likely be a member of the genus *Clostridium* (2). Members of the genus *Clostridium* are anaerobic or microaerophilic sporeforming rods that do not produce spores in the presence of air, are usually gram positive, and do not carry out dissimilatory sulfate reduction (5). Animal bedding contaminated with spores from Tyzzer's bacillus remains infective for over 12 months (25), and although Tyzzer's bacillus has often been reported to be gram negative in tissue sections, it can appear gram variable and gram positive. Many clostridia stain gram variable or even gram negative once they have aged. Because it cannot be grown in acellular medium, it is not known whether Tyzzer's bacillus is an obligate anaerobe. Profiles of its in vivo susceptibility to antimicrobial agents (24) show that neomycin, a drug that is ineffective against obligate anaerobes, is ineffective against Tyzzer's bacillus, suggesting that Tyzzer's bacillus is an obligate anaerobe. Tyzzer's bacillus is an intestinal pathogen, an attribute much more common among the pathogenic clostridia. Furthermore, members of the genus *Clostridium* are much more prevalent in the colon than *Bacillus* spp. Spontaneous epidemics of Tyzzer's disease are frequent, and it is even more common to have activation of latently infected animals or development of disease in previously naive animals following stress. It is well documented that clostridial diseases are induced by antibiotic exposure and change of diet (2).

Sequencing of the small-subunit rRNA (16S rRNA) has become a powerful tool for determining phylogenetic relationships between microorganisms. The sequence of 16S rRNA varies in an orderly fashion across phylogenetic boundaries so that organisms that are phylogenetically related have similar sequences. This property of 16S rRNA is utilized in the study of molecular evolution and systematics (28). An extensive literature now shows that phylogenetic trees generated by 16S rRNA sequence analysis (1, 20, 26) are consistent and agree with relationships inferred from analyses of phenotypic characteristics. Phylogenetic trees constructed from analysis of 16S rRNA sequences have also been shown to agree with evolutionary trees inferred from sequence analysis of other highly conserved genes (17). Using 16S rRNA sequence analysis, we have studied the phylogenetic relatedness of Tyzzer's bacillus to the genera *Clostridium* and *Bacillus*.

MATERIALS AND METHODS

Bacterial strain and culture conditions. Tyzzer's bacillus of rabbit origin was propagated in mouse fibroblast 3T3 cells (22). A pellet that was virtually 3T3 cell free was obtained from Thomas Spencer, Veterinary Research Branch, Na-

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tional Institutes of Health, Bethesda, Md. When grown in this way, the strain remained virulent and produced typical Tyzzer's disease in intravenously challenged laboratory rabbits (22).

16S rRNA analysis. Nucleic acids from Tyzzer's bacillus were extracted as previously described (27). Amounts of DNA ranging from 100 ng to 100 pg were amplified by using primers directed at sequences specific for eubacterial 16S ribosomal DNA (13). The polymerase chain reaction product was reamplified by the plug reamplification method of Zintz and Beebe (30). The amplicon was then transcribed and sequenced (13). Sequencing reaction mixtures were separated on 8% polyacrylamide electrolyte gradient gels (21).

Phylogenetic analysis. The 1,455 nucleotides of inferred sequence of the Tyzzer's bacillus 16S rRNA were aligned on the basis of conserved primary and secondary structures (29) with the 473 corresponding sequences in the first release of the Ribosomal Database Project alignment (19). Regions of the Tyzzer's bacillus sequence that were not inferred (because they were not between the positions of the first and last sequencing primers) or that displayed substantial length variation were excluded from further analysis because of uncertainty of homology. The regions retained for phylogenetic analysis correspond to positions 29 to 70, 98 to 199, 218 to 451, 480 to 837, 849 to 999, 1045 to 1131, 1143 to 1449, and 1459 to 1489 in the *Escherichia coli* sequence (3, 4). These regions included 1,309 unambiguous nucleotides of the Tyzzer's bacillus 16S rRNA sequence.

