

Structural and Genomic Features of Treponemal Architecture

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Abstract

Treponema denticola and *Treponema phagedenis* are important models for deciphering the unique architectural and genetic features of treponemes. Darkfield and electron microscopy have delineated the general structure of treponemes as deduced from these two dimensional techniques. Advances in genetic tools and three dimensional visualization techniques are now linking cell architecture, cell ultrastructures, and genetic data. Ultrastructures are multi-component assemblies within the cell that drive specific functions critical to the cell such as cell division, cell shape and motility. A key treponemal ultrastructure is the cytoplasmic filament ribbon which is involved in the cell division process. Recently, application of new methods to the structural analysis of *T. phagedenis* has revealed novel features of the cytoplasmic filament ribbon. The observed cytoplasmic filament ribbon actually consists of independent filaments connected to each other via bridging components and anchored to the inner membrane. The nature and components of this macromolecular complex are discussed as well as the novelty of the filamentous ribbon structure. The availability of whole genome sequences from two treponemal species also allows the comparative study with other bacterial cytoskeleton-associated structures towards achieving a global picture of spirochetal cell biology.

1. Introduction

Organisms in the genus *Treponema* are prominent members of the Spirochaetaceae, a family that is characterized by a unique cell architecture (Holt et al., 1994; Holt, 1978; Hovind-Hougen, 1972; 1974; 1976; Hovind-Hougen and Birch-Andersen, 1971; Hovind-Hougen et al., 1976). All treponemes have an outer membrane, an inner membrane and a characteristic number of flagellar filaments located in the periplasmic space (**Figure 1**). This basic arrangement is a defining feature of the

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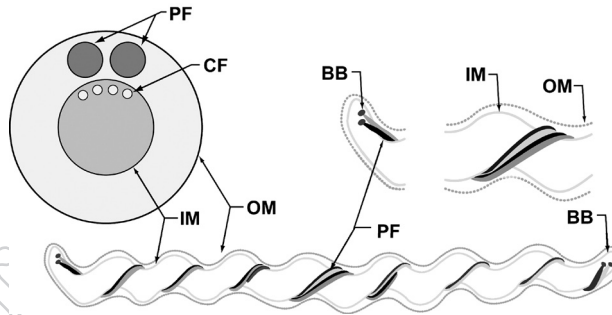


Figure 1. Diagrammatic representation of a *T. denticola* cell, showing the periplasmic location of the flagellar filaments. Also shown is an enlarged region (top right) as well as a cross-sectional view (top left). Although the size of *T. denticola* varies, a typical cell is 0.2 μm in diameter by 6 μm in length. PF= periplasmic flagella, CF=cytoplasmic filaments, BB= basal body, IM= inner membrane, OM= outer membrane. This figure is adapted from Limberger (2004) courtesy of S. Karger AG, Basel.

spirochetes (Smibert, 1984). The overall shape of treponemes appears as coiled, wavelike, helical, or serpentine although the exact structure is difficult to ascertain when viewed under a darkfield microscope. Closer examination of the cell structure reveals the variety of treponemal shapes. Within the treponeme group can be found cells that have a regular helical shape, cells that have irregularly shaped helices, and cells with a flat-wave morphology of the cell cylinder (Charon et al., 1991; 1992b; Charon and Goldstein, 2002; Cox, 1972; Ruby et al., 1997).

Although the coiled or wave-like shape and the periplasmic location of the flagellar filaments define the *Treponema* as spirochetes, the genetic and structural factors that contribute to these distinctive features have not been elucidated. However, recent advances in manipulating spirochete genes and in three-dimensional imaging capabilities have shed new light on treponemal structure. Via the former, creation of stable directed mutants in *Treponema denticola* has become an established technique. More recently, new shuttle plasmids have been developed, thus enabling complementation studies of these mutants. Genetic regulation, as well as structural and functional domain analysis, is now possible using these plasmids (Chi et al., 2002; Slivinski-Gebhardt et al., 2004). The new shuttle plasmids also enable the heterologous expression of treponemal genes, which in turn permits cross-species functionality to be assessed (Chi et al., 1999; Slivinski-Gebhardt et al., 2004). Application of electron tomography has allowed us to study cellular structures in three dimensions, thus providing new insights into treponemal molecular architecture (Izard et al., 2004).

The structure and shape of the treponemal cell are driven by their ultrastructural, multicomponent assemblies. These drive, maintain, or assist in specific functions in the cell and may extend through large regions of the cell. Such structures in *Treponema* include the flagella, the cytoplasmic filaments, the cell division septation ring, the filaments associated with rod cell-shape maintenance, and the peptidoglycan layer. This chapter will describe the contributions of these structures to the distinctive architecture of the *Treponema* and how this architecture determines their cell biology and pathogenicity.

2. Treponemal architecture

We will review the overall structure of this group of bacteria and how the individual components influence the shape of the *Treponema*. Because treponemal cell structure has been extensively detailed in other publications, we will provide only a brief summarization of the major components of the cell (Holt et al., 1994; Holt, 1978; Hovind-Hougen, 1972; 1974; 1976; Hovind-Hougen and Birch-Andersen, 1971; Hovind-Hougen et al., 1976) before focusing on the influence of these structures on cell function.

A typical treponemal cell ranges between 5 and 20 μm in length and between 0.1 and 0.5 μm in diameter, depending on the species. It possesses an outer membrane, a periplasmic space in which the flagellar filaments reside, a peptidoglycan layer, and an inner membrane. The above information was obtained by electron microscopic analysis of fixed and stained cells. New approaches using flash-frozen hydrated unstained cells are resolving those structures in a “caught in the action” state (J. Izard and C.E. Hsieh, unpublished data). This approach combined with tomographic methods can reveal the three dimensional architecture of the treponemal cell without the artifacts due to staining and processing for traditional electron microscopy (Baumeister, 2002; Grimm et al., 1998; McEwen and Frank, 2001).

