

Phylogenetic Foundation of Spirochetes

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Abstract

The spirochetes are free-living or host-associated, helical bacteria, some of which are pathogenic to man and animal. Comparisons of 16S rRNA sequences demonstrate that the spirochetes represent a monophyletic phylum within the bacteria. The spirochetes are presently classified in the Class *Spirochaetes* in the order *Spirochaetales* and are divided into three major phylogenetic groupings, or families. The first family *Spirochaetaceae* contains species of the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, *Spironema*, and *Treponema*. The second family *Brachyspiraceae* contains the genus *Brachyspira* (*Serpulina*). The third family *Leptospiraceae* contains species of the genera *Leptonema* and *Leptospira*. Novel spirochetal species, or phylotypes, that can not be presently cultivated in vitro, have been identified from the human oral cavity, the termite gut, and other host-associated or free-living sources. There are now over 200 spirochetal species or phylotypes, of which more than half is presently not cultivable. It is likely that there is still a significant unrecognized spirochetal diversity that should be evaluated.

Introduction

Based on comparative analysis of 16S rRNA sequences, the spirochetes represent one of about 40 major bacterial phyla (Hugenholtz *et al.*, 1998). The spirochetes are unusual in that they are one of the few phyla whose phenotypic characteristics reflect its natural phylogenetic relationships, *e.g.*, they possess a cellular ultrastructure that is unique among the bacteria (Paster *et al.*, 1991). The biodiversity of spirochetes is marked inasmuch as they are often observed in high numbers in the gastrointestinal tract of man, mammals, insects, and bivalves, as well as in aquatic and marine environments. Some species are pathogenic to man, other mammals and birds. However, the focus of this paper is to review the phylogenetic relationships of the spirochetes, including those that are presently not cultivable.

Phylogenetic Foundation of Spirochetes

Based on 16S rRNA or 16S rDNA sequence comparisons, spirochetes form a coherent phylogenetic phylum, as shown in Figure 1 (Paster *et al.*, 1991). The spirochetes are presently classified in the Class *Spirochaetes* in the order

Spirochaetales that is divided into three families; namely the *Spirochaetaceae*, the *Brachyspiraceae*, and the *Leptospiraceae*. The phylogenetic relationships of representatives of each genus are shown in Figure 1.

The *Spirochaetaceae* are separated into 6 genera—*Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, "*Spironema*", and *Treponema*. New genera of termite spirochetes, such as *Clevelandina*, *Diplocalyx*, and *Hollandina*, have been described on the basis of differences in ultrastructural traits (Breznak, 1984). It has been suggested that they also belong in the family *Spirochaetaceae*, but no sequence information is presently available to determine their phylogenetic position within the spirochetes.

The *Brachyspiraceae* contain the genus *Brachyspira* (*Serpulina*). Due to the close phylogenetic relationship of *B. aalborgi* to species characterized as *Serpulina*, it has been recommended that a single genus be justified. Thus, *Brachyspira* takes precedence over *Serpulina* since the former genus was listed first as a valid name (Hovind-Hougen *et al.*, 1983).

The *Leptospiraceae* are separated into 2 known genera, *Leptonema* and *Leptospira*. The putative misclassified "*Leptospira*" *parvula* may represent a third genus of the *Leptospiraceae*.

Despite the relatively deep branching of the spirochetes, they are also defined by single-base signature analysis (Paster *et al.*, 1991). Thus, bases are found in the 16S rRNA sequences of over 200 species or phylotypes of spirochetes, but are not found (or rarely found) in sequences of approximately 16,000 other bacteria in GenBank and the Ribosomal Database Project (Maidak *et al.*, 1999). These data support the conclusions that spirochetes represent a single monophyletic phylum (Paster *et al.*, 1991) and that they have descended from a common spirochetal ancestor.

Sequences of species of the genera *Treponema*, *Spirochaeta*, and *Leptospira* are unusual in that they possess a 20 to 30 base 5' extension of the 16S rRNA molecule (Paster *et al.*, 1991). The region is highly variable and likely to form helices from 2 to 12 bp. The function of this 5' extension is unknown. These 5' regions were discovered using the older technique of direct RNA sequencing with reverse transcriptase (Paster *et al.*, 1991).

