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# The Bacterial Actin-Like Cytoskeleton

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## INTRODUCTION

The cytoskeleton is a key regulator and central organizer of many eukaryotic cellular processes, including cell shape determination (morphogenesis), division, segregation, polarity, phagocytosis, movement, and macromolecular trafficking. It is a complex and highly dynamic network of protein filaments composed of actin microfilaments, microtubules (MTs [polymers of tubulin]), and intermediate filaments (IFs). These proteins were traditionally thought to be absent in prokaryotes, and the eukaryotic origin of the cytoskeleton was a long-standing dogma of cell biology. For decades, microscopic and biochemical studies failed to detect cytoskeletal elements in bacteria. Moreover, the sequencing of an increasing number of bacterial genomes (352 to date [15 September 2006] [Genomes Online Database, release 2.0 {www.genomesonline.org}]) did not reveal any putative candidates displaying any significant primary sequence similarity to a cytoskeletal protein. However, over the last 15 years, the dogma has been overturned completely, with the identification in bacteria of structural and functional homologues of all three main eukaryotic cytoskeletal proteins: FtsZ and BtubA/B are tubulin orthologues; MreB, ParM, and the recently uncovered MamK (and the archaeal Ta0583) are actin orthologues; and crescentin is an intermediate filament protein.

The first to be identified, in the early 1990s, was the tubulin-like protein FtsZ. FtsZ is a highly conserved cytosolic GTPase (23, 136), present in virtually all eubacteria (and archaea), which forms a ring (namely the Z ring) at the future site of cytokinesis and plays an essential role in cell division (4, 6).

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Although FtsZ's primary amino acid sequence identity to tubulin is low (~17%), their three-dimensional (3D) structures and assembly properties are remarkably similar (106, 124). Two other tubulin homologues, BtubA and BtubB, were recently identified in the bacterial genus *Prosthecobacter* (76). BtubA and BtubB display higher sequence identity (~35%) and closer structural homology to eukaryotic tubulin than to FtsZ (BtubA/B and FtsZ sequences share only 8 to 11% identity) (76, 149). It is believed that FtsZ and tubulin diverged from a common ancestor early in evolution, whereas BtubA/B likely split from eukaryotic tubulin more recently by horizontal gene transfer (76, 149, 157).

Many attempts had been made to isolate actin-like and actinomyosin-like complexes from bacterial cells, but none of these studies was conclusive and correlated with a specific protein. The breakthrough came in 2001, when the MreB and Mbl (*M*re*B*-like) proteins of *Bacillus subtilis* were shown to be required for different aspects of cell morphogenesis and to assemble into helical structures that run along the length of the cell (80). Shortly after, the nature of these helical filaments was revealed when purified MreB from *Thermotoga maritima* was shown to undergo actin-like polymerization and to have a three-dimensional structure remarkably similar to that of actin (164). Two other actin homologues that form cytoskeletal structures in bacterial cells (ParM and MamK) and one archaeal actin (Ta0583) have since been identified.

Finally, in 2003, the *Caulobacter crescentus* coiled-coil-rich protein crescentin was shown to assemble into filaments that play a key role in determining the curved and helical cell shapes of this bacterium and to have biochemical properties and a domain structure similar to those of IFs (3). Furthermore, in addition to homologues of eukaryotic cytoskeletal proteins (actin, tubulin, and IFs), a subclass of filament-forming Walker A ATPases (85) belonging to the large MinD/ParA superfamily was recently categorized as a new class of bacterial cytoskeletal proteins. These proteins, renamed Walker A cytoskeletal ATPases (114), form ATP-induced dynamic filaments in vivo and play important organizing roles in cell division (MinD subgroup) and plasmid/chromosome DNA segregation (ParA/Soj subgroup) in bacteria. Although Walker A cytoskeletal ATPases display no homology to known eukaryotic cytoskeletal elements, they are now considered an additional component of the prokaryotic cytoskeleton (for recent reviews, see references 114 and 142). The discovery of cytoskeletal elements in bacteria opened up new and exciting fields of research, which have evolved rapidly over the last few years. Most of the advances made have arisen from developments in imaging technology and analysis, in particular high-resolution fluorescence microscopy techniques, which previously could not be applied to organisms as small as a bacterium.

Among all prokaryotic cytoskeletal proteins, the field of bacterial actins has developed the most in recent years. The discovery of MreB has led to a continuous flow of new and important findings from several organisms, and the MreB-like proteins have become a major research focus in many laboratories. It is now clear that prokaryotic cells possess actin and that a dynamic actin-like cytoskeleton is involved in a variety of essential cellular processes in bacteria. These functions, like those of the eukaryotic actin cytoskeleton, require the targeting and accurate positioning of proteins and molecular complexes. A series of landmark papers investigating the roles of the actin-like proteins has provided tremendous insights into the mechanisms of cell wall (CW) morphogenesis, DNA segregation, and cell polarity in bacteria.

In this review, I aim to draw together what is known about the cellular, structural, and biochemical properties of MreB (and ParM and MamK) proteins (the bacterial actins). I examine their known roles before considering other possible functions for these cytoskeletal proteins. Currently, interacting proteins for MreB and its relatives remain largely unknown. The quest for them and for a few proteins known to associate with the dynamic bacterial actin-like cytoskeleton is also discussed. The bulk of these studies have been performed with the model organisms *Bacillus subtilis*, *Escherichia coli*, and *Caulobacter crescentus*, although some findings have emerged from other systems (e.g., *Thermotoga*, *Rhodobacter*, and *Streptomyces*), and they are generally thought to be conserved throughout eubacteria. Some perspectives on directions for future research in the field are also provided.

## EUKARYOTIC ACTIN AND THE EUKARYOTIC ACTIN CYTOSKELETON

Actin is one of the most abundant and highly conserved proteins found in all eukaryotic cells. The 43-kDa monomer of conventional actin (globular actin, or G-actin) spontaneously assembles in vitro to form long linear or branched structures (filamentous actin, or F-actin) upon the addition of salt, provided that ATP is present (86). The filaments polymerize noncovalently from both ends, with different affinities for the addition of monomers at each end. This results in an intrinsic polarity in the filament, in the form of a slow-growing end (minus end) and a fast-growing end (plus end). At steady state, the loss of subunits at the minus end and the equivalent gain at the plus end give rise to an effect known as treadmilling (17). Actin microfilaments are thin (3 to 6 nm in diameter) and flexible, and they rarely occur in isolation within the cell but, rather, in cross-linked aggregates and bundles. In vivo, they can form either stable or labile structures. Actin polymerization is a highly regulated process controlled both by nucleotide binding and hydrolysis and by the action of a number of actin-binding proteins that can cross-link, nucleate, cleave, bundle, stabilize, or destabilize the filaments (18, 86, 150). The visualization of the eukaryotic actin cytoskeleton in an unperturbed, close-to-life state was achieved only recently, with the application of cryo-electron tomography to vitrified cells of *Dictyostelium discoideum* (92).