All natural treponemal cells are flagellated. The number of flagella varies by species but typically ranges from 1 to 8. The number of flagella at each cell end in conjunction with cell length, diameter and wavelength, have been proposed as classification and identification criteria (Hovind-Hougen, 1976). The flagella are inserted subterminally at each end of the cell. A given cell usually contains similar numbers of flagellar filaments at each cell end. The flagellar filaments wind around the cell body toward the center of the cell and may or may not overlap with one another in the center. The overall diameter of a filament is usually within the range of 16-25 nm. The typical treponemal filament consists of multiple FlaB polypeptide species which constitute the core filament, and one or two FlaA polypeptides, which make up the filament sheath. The flagellar basal body shows a general morphological similarity to those of other bacteria, although the *Treponema* possess one fewer ring structure due to the periplasmic location of the flagellar filaments. Accordingly, there is no ring creating an anchor in the outer membrane. More detailed analyses of flagellar structure can be found elsewhere in this book as well as in several reviews (Canale-Parola, 1984a; Charon and Goldstein, 2002; Li et al., 2000; Limberger, 2004; Penn et al., 1985; Penn and Luke, 1992). The polypeptides that have been identified as important for the motility of enteric bacteria have identifiable homologues in the treponemes. However, there are additional spirochete-specific polypeptides important for these organisms' unique motility (Limberger et al., 1996).

When observed by darkfield light microscopy or electron microscopy, the treponemal cell appears spiral in shape. Some treponemes, like *T. phagedenis*, possess regular helical waves of consistent amplitude and pitch except at the cell ends which are bent or distorted (Charon et al., 1991). Others, like *T. denticola*, are more irregular in shape, yet still possess primarily a helical form (Ruby et al., 1997). The structure of *T. pallidum* is less clear. Earlier studies of slowly rotating cells by darkfield microscopy have suggested that *T. pallidum* is primarily planar in nature

(Cox, 1972). Subsequently, scanning electron microscopy analysis has suggested that *T. pallidum* is predominantly a left-handed helix (Stepan and Johnson, 1981). In clinical samples, as well as under certain conditions in cell culture, treponemes can assume a spherical shape by association of each cell end to ultimately form a spherical body. However, the formation and significance of these spherical bodies are unknown and remain part of the historical mystique of the biology of treponemes (Cox, 1972; Hampp, 1951). Because *T. pallidum* cannot be manipulated genetically in order to generate specific mutants, it is unknown whether a non-flagellated strain of *T. pallidum* would retain its flat-waved or helical shape. Further work using high resolution microscopic techniques is needed to elucidate the precise structure of *T. pallidum*. As pointed out in the Chapter 6 (Charon et al.) many spirochetes are able to assume different morphological shapes because of the flexible cell cylinder and the influence of the periplasmic flagellum.

The distortions observed at the cell ends of treponemes are due to the exertion of torsional stress by the periplasmic flagella. Non-flagellated mutants of *Treponema* species retain their helical shape and are much more regular at the cell ends (Charon et al., 1991; Ruby et al., 1997). One striking difference between *Borrelia burgdorferi* and the *Treponema* spp. is that, in the absence of flagella, *B. burgdorferi* becomes a straight rod-shaped bacterium (Motaleb et al., 2000; Sadziene et al., 1991). The presence of the flagellar filament in *B. burgdorferi* is directly related to the maintenance of the helical shape, which in this organism is independent of the membrane potential (Motaleb et al., 2000). In the *Treponema*, in the absence of flagellar filaments, there is maintenance of the overall cell morphology. However, there is a loss of the hook shaped end in flagellar filament deficient mutants of *T. phagedenis* (Izard et al., 1999; Limberger and Charon, 1986), and the formation of a more regular helical cell shape in *T. denticola* (Limberger et al., 1999; Ruby et al., 1997); these features are the consequence of the release of tension, modifying the well-defined helical shape of the bacterium.

Treponemal cells possess internal cytoplasmic filaments that consist of one major polypeptide, CfpA. These filaments are smaller in cross-section (5.0 x 6.0 nm) than the flagellar filaments and are found throughout the length of the cell. Their exact function is unknown, but recent evidence suggests that they are involved in the cell-division processes (Izard et al., 2001). The number of cytoplasmic filaments per cell varies by species but it usually is in the range of 4-12 (Izard et al., 1999). In cross sections of the treponemal cell, they are very difficult to discern clearly. They are best visualized by solubilization of the outer membrane followed by negative staining and examination of the entire cell length. Mutants that lack cytoplasmic filaments retain the normal cell shape. The cytoplasmic filaments will be described in detail later in this chapter.

3. Periplasmic flagella, outer membrane, and cell wall

The best-studied structures of the *Treponema* have been the periplasmic flagella (Figure 1). These immunogenic structures are easy to visualize by electron microscopy as well as by high resolution darkfield microscopy. They are uniquely confined to the periplasm, although they can protrude from the cell in some species, under certain circumstances (Charon et al., 1992a). Despite this protrusion, the

flagellar filaments remain covered by what appears to be the outer membrane. The physiological significance of this protrusion is unclear. The details of the flagellar structure are described by our colleagues in this book (See Chapter 6, Charon et al.). The dynamics of the flagellar activities within the periplasm are unknown, but this surely must be a region of high activity given the vigorous rotation of the flagellar filaments within this narrow region.

The outer membrane of the *Treponema* has also been extensively studied using a variety of techniques. The protein components of the membrane are being identified, and their relation to host immune system are being deciphered (Wang et al., 2001). The lipid, glycolipid, and lipoprotein content of treponemal membranes are also under investigation (Haake, 2000; Kesavalu et al., 2002; Martinez-Morales et al., 2003). The details of these outer membrane analyses are presented elsewhere in this book (See Chapter 14, Holt and Ebersole and Chapter 11, Cameron).