Unifying Phenotypic Traits

All spirochetes are helical or spiral shaped microorganisms with internal organelles of motility called periplasmic flagella (Paster and Canale-Parola, 1980). The flagella are inserted subterminally at each end of the cell, wrap around the protoplasmic cylinder, and usually overlap in the center region (Figure 2). Periplasmic flagella are located between the protoplasmic cylinder and an outer membrane-like structure called the outer sheath. The number of periplasmic flagella can range from 2 to 100's per cell depending upon the species. In some species under certain growth conditions, the periplasmic flagella can protrude into the external environment (Charon *et al.*, 1992).

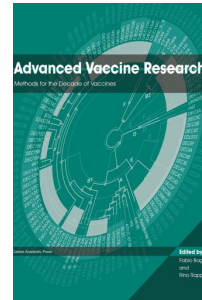
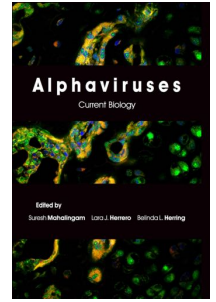
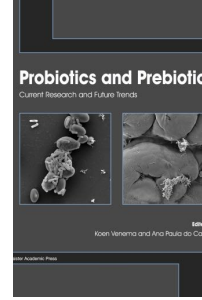
Other unifying characteristics of the spirochetes include

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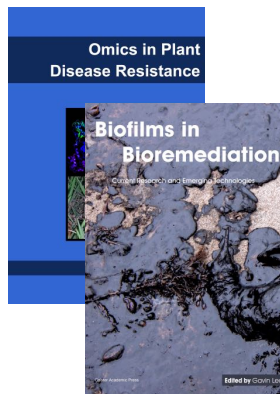
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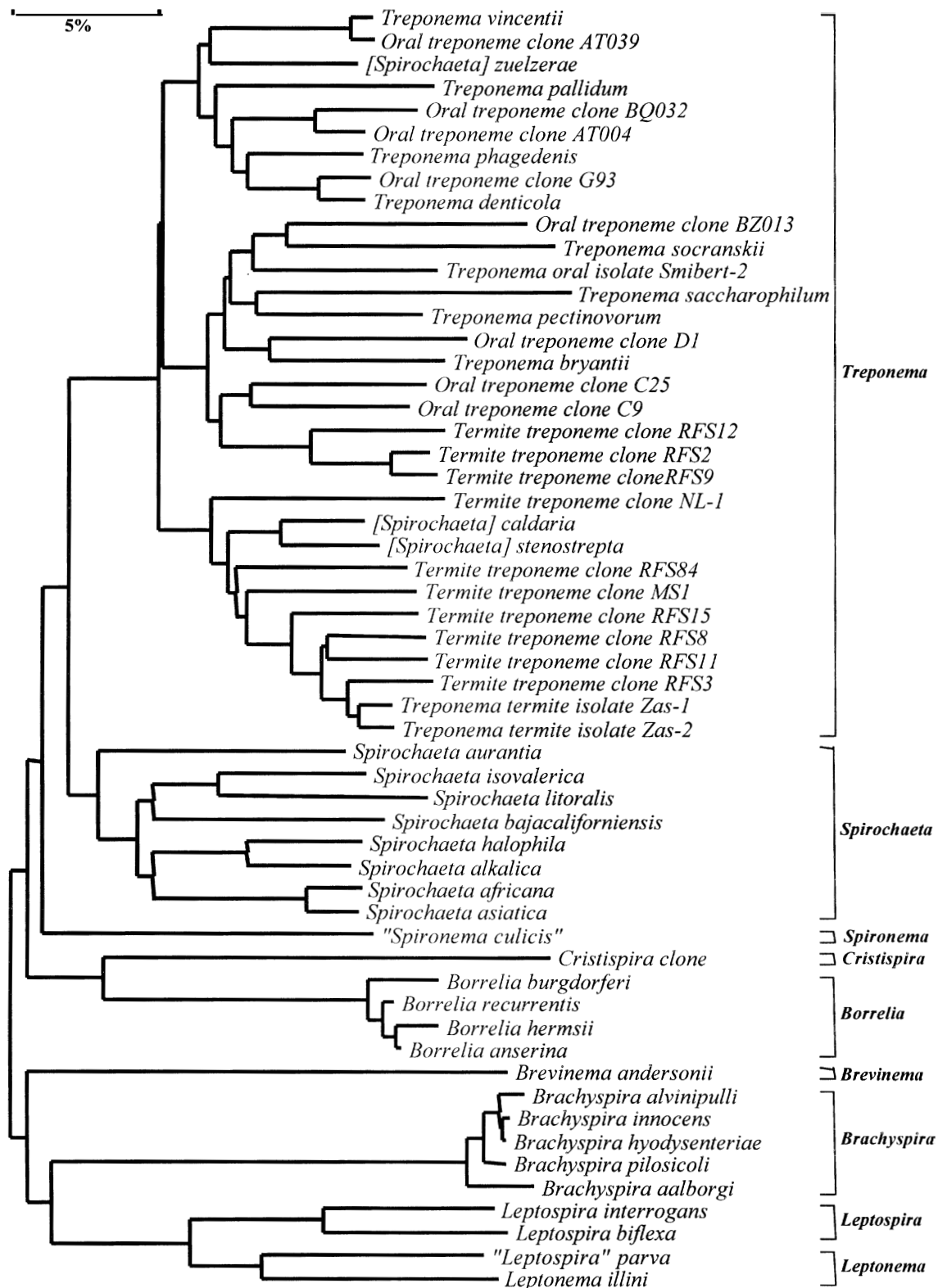