The actin cytoskeleton is highly dynamic in most cells, and F-actin populations continuously assemble and disassemble, with measured half-lives on the order of a few minutes. This turnover is a consequence of the ATPase activity of actin. Irreversible hydrolysis of the bound nucleotide occurs once the monomer is fully incorporated into the filament (86), and thus, like the case for GTP hydrolysis in tubulin polymerization, it is not required to form the actin filaments. Instead, it destabilizes the polymer and promotes depolymerization from its ends since ATP monomers prefer to associate and ADP monomers prefer to disassociate (111). A difference between the two cytoskeletal polymers, i.e., F-actin and MTs, is that GTP-GDP

exchange is very rapid for free tubulin (half-time of seconds) while ATP-ADP exchange is relatively slow for free actin (half-time of minutes).

### The Actin Superfamily

Actin amino acid sequences are extremely conserved across eukaryotes (e.g., there is 100% identity between human and chicken skeletal muscle actin proteins and 88% identity to the yeast *Saccharomyces cerevisiae* actin). The main functional criteria originally used to identify actins included the ability to polymerize spontaneously into thin filaments able to stimulate the ATPase activity of myosin. Most conventional actins can also bind DNase I and drugs such as cytochalasins, phalloidins, and macrolide toxins (e.g., latrunculins). However, there is an emerging subfamily of highly divergent actins that still share significant sequence similarity but have limited functional homology and ligand-binding specificity. In addition, in a landmark publication in 1992, Bork et al. (7) reported on a large group of functionally very different proteins (which included heat shock proteins, sugar kinases, and the following proteins expressed in prokaryotes: the bacterial chaperone DnaK [Hsp70] [176]; the cell division protein FtsA [161]; the plasmid stability protein ParM [StbA] [115, 129]; and the cell shape determinant MreB [36]) that share very limited amino acid sequence identity/similarity (e.g., only ~11% similarity between ParM and actin and ~15% identity between MreB and actin [much less than the 20% identity generally used as a baseline to establish homologues]) but that contain five conserved sequence motifs that were predicted to determine a three-dimensional fold similar to that of actin. This fold consists of two alpha/beta domains (subdomains IA, IB, IIA, and IIB, which correspond to subdomains 1, 2, 3, and 4, respectively, in actin) folding around the central core of the structure, the nucleotide-binding pocket (see Fig. 1I and 5C) (7, 53, 81). Although the primary biological functions of these proteins are diverse and appeared to have little to do with the cytoskeleton, they were all predicted to share with actins the ability to bind and hydrolyze ATP at a structurally equivalent site, suggesting that they were highly diversified groups of descendants from a common ATP-binding ancestor (7). Ten years after Bork et al.'s prediction (which is essentially correct), two of these proteins, bacterial MreB and ParM, were shown to be structural and functional homologues of actin. MreB proteins are closer to actins in overall size and topology than any other superfamily member, while the plasmid-encoded ParM proteins are smaller and more divergent but are also true homologues of actin (see below).

## THE BACTERIAL ACTIN MreB

Bacterial Mre (murein cluster e) proteins have been known for a long time to be cell shape determinants (171). MreB is widespread in bacteria with complex (nonspherical) shapes but is absent from most bacteria displaying coccoid (spherical) morphologies (80). It is present in both gram-positive and gram-negative bacteria (and also in some mollicutes and archaebacteria [see below]). However, multiple copies of the *mreB* gene are conspicuously absent from gram-negative species but often present in gram-positive organisms. The rod-shaped organism *B. subtilis*, for example, has three *mreB*-like genes, namely, *mreB*, *mbl* (*mreB-l*ike), and *mreBH*. MreB appears to be essential in all bacteria studied so far, including *B. subtilis* (80, 166), *E. coli* (88), *C. crescentus* (52), *Rhodobacter sphaeroides* (156), *Salmonella enterica* serovar Typhimurium (19, 20), and *Streptomyces coelicolor* (9, 112). In *E. coli*, *mreB* mutants are spherical, and MreB was inferred not to be essential for many years (170, 171), but a recent report showed that MreB is indeed essential in this bacterium, too (88). In *S. coelicolor*, *mreB* mutants could not be created by conventional gene disruption, and MreB was originally reported as being essential (9). However, a null mutant was recently generated using a PCR-based targeting procedure (112). Although MreB appeared not to play a vital role during vegetative growth, it was revealed to be essential in differentiation and spore formation, which are part of the complex developmental cycle of this organism (see below).

MreB depletion has been shown to induce the formation of enlarged cells with gross morphological defects and, ultimately, cell lysis in *B. subtilis* (Fig. 1B), *E. coli*, and *C. crescentus* (52, 80, 88). *B. subtilis mbl* mutants also display highly distorted morphologies, with bent, twisted, and irregularly shaped cells, a proportion of which are also affected in cell width (Fig. 1C). In light of these findings, an actively determined, MreB-dependent cell shape system was suggested to be conserved across nonspherical microorganisms.

Subcellular localization studies using immunofluorescence microscopy (IFM) and green fluorescent protein (GFP) fusions in several bacteria have shown that MreB-like proteins generally localize to helical filamentous structures that encircle the cytoplasm, just under the cell membrane (see below). In *B. subtilis*, all three isoforms, i.e., MreB, Mbl, and MreBH, form similar helical structures (Fig. 1D to F, respectively) (15, 26, 55, 80). Pioneer localization studies suggested that MreB, Mbl, and MreBH form distinct helical structures with different configurations (26, 80), but these studies were done with separate cell populations with different genotypes and in separate imaging experiments. Colocalization studies (i.e., simultaneous, same-cell imaging) have recently shown that all three of the *B. subtilis* MreBs are in fact in close proximity, in a single apparently helical structure (15). Such filament-like helices could result from the interaction of monomeric MreBs with a preexisting helical structure in the cell or, alternatively, from the noncovalent association of the monomers (i.e., polymerization) into high-order helical forms.