The peptidoglycan layer of treponemal cells has not been sufficiently studied (Caimano et al., 1999; Morioka et al., 1979; Umemoto et al., 1981; Yanagihara et al., 1984). It is clear that treponemes do possess a cell wall that is at least partly responsible for maintenance of the cell shape. Analysis of the treponemal cell wall by electron microscopy has revealed an electron-dense layer of about 5 nm in thickness. The major constituents are glucosamine, muramic acid, D-glutamic acid, L- and D-alanine, ornithine, and glycine. In addition, whole-genome analysis has revealed that most of the genes necessary for the protein and sugar moieties of the peptidoglycan formation are present in *Treponema* (Fraser et al., 1998; Seshadri et al., 2004).

4. A Bacterial cytoskeleton

Traditionally, the term cytoskeleton has been limited to eukaryotic cells. However, given the large number of genomes now sequenced, the analysis of sequence similarity, and the in-depth study of molecular systems in certain organisms, it is now clear that structural and functional homologues of tubulin (FtsZ) and actin (MreB) are present virtually throughout the eubacteria as well as in some *Archaea*. Recent data show that these filaments are dynamic structures in both prokaryotes and eukaryotes.

The cytoskeleton in eukaryotes is generally accepted to be a protein framework within the cytoplasm of the cell. This framework is composed of protein complexes that each have a filament as a central component: actin microfilaments, microtubules composed of tubulin filaments, and intermediate filaments. These filaments are involved in a variety of functions, from providing support for cell extensions such as the small intestinal villi or the axons of neurons, to participating in cell motility and a large array of internal cell functions such as cell division.

The dynamic aspect of the bacterial cytoskeleton has not yet been demonstrated experimentally in spirochetes. However, the evidence for the presence of the septation ring (including FtsZ), and the shape driving filaments (including MreB) has been indirectly documented for years through electron microscopy data and more recently from genomic sequence data. We will describe, in the following paragraphs some of the features of the spirochetal cytoskeleton.

5. The cytoplasmic filament ribbon of *Treponema*

The cytoplasmic filament of *Treponema* is a ribbon-like structure (**Figure 2**) composed of two to six filaments (Holt, 1978; Hovind-Hougen, 1976; Izard et al., 1999). Other cytoplasmic filaments have been described in other spirochete genera. Electron microscopic evidence for this structure has been published for the following genera: *Leptonema*, *Spirochaeta*, *Pillotina*, *Hollandina*, and *Diplocalyx* (Bermudes et al., 1988; 1987; Hollande et al., 1967; Holt, 1978; Hovind-Hougen, 1979).

Electron microscopy has provided a wealth of information about the cytoplasmic filaments. Treponemal cytoplasmic filaments are located beneath the flagellar filament bundle (Hovind-Hougen, 1972; 1974; 1976; Ovcinnikov and Delektorskij, 1970), in close apposition to the cytoplasmic membrane (Eipert and Black, 1979; Hovind-Hougen, 1974; Hovind-Hougen and Birch-Andersen, 1971). The filament bundle is helical inside the cell, and the periodicity of the helix is equivalent to the cell's helical periodicity (Eipert and Black, 1979; Zemper and Black, 1978). The bundle spans the length of the cell, is not transitory, and is detectable at all steps of the cell cycle (Izard et al., 1999). During cell division, the filaments are severed. Roughly half of the length remains in each daughter cell (Eipert and Black, 1979; Izard et al., 1999).

Masuda and Kawata were the first to purify the cytoplasmic filaments of various strains of treponemes. The major constituent of *T. denticola*, *T. phagedenis*, and *T. pallidum* cytoplasmic filaments is an 82-kDa protein (Izard et al., 1999; Masuda and Kawata, 1989; You et al., 1996). Using protein sequencing, PCR, and library hybridization, You and co-workers were able to identify the open reading frame in the *T. pallidum* subsp. *pallidum* genome (Fraser et al., 1998; You et al., 1996). The gene product was named cytoplasmic filament protein A (CfpA). The nucleotide and amino-acid sequences are well conserved among *Treponema* species (Izard et al., 1999). The alignment of *cfpA* nucleotide sequences from *T. denticola*, *T. vincentii*, *T. phagedenis* and *T. pallidum* subsp. *pallidum* and *pertenue* reveals several gaps, from 3 to 15 nucleotides long, at the 3' end of the sequences but no frameshifts, and no mutational hot spots. The amino-acid sequence identity of

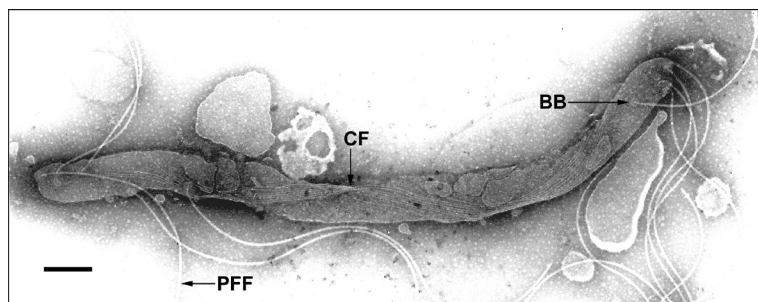


Figure 2. The cytoplasmic filament (CF) ribbon spans the entire cytoplasmic compartment. After removal of the outer membrane, the flagellar filaments are liberated and three helical turns of the cytoplasmic filament ribbon can be seen. *T. phagedenis* cells were stripped of their outer membrane, negatively stained with sodium phosphotungstate, and visualized in the electron microscope. (Izard et al., 1999). BB, flagellar basal body. Scale bar, 250 nm. Photo is reprinted courtesy of ASM Press.