Figure 1. 16S rRNA dendrogram demonstrating the phylogenetic relationships of representatives of spirochetal genera. The sequences of the species shown may be obtained through GenBank or by request from the authors. Designations for source of termite spirochetal clones and isolates: RFS and Zas, *Reticulitermes flavipes*; NL, *Nasutitermes lujae*; MS, *Mastotermes darwiniensis*. Similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (1969). The neighbor-joining method (Saitou and Nei, 1987) was used for phylogenetic tree construction. TREECON, a software package for the Microsoft Windows environment, was used for the construction and drawing of evolutionary trees (Van de Peer *et al.*, 1994). The scale bar represents a 5% difference in nucleotide sequence determined by taking the sum of all of the horizontal lines connecting two species. Vertical distance has no meaning.

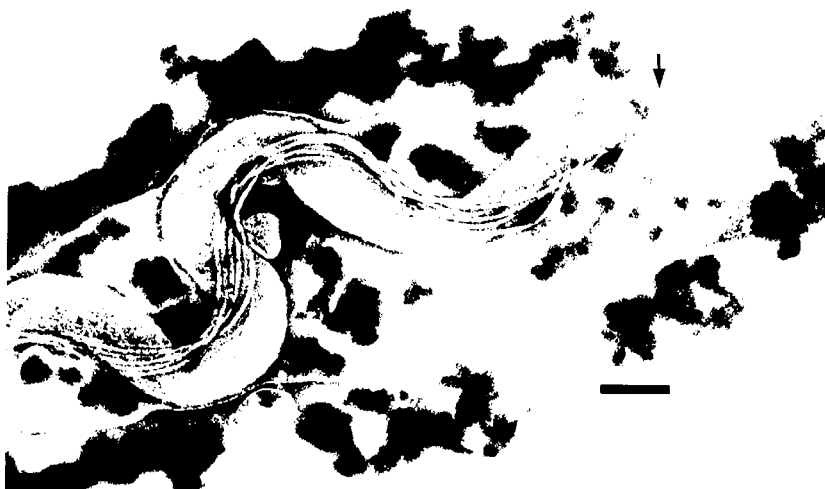


Figure 2. Transmission electron micrograph of negatively stained cell of a spirochete observed directly in a subgingival plaque sample. The species identity is unknown, but likely represents a novel oral treponeme. Six periplasmic flagella are inserted at the end of the cell (arrow head). The outer sheath (arrow), normally tightly associated with the helical protoplasmic cylinder, is swollen in this figure. Bar = 0.2 μm .

their resistance to the antibiotic rifampicin (Stanton *et al.*, 1979; Leschine and Canale-Parola, 1980; Weber and Greenberg, 1981; Leschine and Canale-Parola, 1986; Nelson *et al.*, 1991). Consequently, this antibiotic has been used as a selective agent in the isolation of spirochetes from a variety of environments.

Spirochetes have been classified according to conventional phenotypic and genotypic characteristics including analysis of cellular lipids, carbohydrates, enzymes, cell proteins, cytoplasmic fibrils, metabolites, genome size, structure and base composition, restriction endonuclease analysis, restriction fragment length polymorphism, multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, DNA-DNA hybridization, arbitrarily primed polymerase chain reaction/randomly amplified polymorphic DNA fingerprinting, and gene sequences, including 16S rRNA sequence comparisons as discussed below. Details of most of these analyses and techniques in the molecular taxonomy and chemotaxonomy of spirochetes has recently been reported (Olsen *et al.*, 2000), and will not be presented here.

