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Structure of the Cytoskeleton of *Spiroplasma melliferum* BC3 and Its Interactions with the Cell Membrane

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Spiroplasma melliferum is a wall-less bacterium with dynamic helical symmetry. Taking advantage of the simplicity of this primitive lifeform, we have used structural (electron tomography and freeze fracture of whole cells; cryoelectron tomography and diffraction analysis of isolated cytoskeletons) and proteomic approaches to elucidate the basic organizing principles of its minimal yet functional cytoskeleton. From among ~30 *Spiroplasma* proteins present in a highly purified cytoskeletal fraction, we identify three major putative structural proteins: Fib, MreB, and elongation factor Tu. Fib assembles into a single flattened ribbon that follows the shortest helical line just under the plasma membrane and acts as a linear motor, whereas MreB is present as a matching array of membrane-associated fibrils parallel and associated with the motor. We also identify a prominent previously unknown filamentous network that occupies much of the cytoplasm and appears to cross-link the ribosomes. The abundant potentially filament-forming protein elongation factor Tu may be a component of this network, but the tomography data are most consistent with DNA as the core component. The results provide new information on the minimal organization necessary to support the scaffolding and motile functions of a minimal cytoskeleton.

Keywords: bacterial cytoskeleton; bacterial motility; cryoelectron tomography; linear motor; *Spiroplasma*

Introduction

All eukaryal cells, as well as several prokaryotic (eubacterial and archaeal) organisms, depend on cytoskeletal polymers and molecular motors in determining their shape and in driving motility.[1–3] Much of the molecular machinery underlying these processes arose early in evolution, so that a limited set of general principles, as well as a common set of molecular mechanisms, is thought to underlie cytoskeletal function across the phylogenetic spectrum. Indeed, it is now clear that genes for actin, tubulin, and intermediate filaments arose in prokaryotes (MreB[4,5] FtsZ,[6] and crescentin,[7] respectively), and that eubacterial and archaeal cytoskeletal proteins are remarkably similar to their eukaryal counterparts.[2,3] Such similarities favor a reductionist approach to understanding the cytoskeleton, which in turn recommends *Spiroplasma*[8,9]—a wall-less helical

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Abbreviations used: EF-Tu, elongation factor Tu; 3D, three-dimensional; CBB, Coomassie brilliant blue; 2D, two-dimensional.

bacterium from the class Mollicutes that is one of the simplest free-living and self-replicating forms of life—as an attractive minimal model system. Spiroplasmas are of additional interest because, beyond their well-established role as plant pathogens of significant economic importance,[10] there is some evidence that this bacterium may be involved in certain neurodegenerative diseases.[11]

Consistent with its minimal size and parasitic lifestyle, *Spiroplasma* has one of the smallest known genomes.[12] Despite its limited genetic repertoire, however, *Spiroplasma* has a well-defined cytoskeleton,[13,14] having retained genes for the major cytoskeletal proteins MreB[10,15] and FtsZ,[16] plus the related proteins FtsA[16] and elongation factor Tu (EF-Tu) (this study). Concerning motor functions of the cytoskeleton, *Spiroplasma* presents a unique protein assembly and a linear motor that is linked to at least a part of a filamentous framework, and is closely associated with the cell membrane. Structural analysis indicates that Fib, a unique 59-kDa protein that is the product of the *fib* gene, is the principal component of the linear motor.[17] This motor is organized as a flat monolayered ribbon following, from one end to the other, the shortest helical path of the coiled polar cell,[18] thus also serving a cytoskeletal function. The structural building blocks of the motor core are seven fibrils assembled in parallel with a ribbon of ~10-nm axial and lateral repeats. The latter is thought to reflect pairs of dimers of the Fib protein, assembled into a tetrameric unit cell.[18,19] SDS-PAGE analysis of density-gradient-purified ribbon preparations indicates that a persistent cluster of seven to eight proteins, here shown to include MreB and EF-Tu, copurifies with the ribbon.[18] The nature of the interaction of MreB and EF-Tu with the Fib core of the cytoskeletal ribbon, as well as the identity of the rest of the proteins in the cluster, is as yet unknown.