CfpA among the above treponemes ranges from 83 to 100% (Izard et al., 1999). The high levels of sequence similarity are independent of the habitat or mode of transmission of the organism, or the cultivability of the organism in the laboratory. Analysis of the nucleotide and amino-acid sequences of the various cytoplasmic filament core constituents using bioinformatics has failed to identify the function of these structures.

Expression of the *cfpA* gene is regulated by a σ^{70} recognition sequence (Izard et al., 1999; You et al., 1996). The *T. phagedenis cfpA* σ^{70} promoter differs by only one nucleotide from the *Escherichia coli* consensus σ^{70} sequence (Hawley and McClure, 1983). A similar recognition sequence is used by sigma A (σ^{43}) in *Bacillus subtilis* (Graves and Rabinowitz, 1986; Moran et al., 1982). Little is known about the temporal regulation of genes in *Treponema* (Indest et al., 2000). The expression of *cfpA* is expected to be constitutive. During experimental rabbit infection by *T. pallidum* sp. *pallidum*, *cfpA* is one of the ten most highly transcribed genes (Smajs et al., 2005). This analysis of the *T. pallidum* transcriptome underscores the importance of the cytoplasmic filament ribbon during the infection process.

What is the function of the filamentous cytoplasmic filament bundle? The shape and location of the bundle suggest it is involved in cell structure and shape maintenance, transport, cell motility, and/or cell division. Analysis of the predicted amino-acid sequence of CfpA does not reveal any similarity with proteins of known function, or with any putative protein domain(s). To achieve a better understanding of the relation between cell function and the filamentous structure, we generated a knockout mutant of *cfpA* in *T. denticola* (Izard et al., 2001). The insertional inactivation of *cfpA* was not lethal for the cell. The *cfpA* mutant predominantly forms long chains of cells (Izard et al., 2001). Each such chain is composed of cytoplasmic cylinders of normal length under a single shared outer membrane. Each cytoplasmic cylinder is independent, as determined by electron and fluorescence microscopy. No alteration of the cell shape or structure was observed in the mutant strain (Izard et al., 2001). Consequently, the hypothesis that the cytoplasmic filament ribbon is involved in the maintenance of cell structure and shape could be eliminated. The most probable candidate for such a function is the filament structure composed of MreB, as described later in this chapter.

By dark-field microscopy, it is possible to observe the asynchronous gyration of cell ends from two individual cytoplasmic cylinders facing one another. During the rare event when a single *cfpA* mutant cell of wild-type size was observed, no obvious differences from wild-type movement could be observed, in either liquid or dense medium at 36°C (1% methylcellulose, 15 centipoises). We concluded that motility was not altered at the single cell level. The macroscopic (on agarose plates) and microscopic (in methylcellulose solutions) alteration of motility compared to the wild type could be explained by the asynchronous crankshaft-like movement of the cell. Such a phenomenon can be observed in wild-type cells in early log phase, when they form a long chain. At that time, the rate of cytoplasmic cylinder formation with complete cylinder septation is greater than the rate of the cytoplasmic cylinder detachment (cytokinesis). In the *cfpA* mutant, however, over 90% of the cell population is composed of cells having more than two cytoplasmic cylinders at all stages of cell growth. Therefore, it appears that the cytoplasmic filaments have no direct involvement in treponemal motility, despite their proximity to the flagellar filaments.

The involvement of the cytoplasmic filaments in cell division was revealed when the chromosomal DNA of the CfpA-deficient mutant was visualized using fluorescent dyes (Izard et al., 2001). In wild-type cells, the chromosomal DNA is uniformly distributed throughout the cytoplasmic cylinder (Izard et al., 2001). However, in the CfpA-deficient mutant, the DNA is condensed in distinct areas, and there is an increase in the number of anucleate cells (Izard et al., 2001). As a result of this work, we have hypothesized that the cytoplasmic filaments are involved in the cell-division process, chromosome segregation, or DNA structure localization and maintenance. At present, it is unclear how the filaments would interact with the chromosomal DNA. The evidence for active chromosome segregation after duplication seems unambiguous, even though the model of duplication and separation of the origin of replication region (*oriC*) and the termination region (*ter*) differs for *E. coli* and *B. subtilis* (Glaser et al., 1997; Jensen and Shapiro, 1999; Niki and Hiraga, 1998; Sharpe and Errington, 1999; Webb et al., 1998). The spatial and temporal mechanisms that control and direct *oriC* are unknown. However, it is likely that a hypothetical spindle or a mitotic-like apparatus and “motor” proteins are involved in this process (Sharpe and Errington, 1999). The involvement of the cytoplasmic filaments in cell-division processes and their relationship to the chromosomal DNA are under investigation.

The three-dimensional structure of the cytoplasmic ribbon has been revealed using electron tomography (Izard et al., 2004). As in medical imaging applications, a three-dimensional volume is reconstructed from a series of two-dimensional images of a given structure and recorded at various tilt angles (Crowther et al., 1970; McEwen and Marko, 1999). The advantage of this technique is that it enables one to obtain three-dimensional reconstructions of complex and irregularly shaped structures at moderately high resolution. We used negatively stained *T. phagedenis* cells that had been stripped of their outer membrane to visualize the cytoplasmic filaments. A series of tilt images were recorded in the electron microscope with a 1.5 degree angular interval, over a +/- 60 degree angular range. After alignment of the successive images, a three-dimensional volume was calculated using Fourier transforms (SPIDER program) by weighted back projection methods (Frank et al., 1996). The result of such calculations can be seen as an added feature on the Molecular Microbiology website (Izard et al., 2004). After tracing the different volumes, it was possible to analyze each structure independently (**Figure 3**).