### MreB Filaments Are Generated by Actin-Like Polymerization

**Structure of MreB monomers and MreB protofilaments.** To investigate whether MreB could self-assemble into actin-like filaments to form the helical structures observed in vivo, van den Ent et al. (164) cloned and purified MreB from *Thermotoga maritima*. Biochemical and electron microscopy (EM) analyses showed that the protein polymerized into filaments with a longitudinal repeat similar to that of actin (in MreB, 51 Å; in F-actin, 55 Å). Elucidation of the crystal structure of MreB showed that MreB and actin are very similar in three dimensions, allowing superimposition of the molecules with very little deviation (Fig. 1I). Furthermore, close inspection of

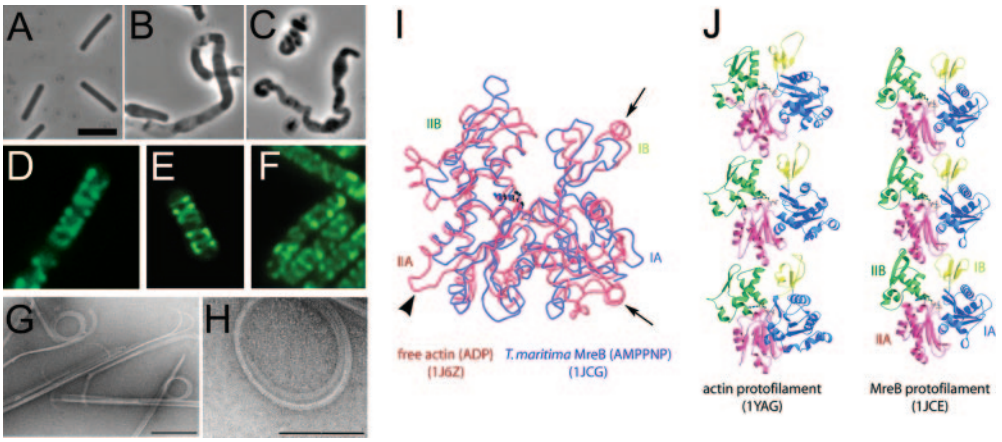


FIG. 1. Properties of MreB-like proteins. (A to C) Effects of mutations of *Bacillus subtilis mreB* and *mbl* on cell shape. Phase-contrast images are shown. Bar, 5 μm. (A) Wild-type cells showing the typical rod shape. (B) Lytic phenotype of cells depleted of MreB. (Panels A and B are reprinted from reference 55 with permission from Blackwell Publishing.) (C) Aberrant morphology of *mbl* mutant cells. (Reprinted from reference 80 with permission from Elsevier.) (D to F) Helical filaments formed in vivo by the three MreB-like proteins of *B. subtilis*. (D) GFP-MreB (courtesy of A. Formstone [unpublished]); (E) GFP-Mbl; (F) GFP-MreBH. (G and H) Electron micrographs of negatively stained MreB filaments. (G) MreB forms filamentous bundles and ring-like structures. An enlarged ring structure is shown in panel H. Bars, 0.2 μm and 0.1 μm, respectively. (Reprinted from *Journal of Biological Chemistry* [47] with permission of the publisher.) (I and J) Comparison of the three-dimensional structures of actin and MreB. (I) Superimposition of uncomplexed actin (purple) and MreB from *Thermotoga maritima* (blue). (Reprinted, with permission, from the *Annual Review of Biophysics and Biomolecular Structure* [107] volume 33, copyright 2004 by Annual Reviews.) Despite having very low amino acid sequence identity (~15%), the two proteins have essentially the same fold. The arrows point to insertions within the actin sequence responsible for binding to DNase I. The arrowhead indicates a loop proposed to make an important interstrand interaction. (J) MreB crystals contain protofilaments that are similar to one strand (protofilament) of modeled F-actin. PDB entry codes are shown in parentheses. (Reprinted from reference 164 with permission from Macmillan Publishers Ltd.)

the crystal packing by high-resolution X-ray crystallography revealed that the protein had crystallized in its polymeric form and that the crystals contained protofilaments of MreB with exactly the same subunit spacing (51 Å). This allowed direct comparison of the MreB polymer to the atomic model constructed for F-actin (67) and showed that they are in remarkably good agreement, with nearly identical molecular orientations and contacts between the monomers of the two proteins (Fig. 1J) (164).

**MreB assembly properties.** Despite their high structural homology, MreB and actin display significantly different assembly properties and nucleotide-binding specificities. Light-scattering and EM studies have been used to explore the basic assembly and mechanical properties of MreB from *T. maritima* (47, 48). As with F-actin, MreB assembly is triggered by ATP in vitro, and the filament ultrastructure and polymerization are temperature and cation dependent (47, 164). Furthermore, MreB catalyzes ATP hydrolysis and releases phosphate ($P_i$) at a similar rate to that of F-actin (47). However, GTP (but not ADP or GDP) can mediate MreB assembly as effectively as ATP (whereas eukaryotic actin assembly is favored in the presence of ATP over GTP), indicating that MreB is an equally effective ATPase and GTPase (48, 164). MreB polymerizes much more rapidly than actin, without nucleation (or nucleation is highly favorable and fast) and with little or no contribution from filament end-to-end annealing (i.e., joining of filaments through the direct association of filament ends [annealing], which contributes significantly to the assembly of actin filaments) (47). MreB exhibits a critical concentration of ~3 nM, which is ~100-fold lower than that of actin. Finally, without the need for accessory proteins, MreB was shown to form predominantly filamentous bundles that display different morphologies and have the ability to spontaneously form ring-like structures (Fig. 1G and H) (47). The presence of both straight and curved filaments was suggested to depend upon the state of nucleotide hydrolysis within the filament (48), a phenomenon that has also been observed in filamentous proteins such as microtubules (125) and FtsZ (108). Using quantitative rheometry, Esue et al. (48) recently showed that MreB filaments possess significant elasticity and mechanical stiffness, also like MTs, and are much less labile than actin filaments in networks. It should be noted that another difference between MreB and actin applies at the filament level, as MreB assembles into single straight protofilaments (164), not into double-helical protofilaments that twist around each other like the case for F-actin (and ParM) (165; see below). Since the polypeptide chain of actin (375 amino acids) is longer than that of MreB (336 amino acids), there are a number of insertions that occur within the actin sequence, and these might account for the differences in the properties of the two proteins. For example, one insertion (arrowhead in Fig. 1I) forms a loop that has been proposed to make an important interstrand interaction that holds the actin filament together, whereas two other insertions (arrows in Fig. 1I) are responsible for the binding of actin to DNase I (41).

## Dynamics of MreB Filaments

Time-lapse microscopy has shown that MreB-like filaments are flexible and highly dynamic structures (like F-actin) that move continuously through the cell. Besides elongation and division, in parallel with cell cycle progression, the Mbl (Fig. 2A) and MreBH helical structures of *B. subtilis* changed curvature and configuration during growth, suggesting that they