Description of Known Genera of Spirochetes

Species of *Treponema* are generally considered as anaerobic, host-associated spirochetes, some of which are pathogenic to man (Smibert 1984). The most notable species is *T. pallidum*, the causative agent of syphilis. Other putative pathogens include some species of oral treponemes; namely *T. denticola*, which has been associated with periodontal disease.

Species of *Spirochaeta* are facultatively anaerobic or obligately anaerobic, free-living spirochetes found in aquatic or marine environments (Canale-Parola 1984). Some have been observed from deep-sea sediments. The type species of the genus, *S. plicatilis*, cannot be grown in pure culture.

"*Spirochaeta culicis*", a new spirochete isolated from the mosquito, is the only species of the genus. Its pathogenicity is unknown. It has not yet been formally named.

Cristispira is a spirochete observed in the digestive tract of bivalves, especially the oyster. Noteworthy

characteristics include its large size (1-3 μm by 100 μm) and its rapid motility. Although it has been observed for over 100 years, it has not yet been grown in pure culture.

Species of *Borrelia* are microaerophilic, host-associated spirochetes that are transmitted to humans and animals via an insect vector (Wang *et al.*, 1999). *Borrelia burgdorferi* and closely related species are the etiological agents of Lyme disease. *Borrelia recurrentis* is the etiological agent of relapsing fever.

Brevinema andersonii, an infectious spirochete isolated from the blood and other tissues of short-tailed shrews and white-footed mice, is the only species of the genus (Defosse *et al.*, 1995).

Species of *Brachyspira* (*Serpulina*) are anaerobic spirochetes that are able to consume O_2 through NADH oxidase (Stanton *et al.*, 1999). Some species are pathogenic. *Brachyspira hyodysenteriae* is the etiological agent of swine dysentery, *B. pilosicoli* is the agent of porcine intestinal spirochetosis and may be pathogenic in man (Trott *et al.*, 1998), and *B. alvinipulli* is enteropathogenic for chickens (Stanton *et al.*, 1998).

The leptospire are obligately aerobic spirochetes, including saprophytic leptospire, such as *L. biflexa* and related species, and pathogenic leptospiras, such as *L. interrogans* and related species (Johnson and Faine, 1984). Leptospirosis is usually a water-borne, zoonotic disease transferred both by sexual and alimentary routes.

Phylogeny of Not-Yet-Cultivated Spirochetes

There are many spirochetes that still cannot be cultivated in vitro. The phylogenetic identity of these "uncultivable" microorganisms can be determined by sequencing cloned 16S rRNA genes that are amplified directly from environmental samples (Hugenholtz and Pace, 1996; Pace *et al.*, 1996). Similar procedures were used to determine the phylogenetic position of *Cristispira* (Paster *et al.*, 1996b), about 50 novel phylotypes of human oral treponemes (Choi *et al.*, 1994; Dewhirst *et al.*, 2000), and 21 novel phylotypes of *Treponema* in one termite species (Lilburn *et al.*, 1999). The phylogenetic position of representative phylotypes from these environments is shown in Figure 1.

One can appreciate the breadth of spirochetal biodiversity in the termite hindgut alone considering that there are over 2,000 species of termites. Similar levels of phylogenetic diversity were observed in five of the seven termite families studied (Lilburn *et al.*, 1999). However, none of the resultant clones was closely related to any previously recognized spirochetal species or phylotype (Berchtold *et al.*, 1994, Paster *et al.*, 1996a; Ohkuma *et al.*, 1999). Based on microscopic observations of other environments, such as aquatic and marine sediments, and the gastrointestinal tract of mammals, birds and insects, it is clear that there are a plethora of additional "uncultivable" spirochetes to be identified.

Perspective and Future Directions

It is important to determine the phylogenetic diversity of spirochetes in order to begin to assess their potential role and significance in the ecosystem. The existence of spirochetal species that cannot be presently cultivated should also be investigated, if only to determine whether cultivation techniques are adequate. Once a complete phylogenetic foundation is established, species-specific or even group-specific DNA probes based on 16S rRNA sequences can be used to rapidly determine the distribution of both cultivable and "uncultivable" species directly in environmental samples (Paster *et al.*, 1998). DNA probes could also be used in studies to follow the effect of nutritional and environmental perturbations on the population dynamics of spirochetes. Data obtained from such studies would help determine the role that spirochetes play in the microbial community and their effect on the environment.

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