The novelty of the organization of the cytoplasmic filaments resides in their unique molecular arrangement. Upon analysis of the tomographic reconstructions, we learned that the filaments are not in contact with one another, *i.e.* there is no interfacial connection. Each filament is composed of one protein, CfpA (Izard et al., 1999; Masuda and Kawata, 1989; You et al., 1996). We were able to provide more accurate measurements of the filaments: the cross-section is $5.0 \times 6.0 \text{ nm} \pm 0.5 \text{ nm}$ (horizontal x vertical, or width x depth) (Izard et al., 2004). Their length is the same as the cell length (Izard et al., 1999). The center-to-center spacing is $10.4 \text{ nm} \pm 1.8 \text{ nm}$. The large standard deviation indicates that there is some variability in the distance between two filaments, even though long stretches of the ribbon have been observed with a uniform spacing.

Another novel feature is that cross-bridging protein(s) are located on the cytoplasmic side of the filament ribbon. They are probably involved in filament

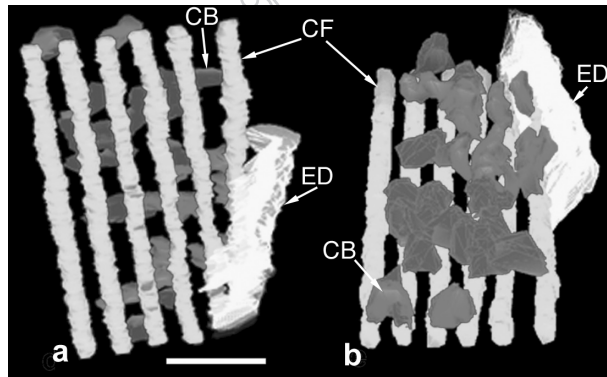


Figure 3. Reconstruction of the treponemal cytoplasmic filaments and filament bridging structures. For simplicity, six filaments were initially traced from axial views over a limited distance. A. Viewed from the periplasmic side. B. Viewed from the opposite side (cytoplasmic side). Note that the connections form an irregular lattice on one side of the filament band. CF, cytoplasmic filaments; ED, unidentified electron-dense material; CB, cross-bridging protein(s). Scale bar, 25 nm. Photo is modified from Izard et al. (2004), courtesy of Blackwell Publishing.

spacing, maintaining the structural integrity of the filament ribbon, and they likely interact with other cytoplasmic components associated with the functional ribbon. The variation in the center-to-center spacing of the filaments could arise from the loss of bridging components during sample preparation; such a loss would impart a greater flexibility to the filament organization.

Finally, anchoring protein(s) are located on the periplasmic side of the cytoplasmic filaments. They are associated with the cytoplasmic membrane, creating a stable link between the membrane and the ribbon of filaments. The anchoring protein may be a lipoprotein or an integral membrane protein. The membrane-connector width is approximately the same as the width of the filaments.

On the basis of these data, we could estimate the extent of the coverage of the cytoplasmic surface of the inner membrane by the cytoplasmic filament ribbon. For *T. phagedenis*, based on the center-to-center spacing of the filaments, the horizontal cross-section of the filaments, and an estimated cytoplasmic cylinder diameter of 200 nm, an estimated 3-18% (average, 7-9%) of the surface is covered (Izard et al., 2004). The membrane-associated region of the cytoplasmic filament structure occupies a significant space proximal to the inner cell membrane. Although, the abolition of the cytoplasmic filament structure by gene knockout is not lethal, cell growth, motility and cell division processes are profoundly altered, thus highlighting the physiological importance of such a structure (Izard et al., 2001).

Study of the cytoplasmic filaments and their protein partners will provide critical data on cell division processes and maintenance of chromosome structure.

6. Cell septation and filamentous structures in *Treponema*

On the basis of our work (Izard et al., 1999) (Izard J., Samsonoff W. A., Slivinski-Gebhardt L. L., and Limberger R. J., unpublished data) and data published by other groups on *Treponema* (Hovind-Hougen et al., 1976; Izard et al., 1999; Listgarten and Socransky, 1964; Ryter and Pillot, 1963), and on other spirochetes (Holt, 1978;