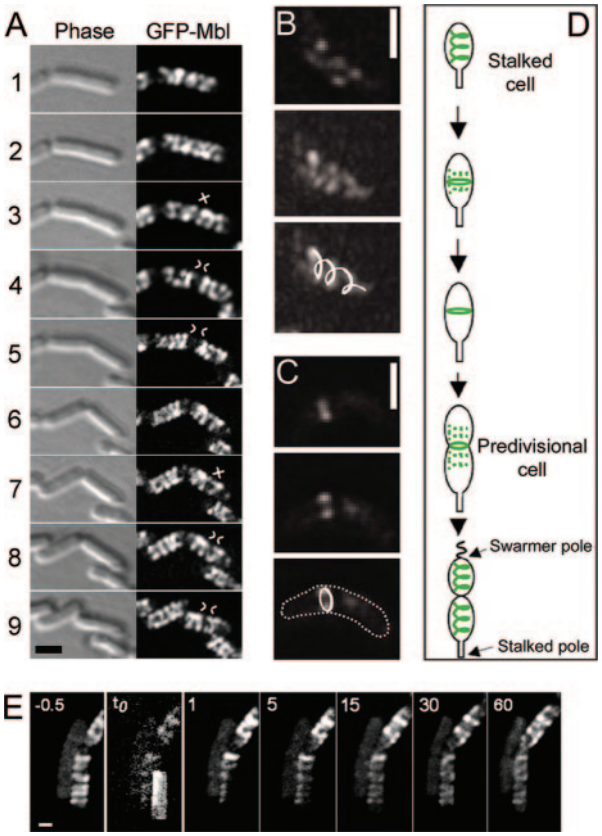


FIG. 2. In vivo dynamics of MreB filaments. (A) Time-lapse microscopy of GFP-Mbl dynamics in *Bacillus subtilis*. A 4-h time course was monitored every 30 min. The crosses indicate fragmentation of the helical structures at about the time of division. Bar, 2 μm. (Reprinted from reference 13 with permission from Elsevier.) (B to D) MreB forms a contracting and expanding spiral in *Caulobacter*. (B) Helical pattern of GFP-MreB in a stalked cell. The panels show, from top to bottom, a single deconvolved optical section, a volume projection of 15 optical slices, and a cartoon interpretation of the spiral pattern overlaid on the image from the middle. (C) Hollow ring organization of division-plane GFP-MreB localization. The ring is visualized as a continuous band in one optical section (top) and as two separate points in an optical section 300 nm deeper into the cell (middle). The bottom panel shows a cartoon representation of the ring and cell outline overlaid on the image from the panel above. Bars, 1 μm. (D) Schematic representation of the dynamic behavior of GFP-MreB during the *Caulobacter* cell cycle. (Reprinted from reference 61 with permission of the publisher. Copyright 2004 National Academy of Sciences, U.S.A.) (E) FRAP analysis of GFP-Mbl in live *B. subtilis* cells. The first panel shows the helical cables of Mbl 0.5 min before photobleaching. The next frame shows the laser photobleaching of a rectangular region covering the lateral half of a cell ($t_0$). The following image sequence monitors fluorescence recovery in the bleached Mbl cables at the indicated times (in min). The recovery of fluorescence occurred at the expense of fluorescence in the unbleached cables (indicating an exchange of subunits), with a half-time of ~8 min. Bar, 1 μm. (Reprinted from reference 13 with permission from Elsevier.)

may move inside simultaneously or together with the cell wall (13, 15). Similarly, a variety of arrangements have been observed for the MreB helices in live cells of *B. subtilis* (26, 55) and *E. coli* (153), suggesting that their organization also changes dynamically throughout the cell cycle. In *E. coli* (153) and *C. crescentus* (52), MreB filaments were reported to form helical structures following the long axis of the cell and/or single loops or rings at midcell (Fig. 2B and C, respectively). Time-lapse microscopy of synchronized *C. crescentus* cells showed that such patterns result from the dynamic localization of MreB into a contracting and expanding spiral (Fig. 2D) (61). The switch to midcell localization occurs at a time that coincides with the initiation of cell division and depends upon the cell division protein FtsZ (the tubulin homologue) (52, 61), which forms a cytokinetic ring that recruits the division machinery at the future sites of division (44, 109). A similar localization pattern was reported for *R. sphaeroides* cells, in which MreB localized predominantly as a midcell ring (or a one-turn helix) at the time of division and seemed to disassemble before the completion of septation (156). The different localization patterns (helical/septal) displayed by MreBs might reflect the diverse cellular functions that they are involved in during the cell cycle. Gene duplication would be an alternative means to account for functional variability. *E. coli*, *Caulobacter*, and *Rhodobacter* (the three organisms where division-plane MreB rings have been observed) are gram-negative bacteria, and each has only one MreB homologue (80, 156). Midcell localization has not been reported so far for either MreB or Mbl in *B. subtilis* (13, 26, 55, 80). However, the third homologue, MreBH, was found to localize in helical structures along the cylinder but also occasionally in very intense bands and spotty ring-like structures at cell separation sites, and a dynamic switch between the septal and helical localization patterns could be observed via time-lapse imaging (15). The fact that the three isoforms colocalized in a single apparently helical structure in independent imaging experiments (15; see above) means that any function(s) associated with the three isoforms could be spatially and temporally coordinated. However, although the significance of and factors triggering the midcell localization patterns of MreBH are not yet clear and it remains to be elucidated whether MreB and Mbl exhibit the same behavior under similar conditions, the differences observed in the dynamics of MreBH compared to those of MreB and Mbl strengthen the idea of specialized functions of the three homologues (thereby paralogues, i.e., results of gene duplications allowing new functional divergences) in *B. subtilis*.

Fluorescence recovery after photobleaching (FRAP) experiments using a functional GFP-Mbl fusion showed that Mbl cables (referred to as such by analogy to eukaryotic actin cables) are continuously remodeled, i.e., assembled and disassembled, as the cell cycle progresses (13). Under the conditions tested, turnover occurred along the Mbl helices, with no obvious polarity and a half-time of recovery of about 8 min (Fig. 2E) (13). In a different study, a number of separate filaments of GFP-MreB and GFP-Mbl appeared to move rapidly through the cell, toward and away from the cell poles, respectively, along helical tracks (26). Movement of the filaments was dependent on active cell growth, and it was not continuous over a prolonged period; frequently, full turns or 1.5 turns were observed, after which movement seemed to pause or cease. MreB filaments traveled a turn around a *B. subtilis* cell within 50 to 60 s, giving an average speed of 0.07 μm/s (26) (which is in good agreement with the measured speed of extension [polymerization] of the growing end of isolated F-actin [0.1 to 1 μm/s] [118]) and a net speed relative to the cell length of 4.2

nm/s (0.24 μm/min) (26). The general helical movement and the speed of migration of the Mbl filaments were similar, although their general direction seemed to be opposite that of MreB filaments. It was concluded that a potential poleward or centerward pushing velocity of 0.24 μm/min was generated by MreB or Mbl, respectively, possibly through a treadmilling mechanism (26). The treadmilling behavior of MreB filaments in vivo was indeed confirmed recently, using quantitative imaging of single molecules of fluorescent MreB-yellow fluorescent protein fusions in living *Caulobacter* cells (82). In this study, both polymerized MreB (filamentous MreB [fMreB]) and unpolymerized MreB (globular MreB [gMreB]) populations could be distinguished and were shown to display different dynamics: gMreB moved rapidly in a random fashion, whereas fMreB displayed slow, directed motion. The fast motion of unpolymerized monomers (gMreB) had a rate of diffusion that was restricted compared to that of cytoplasmic proteins but that appeared similar to that of membrane-bound proteins, suggesting that gMreB may associate with an additional factor(s), possibly (in) the cytoplasmic membrane (82). By analyzing the rate, distance, and direction of labeled MreB in the polymers, it was shown that the slow directional movement of fMreB did not result from whole-filament translocation but from treadmilling of the MreB monomers through short MreB filaments with fixed ends, i.e., by preferential polymerization at one end and depolymerization at the other end of a filament (82). Therefore, MreB, like F-actin (17), exhibits treadmilling behavior in vivo and thus assembles in a polarized manner. From these treadmilling observations, it was also extracted that the steady-state rate of MreB monomer addition was 1.2 $s^{-1}$, that the average MreB filament length was ≈400 nm (much shorter than the cell length of 3.5 μm), and that the polarized assembly of individual MreB filaments was random relative to the overall cell polarity (82).