Sequence similarities were used to select organisms for inclusion in more detailed phylogenetic analyses. Also included were bacteria that had been previously proposed to be relatives of Tyzzer's bacillus and a diverse sample of low-G+C-content gram-positive bacteria. The sequence from *E. coli* was included as an outgroup. The data were analyzed by using several methods, and the results were compared. Parsimony analysis (11) was performed with PAUP version 3.0s (23). Least-squares distance analyses were performed with the distance correction of Jukes and Cantor (15) by using programs described by Olsen (18). Maximum-likelihood analyses as described by Felsenstein (7, 9) were performed by using the fastDNAml program, version 1.0.5 (18a). The equilibrium base composition was estimated from the sequences (empirical base frequencies option), and the assumed ratio of transition to transversion substitutions was 2.0 (the default of the program). The confidence limits in the trees were estimated by using the bootstrap method (8).

Nucleotide sequence accession number. The sequence of the *Clostridium piliforme* 16S rRNA gene has been deposited in the GenBank data bank under accession number L07416.

RESULTS

The levels of 16S rRNA similarity (fractions of sequence identity) between Tyzzer's bacillus and the bacterial sequences in the Ribosomal Database Project alignment (19) were calculated and sorted. All of the highest levels of similarity were with members of the low-G+C-content gram-positive group (28). A representative collection of levels of sequence similarity and corresponding evolutionary distance estimates is presented in Table 1.

More detailed phylogenetic analyses were performed on the 16S rRNA sequence from Tyzzer's bacillus, the sequences most similar to it, and a diverse collection of other sequences from the same general region of the phylogenetic tree available from the Ribosomal Database Project (19). These sequences were subjected to analysis by the maximum-likelihood method (Fig. 1) and the parsimony method (data not shown). A smaller group of sequences were analyzed by the least-squares pairwise distance analysis method (data not shown). In all cases, the sequences from Tyzzer's bacillus, *Clostridium coccoides*, *Clostridium oroticum*, *Clostridium clostridiiforme*, *Clostridium symbiosum*, *Streptococcus hansenii*, *Clostridium aminovalericum*, and "*Acetitomaculum ruminis*" formed a distinct group. Although the maximum-likelihood tree (Fig. 1) suggests that *C. aminovalericum* and "*A. ruminis*" might be viewed as peripheral to the group, other methods of analysis or changes in the collection of sequences included can move Tyzzer's bacillus to a branch point deeper than that of these two organisms. The tree shown is the best estimate given by the maximum-likelihood analysis.

The statistical reliability of this group as a whole was examined by using the bootstrap method (8), which estimates the uncertainty in a measurement due to a finite sample size (in this case, the limited length of the sequences). When parsimony was used, 92% of the 100 bootstrap resamplings retained all eight organisms of the proposed group, whereas when maximum-likelihood analysis was used, 99% (119 of 120) of the bootstrap resamplings retained this group. Our inclination is to accept the support offered by the maximum-likelihood analysis as a reflection of additional statistical power of the method and therefore to interpret the inferred grouping of Tyzzer's bacillus as strongly supported. Neither parsimony nor maximum-likelihood analyses indicated a significant resolution of the order in which Tyzzer's bacillus, *C. aminovalericum*, and "*A. ruminis*" separate from the remaining group of five sequences (data not shown).

There are two oligonucleotides which when used together are a signature of this phylogenetic group. These are GAAG TATYTCGGTATGTAAA at positions 413 to 432 and TCC ACCTGGGGAGTA at positions 878 to 892 on the *E. coli* numbering system (3, 4).

DISCUSSION

When Tyzzer first described a fatal disease of the Japanese waltzing mouse, he named the causative agent "*Bacillus piliformis*" on the basis of its morphology and reported that it appeared to be a stiff, inflexible, slender, rod-shaped organism (25). This classification has not been revised since. By sequencing the 16S rRNA of Tyzzer's bacillus we have demonstrated its phylogenetic relationship with other bacteria in the Ribosomal Database Project (19) and assigned it to a phylogenetic position among organisms that are primarily members of the genus *Clostridium*. We propose that Tyzzer's bacillus be reclassified as *Clostridium piliforme*, and the definition of the species is its 16S rRNA sequence.