Regardless of the exact composition, it is clear that the ribbon's close apposition to the plasma membrane provides the molecular arrangement necessary to function as a coordinated linear motor, in that the Fib-based infrastructure is well positioned to couple to integral membrane proteins that might act as anchors and force generators. Exhaustive purification of the cytoskeletal ribbon results in preparations characterized by a strong affinity for membrane patches and occasionally by breakdown products such as ribbons with frayed ends or individual fibrils. This suggests strong bonding within and between fibrils, as well as with the membrane zones adjacent to the ribbon.

Previous analyses of the *Spiroplasma melliferum* cytoskeleton were carried out on a variety of isolated and progressively disassembled components.[18,19] Although this has evident advantages for data collection and analysis at the macromolecular level, electron tomography is the tool of choice for elucidating the organization of the entire cell. Here, we have used electron tomography of rapidly frozen whole cells, together with freeze fracture, electron diffractogram analysis, and proteomics, to determine the *in situ* composition and spatial organization of the *Spiroplasma* cytoskeleton. The results provide, at 4–5 nm resolution, a three-dimensional (3D) view of the intact *in situ* cytoskeletal ribbon, its path within the cell, and its associations with the membrane and other cytoskeletal elements. This analysis is complemented by tomograms of highly purified vitrified cytoskeletal ribbons and by electron diffractograms from negatively stained ribbons. We also provide proteomic data on the protein cluster that copurifies with the cytoskeletal ribbon, showing the presence of canonical (Fib and MreB) and putative (EF-Tu) cytoskeletal proteins, as well as several unique and as yet unidentified *Spiroplasma* proteins, some of which will likely prove to be motor ATPases or membrane anchors.

Results

Structural preservation in cryofixed freeze-substituted preparations: Suitability for tomographic reconstruction

Our main goal here is to resolve the spatial organization of the cytoskeleton and its interactions

Fig. 1. Structural preservation in impact-frozen *Spiroplasma* cells freeze-substituted in acetone/glutaraldehyde. (a) An axial projection of an aligned double-axis tilt series. Fiducial 10-nm gold particles (G; black dots between cells) are scattered on both surfaces of the section. Membranes (a; arrows) are unusually thick. (b) A corresponding one-voxel-thick (0.6 nm) slice through the tomogram. Ribosomes (r) are visible, as are thin well-defined lines traversing the cytoplasm, some of which appear to connect ribosomes (b; arrow (F)). Some of the lines approach the membrane or ribbon. In each cell, there is only one uniform ribbon (a; arrows (R)). (c) An axial projection of cells freeze-substituted in acetone containing osmium tetroxide [arrowheads point to face views of the ribbon (R); an arrow (m) points to the thick membrane]. (d) Contains a corresponding single-voxel tomographic slice. (e and f) Axial projections and tomographic slices, respectively, from cells freeze-substituted in acetone/uranyl acetate. In the osmium-tetroxide-substituted preparation, a uniform coat is seen on the outer surface of the well-contrasted membrane (c; arrow). This layer is likely due to the lipoprotein spiralin that is known to coat the cell surface.[20] The outer leaflet of the membrane appears denser and thicker than for the cells substituted in glutaraldehyde. This difference may reflect an external carbohydrate layer that ruthenium red staining suggests is present (data not shown). In addition, the space between leaflets is relatively wide, perhaps due to the high content of cholesterol, which constitutes 20% of the total membrane lipids[21] or 60% of the total neutral lipids.[22] As with the glutaraldehyde substitution regime (a and b), osmium tetroxide freeze substitution presents a dense cytoplasm that reduces the visibility of the thin ribbons, particularly for face-on views that are a projection of the ribbon's monomolecular layer (arrowhead). For cells that are freeze-substituted in acetone/uranyl acetate, the membrane and the cytoplasm are weakly contrasted, whereas the ribosomes (r) exhibit strong contrast due to the high affinity of uranyl ions for RNA. The cytoskeletal ribbons (R) are also highly contrasted. Clear edge-on views (arrows) and face-on views (arrowheads) of the cytoskeletal ribbon reveal its curvature, twist, thickness, and discrete and uniform fibrillar composition on a background of low cytoplasmic contrast. Scale bar represents 100 nm.