### Ultrastructural Organization of MreB Filaments In Vivo

The important implications of the dynamic behavior described above are still hampered by our lack of knowledge of the ultrastructure of the high-order filamentous forms displayed by the MreB-like proteins and of the mechanisms that regulate their dynamics in vivo. Nevertheless, in view of (i) the turnover detected by FRAP experiments (13), (ii) the fluorescence intensity and low background of inducible GFP fusions (13, 15, 26), (iii) the average MreB filament length (significantly shorter than the overall MreB helices) (82), and (iv) the recent biochemical data on MreB assembly (ATP/GTP hydrolysis, no or little filament end-to-end annealing, and filamentous bundles observed by EM) (47, 48), it is likely that in vivo the helical structures are composed of, and exchange subunits in the form of, lateral bundles of protofilaments (rather than monomers). According to this model, the dynamics of MreB helical cables involve the exchange of short polarized protofilaments (which assemble randomly, i.e., with no uniform global polarity) to the sides of a multistranded polarized structure. Like in the case of eukaryotic actin, the mechanism may be controlled by both nucleotide binding and hydrolysis. The structural basis for the colocalization of the three MreB isoforms of *B. subtilis* is still unknown, but it seems likely that they form a triplex helical structure composed of some kind of mixed heteropolymeric bundles (15). It still remains to be elucidated whether this model is correct and whether direct interactions between the MreB protofilaments or cross-linking through accessory proteins occurs to form such bundled structures.

## MreB-LIKE PROTEINS AND CELL MORPHOGENESIS

Prokaryotic cells display a wide diversity of shapes. In all eukaryotic cells, shape is determined primarily by cytoskeletal structures, particularly actin filaments. Since such structures were traditionally thought to be absent from bacteria, the tough external peptidoglycan (PG) cell wall was assumed to be the primary determinant of bacterial cell shape. Indeed, isolated PG sacculi comprise a single huge molecule that retains the shape associated with their original cell (69), and conversely, removal of the wall results in spherical protoplasts (i.e., loss of shape). Moreover, most genes identified to be involved in cell shape (morphogenes) were associated with CW biosynthesis. For decades, research on cell shape in bacteria focused on cell wall synthesis and structure (for a recent review, see reference 133). However, the discovery of a morphogenetic actin-like cytoskeleton brought about a radical change in the context in which bacterial cell shape is studied.

### MreB Filaments Govern Cell Morphogenesis by Actively Directing Lateral Wall Biogenesis

MreB proteins play an essential role in the control of cell morphogenesis in nonspherical bacteria (see above). It seemed likely that the MreB cytoskeletal structures would control cell shape by determining CW architecture (13, 80).

**Mbl filaments direct lateral wall synthesis in *Bacillus subtilis*.** A range of studies with rod-shaped bacteria suggested that PG insertion occurs at the nascent septum and randomly all over the surface of the lateral wall during growth (10, 27, 30, 113, 117). However, the sensitivity and resolution of the methods used in these studies were limited: they did not provide adequate spatial resolution to determine the underlying patterns of PG incorporation. A more sensitive, high-resolution probe for nascent PG insertion in nonfixed cells was recently developed by Daniel and Errington (22). These authors used a fluorescent derivative of the antibiotic vancomycin (a cell wall synthesis inhibitor that binds specifically to PG intermediates) to label nascent PG in gram-positive bacteria by fluorescence microscopy (note that gram-negative bacteria do not stain because their outer membrane presents a permeability barrier to vancomycin) (169). This novel staining method revealed that, at least in *B. subtilis*, synthesis of the wall occurs in a helical pattern over the cylindrical part of the cell and also specifically at the septum in dividing cells (Fig. 3A). The lateral helical pattern of fluorescein-labeled vancomycin (Van-FL) was reminiscent of the helical localization of MreB and Mbl, both of which are required for cell shape determination in *B. subtilis* (1, 80). Strikingly, the helical staining was abolished in a strain lacking Mbl (Fig. 3C) and not in a strain lacking MreB (Fig. 3D) (22, 55). The septal insertion was dependent on cell division (FtsZ), as expected (Fig. 3E) (22). Recently, in a similar independent study that used fluorescent derivatives of vanco-

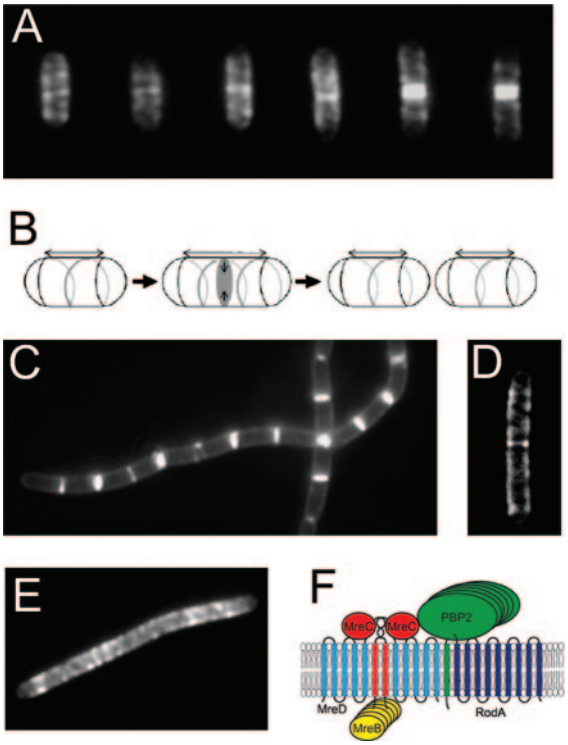


FIG. 3. Control of lateral wall synthesis by MreB-like proteins. (A) Van-FL staining of nascent cell wall synthesis in wild-type cells of *Bacillus subtilis*. A compilation of images of typical cells during the cell cycle progression is shown from left to right. (B) Model for separate systems for septal and cylindrical PG synthesis in *B. subtilis*. Gray lines and ovals show the sites of PG insertion during elongation and division, respectively. Arrows show the directions of elongation or division driven by cell wall biosynthesis. (C to E) Effects of Δ*mbl* null mutation (C), Δ*mreB* null mutation (D), and FtsZ depletion (E) on Van-FL staining. (Panels A, B, C, and E were reprinted from reference 22 with permission from Elsevier. Panel D was reprinted from reference 55 with permission from Blackwell Publishing.) (F) Schematic representation of interactions within the MreBCD complex in *E. coli* and the possible interaction of MreBCD with periplasmic proteins involved in peptidoglycan synthesis (PBP2 and RodA). (Reprinted from reference 88 with permission from Blackwell Publishing.)

mycin and ramoplanin (another PG-binding antibiotic), insertion of nascent PG along the lateral wall of *B. subtilis* was confirmed to be helical (160). However, in this study, a sidewall helical staining pattern qualitatively similar to that observed in wild-type cells (although less regular) was observed in cells lacking Mbl (160); the authors of that study concluded that Mbl plays an indirect role in directing PG synthesis but that it is not essential for the incorporation of sidewall PG.