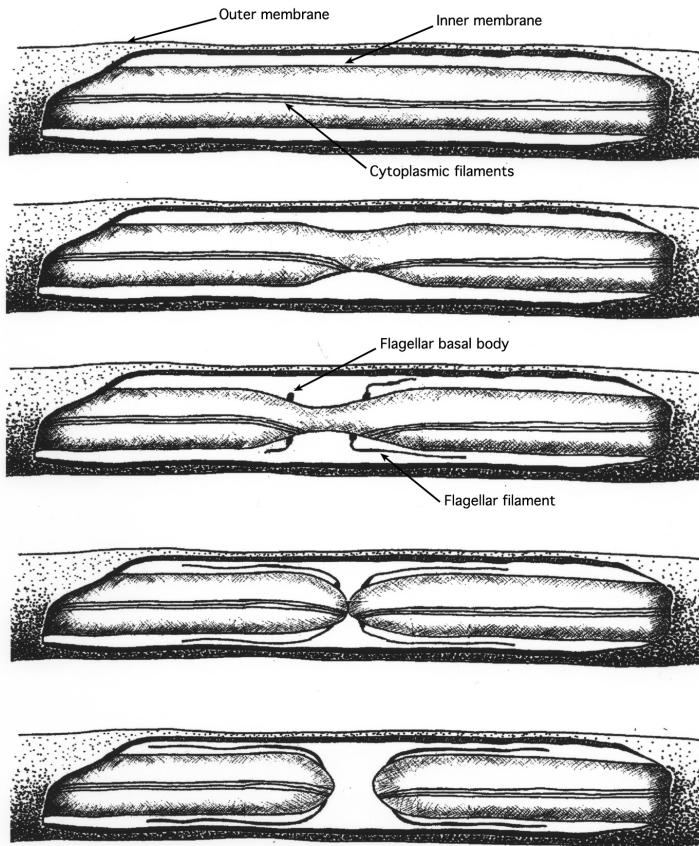


Figure 4. Temporal model of treponemal cytokinesis showing the severing of the cytoplasmic filament bundle and the formation of the flagellar apparatus. This cut-away view of the model is based on published observations (Izard et al., 1999) of treponemal cells undergoing cell division, using *T. phagedenis* cells stripped of their outer membrane, negatively stained with sodium phosphotungstate, and visualized in the electron microscope. The actual attachment site of the cytoplasmic filaments to the cell body is unclear.

Hughes et al., 1975; Nauman et al., 1969; Ritchie and Ellinghausen, 1965), we can establish a probable sequence of events triggered by the start of the cell division cycle, and ending with the separation of two individual cells. The model is shown in **Figure 4**.

During the cell cycle, the cytoplasmic cylinder extends to about twice the length of a single cell, and a septum of cell division becomes positioned at the mid-cell region. During or before the positioning and formation of the FtsZ ring, the cytoplasmic filaments are severed, and their ends are anchored into the membrane. When the septum of cell division is viewed under the microscope, two independent bundles of filaments can be observed on each side. The number of filaments is the same on either side of the septum. At this stage, a nascent flagellar apparatus can be observed, with a basal body, a hook, and a short flagellar filament. While the septum is constricting, the flagellar filaments are elongating. When the septum

of cell division is closed and the two cytoplasmic cylinders are separated under a common outer membrane, the flagellar filaments may continue to elongate. The final step is the separation of the two cells.

We hypothesize that the filaments within the ribbons are cut prior to the formation of the FtsZ ring. It is expected that a dedicated machinery exists to sever the cytoplasmic filament bundle and to anchor them on both sides of the septum of cell division. In the case of Mbl filaments, in *B. subtilis*, experiments have shown that the septum of division is required for the severing of the filaments to occur. The severing occurs at a very late stage of septum constriction (Carballido-Lopez and Errington, 2003a). How the treponemal cytoplasmic filaments are anchored to the inner cell membrane, and how they are severed, are remaining questions to be addressed experimentally.

7. The septation ring and FtsZ, a tubulin homologue

Treponema cells divide symmetrically during normal growth, constricting in the middle. The cytokinesis is a constriction process, as in *E. coli*. It involves the outer membrane, the peptidoglycan layer, and the inner membrane. Rupture of the outer membrane is the final event occurring in cytokinesis. In *Treponema*, during the early and log phases of bacterial growth, it is common to observe cells with two independent cytoplasmic cylinders that are rotating, showing the autonomy of the motility apparatus of each cytoplasmic cylinder, even though they are located under the same outer membrane. Before that step, the periplasmic peptidoglycan layer will have been broken down and reconstituted, providing each daughter cell with its own exoskeleton. The first step of cytokinesis is the formation of a constriction ring.

The closest bacterial model that may help to understand *Treponema* cytokinesis is *E. coli*. Both stain as Gram-negative (Canale-Parola, 1984b). They have similar membrane-periplasm-membrane-cytoplasm organizations, the major difference being the periplasmic location of the flagellar filaments in spirochetes (Holt, 1978). They are relatively distant from one another phylogenetically (Paster et al., 1991). Numerous proteins are associated with the cell division processes, a subset is associated with cytokinesis, and a group is involved in forming a filamentous structure at the septum of cell division. The key component of such a filamentous structure is FtsZ (Bi and Lutkenhaus, 1991). The FtsZ protein and associated components form the divisome (Nanninga, 2001). The divisome's functions are integrated in the regulatory network of the overall cell division processes; consequently alterations to the divisome will alter other cell division-related events. In addition to its constriction of the septum, the divisome has another critical function: it positions a specialized penicillin-binding protein required for synthesis of the wall material at the new cell poles.

FtsZ, the first component of the septum ring, is required for the targeting of other proteins to the cell division site. It can be found in virtually all eubacteria, in most archaea and in organelles of many eukaryotes (Beech et al., 2000; Kiessling et al., 2000; Margolin, 2000). *In vitro* FtsZ is able to form various types of filaments (Bramhill and Thompson, 1994; Erickson et al., 1996; Romberg et al., 2001). The structure of FtsZ is similar to that of tubulin (Lowe and Amos, 1998). The ring seems to be highly dynamic, with the exchange between the cytosol and the filament

Table 1. Proteins located at the septum of division and associated with the formation of the septal ring¹

Genome	<i>Treponema pallidum</i> ²	<i>Treponema denticola</i> ³	<i>Borrelia burgdorferi</i> ⁴	<i>Leptospira interrogans</i> serovar <i>Lai</i> ⁵	<i>Escherichia coli</i> ⁶	<i>Bacillus subtilis</i> ⁷
Gene Name						
<i>ftsZ</i>	Yes	Yes	Yes	Yes (chr I)	Yes	Yes
<i>ftsA</i>	Yes	Yes	Yes	Yes (chr I)	Yes	Yes
<i>zipA</i>	No	No	No	No	Yes	No
<i>ftsE</i>	No	No	No	No	Yes	Yes
<i>ftsX</i>	No	No	No	No	Yes	Yes
<i>ftsK</i>	Yes	Yes	Yes	Yes (chr I)	Yes	No
<i>ftsQ</i>	Yes	Yes	No	Yes (chr I)	Yes	No
<i>ftsL</i>	No	Yes	No	No	Yes	Yes
<i>ygbQ</i>	No	No	No	No	Yes	No
<i>ftsW</i>	Yes	Yes	Yes	Yes (chr I)	Yes	Yes
<i>ftsI</i>	Yes	Yes	Yes	Yes (chr I)	Yes	Yes
<i>ftsN</i>	No	No	No	No	Yes	No
<i>AmiC</i>	No	No	No	Yes (chr I)	Yes	No
<i>divIB</i>	No	No	Yes	No	No	Yes
<i>divIC</i>	No	No	No	No	No	Yes