Classification of the clostridia on the basis of phenotypical characteristics alone clearly demonstrates that phylogenetically the genus is diverse (5). The 16S rRNA sequences of many clostridial species are known, and analysis of these sequences also indicates that the clostridia form a diverse group with many branches. If, as has been suggested many times, reclassification of the clostridia occurs, *C. piliforme* should be grouped with the five species with which it exhibits the highest levels of similarity, (*C. coccoides*, *C. oroticum*, *C. clostridiiforme*, *C. symbiosum*, and *S. hansenii*) and possibly with *C. aminovalericum* and "*A. ruminis*," but not with the type strain of the genus, *Clostridium butyricum*. Both the early study of Johnson and Francis (14)

TABLE 1. Levels of similarity and evolutionary distances between small-subunit rRNA sequences

Species	Similarities and evolutionary distances[a]														
	Escherichia coli	*Clostridium symbiosum*	*Clostridium clostridiiforme*	*Clostridium coccoides*	*Streptococcus hansenii*	*Clostridium oroticum*	Tyzzer's bacillus	*Clostridium aminovalericum*	*"Acetitomaculum ruminis"*	*Lactobacillus casei*	*Bacillus subtilis*	*Clostridium thermosaccharolyticum*	*Clostridium leptum*	*Clostridium butyricum*	*Clostridium barkeri*
Escherichia coli		0.230	0.235	0.240	0.241	0.224	0.253	0.221	0.224	0.230	0.237	0.242	0.258	0.246	0.247
Clostridium symbiosum	0.802		0.050	0.066	0.077	0.056	0.137	0.082	0.110	0.174	0.183	0.201	0.185	0.177	0.205
Clostridium clostridiiforme	0.798	0.952		0.057	0.070	0.058	0.133	0.086	0.109	0.184	0.191	0.192	0.181	0.181	0.207
Clostridium coccoides	0.794	0.937	0.945		0.028	0.060	0.124	0.087	0.094	0.188	0.184	0.186	0.178	0.176	0.191
Streptococcus hansenii	0.794	0.927	0.933	0.972		0.066	0.129	0.094	0.101	0.194	0.197	0.193	0.182	0.176	0.192
Clostridium oroticum	0.807	0.946	0.944	0.943	0.937		0.123	0.071	0.111	0.183	0.185	0.196	0.181	0.158	0.188
Tyzzer's bacillus	0.785	0.875	0.878	0.886	0.881	0.886		0.126	0.148	0.210	0.206	0.190	0.210	0.189	0.197
Clostridium aminovalericum	0.809	0.923	0.918	0.918	0.912	0.932	0.884		0.096	0.180	0.182	0.190	0.182	0.164	0.185
"Acetitomaculum ruminis"	0.806	0.898	0.898	0.912	0.905	0.897	0.866	0.910		0.196	0.193	0.178	0.151	0.187	0.178
Lactobacillus casei	0.802	0.845	0.837	0.834	0.829	0.838	0.817	0.840	0.828		0.117	0.194	0.186	0.182	0.195
Bacillus subtilis	0.797	0.838	0.831	0.837	0.827	0.836	0.820	0.839	0.830	0.892		0.173	0.207	0.172	0.173
Clostridium thermosaccharolyticum	0.793	0.824	0.831	0.836	0.830	0.828	0.832	0.832	0.842	0.829	0.845		0.192	0.183	0.176
Clostridium leptum	0.781	0.836	0.839	0.842	0.838	0.839	0.816	0.838	0.863	0.835	0.819	0.830		0.188	0.189
Clostridium butyricum	0.790	0.843	0.839	0.844	0.843	0.858	0.833	0.853	0.835	0.838	0.846	0.838	0.834		0.185
Clostridium barkeri	0.789	0.820	0.819	0.832	0.830	0.833	0.827	0.836	0.841	0.829	0.846	0.843	0.833	0.836	

[a] The values on the lower left are the levels of fractional sequence identity within the regions of unambiguous alignment. The values on the upper right are the average numbers of substitutions per sequence position (evolutionary distances), adjusted as described by Jukes and Cantor (15) for multiple substitutions at individual sequence positions.