These staining methods cannot be applied to gram-negative bacteria, but it is interesting that murein deposition patterns in the sacculi of growing *E. coli* cells (31) (by the incorporation of D-cysteine [D-Cys] [30]) also suggest a helical pattern of PG insertion into the lateral CW, although this remains to be confirmed. In contrast, Van-FL staining of two spherical bacteria (which do not possess MreB homologues) (*Streptococcus pneumoniae* [22] and *Staphylococcus aureus* [130]) and of a rod-shaped bacterium with no MreB/Mbl system (*Corynebacterium glutamicum* [22]) was highly specific for the division septum (and its derived poles in *C. glutamicum* cells) and absent in the lateral walls, indicating that PG insertion in these organisms occurs exclusively in zones specified by the FtsZ-dependent division machinery.

Taken together, these findings have several important implications. First, insertion of PG along the cylindrical walls of *B. subtilis* (and possibly *E. coli*) cells occurs in a helical pattern. Second, there are two spatially specialized systems for PG synthesis in *B. subtilis* (and probably in all rod-shaped bacteria with an MreB system): dispersed helical insertion of PG throughout the lateral wall during growth results in rapid cylindrical extension (elongation), and cell division-directed PG synthesis allows septum formation (division) (Fig. 3B). Third, Mbl may be the MreB homologue required mainly, or exclusively, for lateral (helical) wall biosynthesis. This has raised interesting questions about the role of MreB in *B. subtilis* morphogenesis. MreB is essential under normal growth conditions and has an important role in the control of cell width (55, 80). Consistent with a role in CW integrity, the lethal *mreB* mutant phenotype could be ameliorated by high concentrations of magnesium ($Mg^{2+}$) (55), like the phenotypes of several *B. subtilis* mutants thought to be required for different aspects of PG synthesis (94, 95, 121, 138, 139; see below). The mechanism by which $Mg^{2+}$ is able to rescue the phenotypes of these mutants is currently unknown. It also remains unclear how MreB controls cell width, but it might influence the synthesis or structure of the cylindrical and/or septal CW. Van-FL staining showed that MreB was not primarily required for lateral PG synthesis (22, 55; see above), although a redundant role in this process cannot be excluded. Alternatively, MreB could be required for insertion of teichoic acids or of autolysins into the CW, but all of these hypotheses remain to be tested. It has been suggested that MreB might act either continuously to restrain the diameter during elongation or discontinuously to reset the correct diameter when the cell divides (55). Finally, it is exciting to mention that a new, distinct role in cell wall morphogenesis has recently been uncovered for MreBH, the third MreB isologue in *B. subtilis* (15; see below).

**PBP localization and the MreB cytoskeleton.** The Van-FL findings provided strong support for the view that at least the cables of Mbl direct the synthesis of lateral PG in a spatially controlled manner. Conceivably, this might involve the localization of PG-synthesizing enzymes, named penicillin-binding proteins (PBPs), that incorporate the PG precursors into the growing CW sacculus. Two factors (or rather a combination of them) are thought to be critical for PBP localization, and they are protein-protein interactions and substrate recognition (147). The PG-synthesizing septal machinery (i.e., septal PBPs) has been shown to be recruited by the FtsZ ring (44, 109). However, no factors for targeting of PBPs to the lateral wall have yet been identified. Hence, the helical MreB scaffolding structures could direct PBP localization either by providing a substrate(s) that can be recognized by elongation-specific PBPs or by providing a track for protein-protein interactions that target PBPs to their site(s) of action (see below).

A putative candidate for MreB-directed targeting is the product of *pbpA*, PBP2, a high-molecular-weight PBP that displays transpeptidase activity (i.e., catalyzes PG cross-linking) and has classically been associated with sidewall synthesis during elongation (158). In *E. coli*, a functional GFP-PBP2 fusion localized preferentially in a spot-like pattern over the

cylindrical part of the envelope, and also at midcell during cell division (28). The localization of GFP-PBP2 over the lateral wall was suggestive of a helical pattern strikingly similar to that of MreB. Consistent with this, PBP2 formed a banding pattern reminiscent of that formed by MreB filaments in *C. crescentus* cells, as shown by both IFM (35, 52) and the use of a GFP-PBP2 fusion (40). Since the distinct banding pattern of PBP2 was lost (although PBP2 foci were still present) in *C. crescentus* cells that had been depleted of MreB for 10 h, it was originally suggested that PBP2 localization was dependent on MreB (52). Under such conditions, the aberrant localization pattern observed could indeed be attributed to the lack of MreB or, alternatively, to a secondary effect resulting from the severe shape defects resulting from the long-term absence of MreB. The latter hypothesis was supported by a recent IFM study, where rapid disruption of the MreB filaments by treatment with A22 did not affect the helical pattern of PBP2 (35). A22 is a small molecule that specifically and rapidly (<1 min) delocalizes MreB in *Caulobacter* cells, allowing the effects of the absence of MreB helices to be assessed prior to observable deformations in shape (62; see below for more details). However, in an independent study, GFP-PBP2 was reported to mislocalize to the division plane upon A22 treatment (40). Interestingly, only newly synthesized GFP-PBP2 seemed to relocalize to the septum, and thus it was suggested that (MreB-dependent) helical localization of PBP2 was regulated at the level of insertion and that established helical PBP2 patterns are stable in the absence of MreB (35). Although this finding may explain the differences between the two reports, whether PBP2 localization depends on MreB in *C. crescentus* remains to be unambiguously demonstrated. (Indeed, in the GFP-PBP2 study [40], cells were treated with 10 μg/ml A22 for 6 h, whereas in the IFM study [35] cells were treated with 50 μg/ml A22 for 2 h. After 2 h of A22 treatment at 50 μg/ml, growth is arrested but shape defects are not yet visible [62], and thus the previously inserted PBP2 patterns could be stable. On the other hand, *C. crescentus* cells grown in 10 μg/ml A22 for 6 h keep growing [albeit slowly] but already display significant cell shape deformations [62], which could indirectly affect PBP2 localization.)