¹The protein list is based on published literature (Errington et al., 2003; Schmidt et al., 2004), and the following Web databases: the STD sequence database for *T. pallidum* (www.stdgen.lanl.gov), the Baylor College of Medicine database for *T. denticola* (www.hgsc.bcm.tmc.edu/microbial/Tdenticola/), and from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

²Fraser et al. 1998.

³Seshadra et al. 2004.

⁴Fraser et al. 1997

⁵Ren et al. 2003.

⁶Blattner et al. 1997.

⁷Kunst et al. 1997.

(chr I): gene located on chromosome I.

closely apposed to the inner surface of the cytoplasmic membrane (Stricker et al., 2002). The mechanism of constriction has not yet been resolved, and models need to be tested *in vivo* and *in vitro* (Bramhill, 1997; Gonzalez et al., 2003; Romberg and Levin, 2003). However, the closure of the gap between the two new cells does not require a complete closure of the FtsZ ring (Addinall and Lutkenhaus, 1996).

The proteins associated with the FtsZ ring are listed in **Table 1**. In *E. coli*, the pathway for assembly is linear: FtsZ → [ZipA, FtsA] → [FtsE, FtsX] → FtsK → FtsQ → [FtsL, YgbQ] → FtsW → FtsI → FtsN → AmiC (Bernhardt and de Boer, 2003; Errington et al., 2003; Schmidt et al., 2004). FtsA is in contact with the FtsZ monomers, and their ratio is important for correct cytokinesis (Dewar et al., 1992). The primary sequence and the crystal structure both show similarities to actin (Bork et al., 1992; Van den Ent et al., 2001); however, *in vitro* polymerization

Table 2. Presence of *mreB* and *mreB*-like (*mbl*, *mreBH*) genes in different bacteria, including spirochete genomes¹

Genome	<i>Treponema pallidum</i>	<i>Treponema denticola</i>	<i>Borrelia burgdorferi</i> ²	<i>Leptospira interrogans</i> serovar <i>Lai</i>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>
Gene Name						
<i>mreB</i>	Yes	Yes	Yes (chr) ³	Yes (chr I) ⁴	Yes	Yes
<i>mbl</i>	No	No	No	No	No	Yes
<i>mreBH</i>	No	No	No	No	No	Yes

¹The data were collected from the STD sequence database for *T. pallidum* (www.stdgen.lanl.gov), the Baylor College of Medicine database for *T. denticola* (www.hgsc.bcm.tmc.edu/microbial/Tdenticola/), and the database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

²*B. burgdorferi* has a gene annotated *mreB-2*, which is a true homologue of *rodA*.

³(chr): present on the chromosome.

⁴(chr I): present on chromosome I.

of FtsA has not been achieved. FtsA is a member of the actin/Hsp70/sugar kinase ATPase superfamily, like another protein present in spirochetes, MreB (see below) (Table 2) (Bork et al., 1992). ZipA in *E. coli* is suspected to be the anchor to the membrane of the FtsZ ring. The *zipA* gene is not present in any spirochete genome available for searches. Spirochetes may use an alternative protein, like EzrA or ZapA, to perform such a function (Gueiros-Filho and Losick, 2002; Levin et al., 2001). With the exception of FtsI, none of the other proteins yet has a precisely delineated function. All are transmembrane proteins. FtsK is present in all spirochetes (see Table 1). FtsQ is present in *Treponema*. *B. burgdorferi* most probably uses DivIB, the homologue of FtsQ in *B. subtilis* (Harry et al., 1994). *Leptospira interrogans*, has no homologues for these two proteins. FtsL and its functional homologues DivC and YgbQ are absent from most spirochetes except *T. denticola*. FtsW is present in all spirochetes. FtsW is part of the SEDS family of proteins (shape, elongation, division and sporulation) that includes RodA and SpoVE. The latter has a specialized role in sporulation in *B. subtilis* (Henriques et al., 1998; Ikeda et al., 1989). Septation requires a dedicated penicillin-binding protein (FtsI or PBP-3 in *E. coli*), which is also present in all spirochetes. FtsN is a poorly conserved protein, with clear homologues in enteric bacteria and in *Haemophilus* species. (Dai et al., 1996).

In the case of FtsN and other proteins involved in cell division, the absence of a detectable homologue may be due to a poor conservation of the protein, the functional replacement by another protein, or compensatory mutations that eliminate the requirement for such a protein. Poor sequence similarity has complicated the naming and functional assignments of certain genes (Limberger et al., 1999). Detailed analysis of *in vivo* mechanisms is required to decipher such issues. Although the overall mechanism of cell division is similar, specific differences can be observed among spirochetes. However, insufficient genomic information is available to understand the roots of those differences.

8. Cell shape and MreB, an actin homologue

The shape of a bacterium is a stable characteristic that has been used extensively as a taxonomic criterion. For the spirochetes, cell shape is a crucial component of their mode of propagation. The helical nature of most spirochetal cells is evident from microscopic observations. The maintenance of such a shape and the presence of periplasmic flagellar filaments together form the basis of the ability of spirochetes to penetrate dense media (Klitorinos et al., 1993; Ruby and Charon, 1998), or cell monolayers and tissues, as demonstrated *in vitro* (Lux et al., 2001; Thomas et al., 1988).