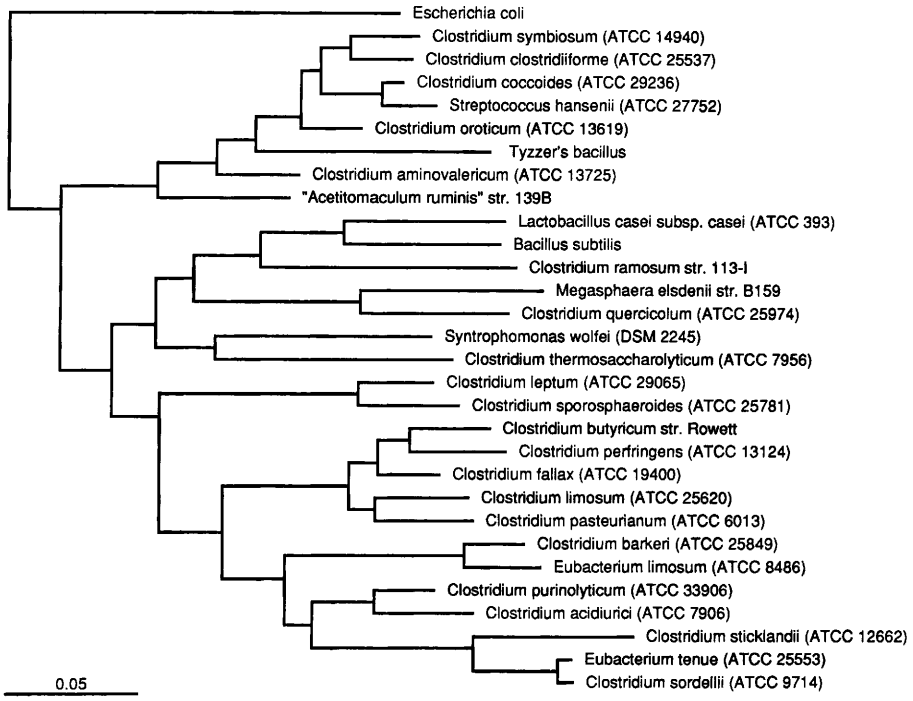

FIG. 1. Maximum-likelihood phylogenetic tree of small-subunit rRNA sequences showing the relationships of Tyzzer's bacillus (*C. piliforme*) and a sample of other low-G+C-content gram-positive organisms. The sequence from *E. coli* was used as an outgroup to root the tree. The length of the horizontal branches in the tree indicates the estimated number of substitutions per sequence position that occur along the line. Scale bar = 0.05 substitution per position.

and the review of Cato and Stackebrandt (6) agree with the above conclusion and would put *C. piliforme* into the clostridial group designated group III.

It is of interest to note that bacteria able to produce spores do not all cluster together, suggesting that this ability was lost at different stages during evolution. Many members of the genus *Eubacterium* are closely related to the clostridia, the major difference being that the former are not known to produce spores. Our results enable us to reject the previously proposed affinity of Tyzzer's bacillus, namely the genus *Bacillus* (25).

Knowing the 16S rRNA sequence of the organism opens many new avenues for research and treatment of the disease. The knowledge that Tyzzer's bacillus is a *Clostridium* sp. will help in determining the most appropriate treatment or management of the disease. For example, bowel diseases caused by clostridia are best treated with oral vancomycin, bacitracin, or metronidazole (10). Oligonucleotide primers specific for Tyzzer's bacillus may be used to diagnose the disease by the polymerase chain reaction, enabling more timely diagnosis and thus greater scope for treatment or isolation of the infected animal. Alternatively, an oligonucleotide probe specific for Tyzzer's bacillus could be generated and used for rapid in situ analysis of intestinal material.

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