IFM studies of *R. sphaeroides* cells showed that MreB colocalized with PBP2 in a cell cycle-dependent manner (155). PBP2 localized in partial rings (which presumably represent unresolved helices) at the middle of elongating cells and at the one-quarter and three-quarter positions in septating cells. MreB colocalized with PBP2 during elongation only; during septation, MreB remained at the septation site, whereas PBP2 relocalized to the midcell sites of the forming daughter cells. It was concluded that MreB and PBP2 interact during elongation to synthesize PG at or near midcell but are involved in different cellular roles during septation (155). The possible dependence of PBP2 localization on MreB in *R. sphaeroides* (and in *E. coli*) remains to be tested, and this point needs to be resolved for *C. crescentus* (see above). Work with *B. subtilis* showed that some PBPs fused to GFP also localize specifically to the sidewall in distinct foci and bands around the cell periphery, again reminiscent of the helical distribution of MreB and Mbl (146). This further suggested that PG synthesis occurs at distinct regions of the lateral wall and that new PG is inserted in a helical manner during the elongation-specific phase. In this study, however, the patterns of GFP-PBP localization were not detectably altered in the absence of either MreB or Mbl (146). Nevertheless, in light of the recent finding that MreB, Mbl, and MreBH colocalize in a single helical structure in *B. subtilis* (15), it remains plausible that PBP localization depends on more than one MreB isoform in this organism. The link between the MreB cytoskeleton and the cell wall synthesis machinery undoubtedly remains a major challenge for future work.

**MreBH filaments direct lateral wall hydrolysis in *Bacillus subtilis*.** It was recently shown that MreBH also has an important role in *B. subtilis* cell morphogenesis and that this function is effected, at least in part, by controlling the autolytic activity over the lateral wall by direct interaction with LytE, a cell wall hydrolase (15). Depletion of MreBH led to a mild cell shape defect, in contrast to the case for MreB- and Mbl-depleted cells (24). Similarly, under normal growth conditions, *mreBH* mutant cells displayed a mild phenotype; they were slightly affected in length and width and were bent at points that appeared correlated with abnormal thickening of the CW (15). However, all aspects of the *mreBH* mutant phenotype were strongly affected by the $Mg^{2+}$ concentration, and *mreBH* mutants required higher levels of $Mg^{2+}$ for viability than did the wild type (15). A similar dependence on $Mg^{2+}$ concentration has been observed for *mreB* (55; see above), *mbl* (15), and *mreCD* (95; see below) mutant cells and for several other *B. subtilis* mutants thought to be affected in different aspects of CW synthesis and structure, as mentioned before (94, 121, 138, 139). Strikingly, MreBH was found to specifically interact with LytE (previously thought to be a cell septum-specific autolysin [72, 127]) in a genome-wide two-hybrid screen, and the $Mg^{2+}$ dependence and shape defetcts of *lytE* mutants appeared remarkably similar to those of *mreBH* mutants (15). MreBH, like Mbl and MreB, forms dynamic helical filaments (see above and Fig. 1F) (15, 26). A functional LytE-GFP fusion localized to ongoing division sites and to cell separation sites, as previously observed by IFM (178), but surprisingly, it also localized as punctate fluorescence over the external lateral wall (15). Targeting of LytE to the sidewall of the cell was dependent on MreBH (and not on MreB or Mbl), while targeting of LytE to the sites of division was dependent on early (FtsZ) and late (PBP 2B) division proteins (15).

On the basis of the similar phenotypes, the direct protein-protein interaction in vivo and in vitro, and the MreBH-dependent localization of LytE along the lateral wall, it was concluded that MreBH influences lateral wall maturation by directing the localization of LytE (15). In light of these findings, together with the colocalization of all three MreB isoforms, an MreBH-dependent helical mode of cell wall hydrolysis that is coordinated with an Mbl-dependent helical mode of cell wall insertion has been suggested to control elongation of the rod-shaped *B. subtilis* cells during growth (15).

In summary, it is currently believed that the MreB helical structures are spatial regulators of cell wall biogenesis. How MreB-like proteins direct the insertion and maturation of CW material remains to be elucidated, but the current model is that they target/position cell wall enzymes (synthases and hydrolases) and/or membrane-associated and extracellular proteins, such as MreC and MreD (see below), that are involved in morphogenesis. It is interesting that the same genomic ar-

rangement of the *mre* gene cluster (*mreBCD*) upstream of the *mrd* gene cluster (*pbpA* and *rodA*, both of which are associated with lateral wall elongation), transcribed colinearly with the direction of replication, is found in *C. crescentus* (52), *R. sphaeroides* (156), *S. coelicolor* (9), *Magnetospirillum magneticum* AMB-1, and *Magnetospirillum magnetotacticum* MS-1 (NCBI and GenBank accession no. AP007255 and AAAP00000000 [unfinished sequence], respectively), suggesting a functional interaction between the encoded proteins and further supporting the concerted action of the MreB cytoskeleton and PG synthesis. A complex consisting of MreB, MreC, MreD, PBP2, and RodA has been proposed to function in the extension of the lateral CW (Fig. 3F and see below).

### Is Transmission of Shape Mediated by MreB-Directed Peptidoglycan Factories?

Over the last decade, findings on the growth and division of the PG sacculus have suggested the existence of a large multienzyme complex, consisting of both PG synthases and hydrolases, responsible for controlled growth of the sacculus. In the model proposed (for *E. coli*) by Höltje (68–70), this machine—a holoenzyme of peptidoglycan replication—uses the inner layers of the sacculus as a template (thereby ensuring the maintenance of cell width) at sites of growth and synthesis randomly distributed along the length of the cell (thereby ensuring smooth elongation). The complex proceeds along the PG strands, copies their length precisely, and catalyzes crosslinkage to the acceptor sites determined by the existing sacculus. As more strands are added, the layers of the cell wall are pushed apart and stretched, aiding their turnover (69).

Although biochemical evidence that PBPs are present in a complex is more and more compelling (2, 52, 148, 168) and a complex between a PG polymerase and a PG hydrolase was isolated in the presence of a scaffolding protein in *E. coli* (168), the model of Höltje has yet to be demonstrated conclusively. Nevertheless, it is extremely attractive to suppose that such multienzyme complexes exist and that they function as organized PG-synthesizing factories that are spatially controlled by the MreB helical scaffold to generate a functional CW architecture, and thereby the final three-dimensional structure of the cell. Several PBPs have been shown to display a putative helical distribution over the lateral cell wall, but the determinants of their localization remain unclear (28, 35, 146). However, the lateral (presumably helical) localization of one PG synthase (PBP2) and one PG hydrolase (LytE), in *C. crescentus* (40) and *B. subtilis* (15), respectively, was recently shown to be directly dependent upon MreB-like helices. These are the first (and almost certainly not the last) specific effectors of cell wall morphogenesis shown to be controlled by an MreB homologue in bacteria.