In straight-rod-shaped bacteria like *E. coli* and *B. subtilis*, a number of mutants with spherical morphology have been isolated. The mutations are known to affect various cell processes including their association with the peptidoglycan layer (*pbpA*, *rodA*) (Henriques et al., 1998; Spratt, 1975; Tamaki et al., 1980), their participation in teichoic acid synthesis (*rodC*) (Honeyman and Stewart, 1989); or their direct involvement in cell shape control (*mreB*, and *mbl*) (Abhayawardhane and Stewart, 1995; Levin et al., 1992; Wachi et al., 1987). MreB (present in all spirochetes; Table 2) and Mbl (MreB-like) proteins in *B. subtilis* have been shown to form filamentous helical structures in the cytoplasm of the bacteria. A second homologous gene to *mreB* in *B. subtilis* is *mreBH*, that can be disrupted with no obvious phenotypic consequence (Carballido-Lopez and Errington, 2003b). In *B. subtilis*, MreB is essential for viability (Abhayawardhane and Stewart, 1995; Jones et al., 2001). In *E. coli*, the inactivation of the *mreB* gene results in spherical cells (Wachi et al., 1987). In both organisms, the filaments extend for the length of the cell (Jones et al., 2001; Shih et al., 2003). With a few exceptions, MreB orthologues are present only in species with rod- or helical-shaped cells (Daniel and Errington, 2003) (Table 2); they are usually absent in species with spherical-shaped cells (Daniel and Errington, 2003).

The first approach to an understanding of the mechanisms beyond filament formation that maintain the cell shape was to create mutations of MreB in *B. subtilis* (Jones et al., 2001; Wachi et al., 1987). The filaments control the cell length and the width of the bacterium. The first capability is linked to the ability of the cell to divide in such a way to produce identical daughter cells. The second capability is also tightly controlled (Sharpe et al., 1998; Woldringh et al., 1985). The crystallization of the MreB protein from *Thermotoga maritima* brought a new perspective on the system (Van den Ent et al., 2001). MreB and actin have highly similar three-dimensional folds. The crystallization revealed an actin-like strand at atomic resolution, thus elucidating the monomer-monomer interaction. *In vitro*, MreB forms a large fibrous spiral in the presence of ATP (Van den Ent et al., 2001); this supports the ability of the protein to form helical filaments, *in vivo*, as detected by fluorescence microscopy (Jones et al., 2001). The structures formed by Mbl (MreB-like) filaments are highly dynamic; they re-model continuously during cell elongation (Carballido-Lopez and Errington, 2003a). The Mbl filaments probably constitute only one of the factors influencing cell shape. Another factor is the formation of the peptidoglycan layer. Mbl has a direct influence on the formation of new peptidoglycan (Carballido-Lopez and Errington, 2003a; Daniel and Errington, 2003). In *E. coli*, the alteration of cell shape in mutant forms of the MreB protein

was seen to influence cell-division processes, including chromosome segregation (Kruse et al., 2003). Those observations underline the necessity of cell-shape maintenance to other complex functional processes of the cell.

In *Treponema* species, whole-genome analysis has demonstrated the presence of the genes that regulate the cell shape in other bacterial models, including MreB and RodA. As yet, however, functional evidence needed to confirm the functions of these genes is lacking in *Treponema*. As indicated earlier in this chapter, one striking difference between *B. burgdorferi* and the *Treponema* species is that, in the absence of flagella, *B. burgdorferi* becomes a straight rod shaped bacteria (Motaleb et al., 2000; Sadziene et al., 1991). The presence of the flagellar filament in *B. burgdorferi* is directly related to the maintenance of the helical shape (Motaleb et al., 2000). In contrast, in *Treponema* spp., the absence of a flagellar filament does not alter the overall cell morphology (Izard et al., 1999; Limberger et al., 1999). Consequently, other factors must be involved, working together with MreB and associated proteins, to maintain the helical nature of the cell.

9. Hyperstructure, filaments and compartmentalization

The conceptual difference between ultrastructure (cytoskeleton and flagella) and hyperstructure resides in the fact that hyperstructures are not visible by microscopy. However, with the advances of microscopic techniques, including the design of new fluorophores and tagging systems, this difference may soon disappear. A hyperstructure occupies a large portion of the cell's volume and contains many of the cell's constituents, and has a readily assigned function (Norris et al., 1999). Such a structure has many components and requires a flow of building blocks (amino acids, nucleotides, etc.), proteins (enzymes, monomers of protein complexes, etc.), and other components (tRNAs, precursors, etc.). Hyperstructures are activity-based, and they form only when their constituents are actively engaged in carrying out their function. Examples include; the lipid preference of the replisome and the environment surrounding the multi-protein complex (30-40 nm in diameter) associated with the glycolysis (Baker and Bell, 1998; Dowhan, 1997; Lemon and Grossman, 1998; Mileykovskaya et al., 1998; Mowbray and Moses, 1976; Velot et al., 1997). As we begin to understand the dynamic aspect of the cytoskeleton, the concept of dynamic compartmentalization has to be integrated in the temporal studies of bacterial cell biology.

In *Treponema*, the cytoplasmic filament ribbon, the MreB helical filament, the flagellar basal body and filament are hyperstructures. Their volume occupancy and their requirement for helper proteins to assist in their function, create and define volumes within the cell where activities are primarily related to the filaments. Moreover, functions associated with formation, anchoring, maintenance, and severing require another set of proteins that are most probably in close proximity to the filaments for a short period of time. Those structures and associated components define, on the temporal scale, volumes that can be transient but create a specific type of compartmentalization. The confluence of genetic, comparative genomic, biochemical, comparative proteomic and biophysical findings will provide a clearer picture of this dynamic architecture.

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