### The Essential MreBCD Complex and Lateral Wall Synthesis

In many bacteria, the *mreB* gene lies immediately upstream of the conserved *mreC* and *mreD* morphogenes, and it has been shown that *mreB* is cotranscribed with *mreC* in an operon in *B. subtilis* (55). In both *E. coli* and *B. subtilis*, the three cell shape determinants encoded by *mreBCD* are essential and required for the maintenance of the rod shape of the cell, and depletion of each *mre* gene separately confers similar gross changes in cell morphology (24, 88, 95, 96). *mreC* has also been shown to be essential in *C. crescentus*, where MreC-depleted cells also displayed morphological defects identical to those of cells depleted of MreB (or treated with A22) (40). Thus, it is currently believed that MreBCD proteins function in the same morphogenetic pathway (14, 35, 40, 88, 95, 96). Consistently, MreC and MreD in *B. subtilis* (95) and MreC in *C. crescentus* (35, 40) have been shown to localize along the cell length in banded patterns that resemble those of the helical cables formed by the MreB homologues. Cytological and biochemical fractionation experiments have shown that, in *E. coli*, MreC and MreD are associated with the cytoplasmic membrane (88), in agreement with predicted transmembrane domains in the two proteins. MreC is predicted to have a single transmembrane span near the N terminus and a large C-terminal domain outside the cytoplasmic membrane; MreD is highly hydrophobic and is predicted to have four to six transmembrane spans (Fig. 3F). GFP-MreC and GFP-MreD fusions were also clearly associated with the membrane in *B. subtilis* (95), whereas subcellular fractionation experiments showed that MreC is a periplasmic protein in *C. crescentus* (35).

Thus, the MreC and MreD proteins (with as yet unknown biochemical functions) were excellent candidates for proteins that interact with MreB (and/or Mbl). In a model in which the membrane-associated MreBCD complex directs lateral cell wall synthesis, in a process essential to maintain cylindrical elongation of rod-shaped cells, MreC/MreD could anchor the cytoplasmic MreB filaments to the membrane and couple them to the extracellular cell wall synthetic machinery (14, 88, 95). Using a bacterial two-hybrid system, it was found that *E. coli* MreC interacted with both MreB and MreD and with itself. MreB also self-interacted, consistent with its polymerization into filaments (88). These results suggested that the *mre*-encoded proteins might form a multiprotein higher-order complex in which MreC interacts with both MreB (and/or Mbl in *B. subtilis* [95]) and MreD (88). Further supporting this suggestion, MreB localization is perturbed in cells depleted for either MreC or MreD in both *E. coli* (88) and *B. subtilis* (25). In light of these and other findings, a model of the MreBCD complex and how it communicates with enzymes in the periplasm to direct lateral CW synthesis was proposed and is shown in Fig. 3F (88).

In *C. crescentus*, MreB helices and rings were still observed in MreC-depleted cells (40), and the helical or banded patterns adopted by MreC along the cell length were not affected by rapid disruption of MreB upon A22 treatment (35, 40). The mechanism by which MreC localizes helically remains unclear. MreC did not colocalize with MreB, suggesting that they form independent helical structures in *Caulobacter* (40). However, they anticolocalized, i.e., when both proteins formed helices, they interdigitated, but when MreB was a ring, MreC was absent from this site, and this requires there to be some communication between the two (40). PBP2 has been shown to partially colocalize with MreC (40), and biochemical evidence has been provided for a direct interaction between the two (35). In light of these findings, it was proposed that MreC promotes helical PBP2 localization along the lateral wall, i.e., lateral wall synthesis (40). Consistently, IFM studies of *R. sphaeroides* showed that MreC colocalized with PBP2 through-

out the cell cycle (while MreB colocalized with PBP2 only at certain stages of the cell cycle [see above]), again suggesting that MreC and PBP2 function in concert in PG synthesis during elongation (155). Interestingly, several PBPs (including PBP2 and PBP1) and outer membrane proteins were isolated from *C. crescentus* cell extracts by affinity chromatography using Sepharose-bound MreC (35). Undoubtedly, elucidation of the biochemical functions of the MreC and MreD proteins remains a major question for future research.

### MreB Proteins and Spore Lateral Wall Formation in Actinomycetes

In actinomycetes (*Mycobacter*, *Actinomyces*, *Nocardia*, and *Streptomyces* spp.), *mreB* homologues are present (and highly conserved) only in genera that form aerial mycelia and sporulate (112). *Streptomyces* spp. are gram-positive filamentous soil bacteria with a complex developmental cycle. This involves a phase of vegetative growth as branching hyphal filaments followed by the development of aerial hyphae, which undergo multiple septation and eventually differentiate into spores (for a review, see reference 54). Van-FL staining confirmed the previous finding that vegetatively growing *S. coelicolor* grows specifically by CW extension at the tips of the branching mycelia (22). However, *S. coelicolor* has two MreB isoforms, namely, MreB and Mbl, whose roles (in a bacterium that does not undergo cylindrical wall expansion) appeared particularly intriguing. In a recent study, Mazza et al. (112) addressed the role of MreB in the morphogenesis of *S. coelicolor* and demonstrated that MreB is dispensable for vegetative growth but essential for spore formation and assembly of the spore lateral cell wall. Previous attempts to create *mreB* mutants of *S. coelicolor* had been unsuccessful, leading to the suggestion that the gene is essential (9), but Mazza et al. (112) were able to generate a viable *mreB* null mutant. Surprisingly, growth and morphology of the substrate mycelium of the *mreB* mutant were normal, but aerial hyphae were irregularly shaped, swelled, and lysed, and spores doubled their volume and lost their cell wall consistence (112). MreB specifically localized at the septa and their derived poles in prespore chains, and it completely surrounded more mature spores (112). Although a role for MreB in vegetatively growing *S. coelicolor* cannot be excluded and although MreB and Mbl could display some functional redundancy in this organism, it was concluded that MreB primarily affects lateral wall formation, but exclusively during the sporulation process. The lateral CW of streptomycetes thickens and undergoes structural changes during spore maturation (54). Thus, the PG biosynthetic activity may be reorganized in sporogenic aerial hyphae, and MreB might play a key role in this process. Sporulating bacteria activate separate programs of gene expression and undergo different morphological changes during vegetative growth and during sporulation. However, spatial and temporal control of cell morphogenesis is required during the two independent developmental cycles. The work by Mazza et al. (112) suggests that the primary role of MreB in directing lateral CW formation might be conserved during sporulation, at least in *Streptomyces*. It will be interesting to see if MreBs are involved in spore formation in other bacteria. Interestingly, in *B. subtilis*, *mbl* lies immediately downstream of the sporulation gene *spoIIID*. It has been reported that *mbl* is constitutively transcribed from its own promoter during vegetative growth but also from a promoter located upstream of *spoIIID* and controlled by the sporulation-specific transcription factor $\sigma^E$ (43, 51). Whether MreBs play a role in sporulation in addition to their roles during vegetative growth is an important question for future work.

### MreB Proteins and Cell Shape Determination in Wall-Less Prokaryotes

As mentioned above, the cell wall is the major structure that defines the shape of most bacterial cells, and MreB proteins control cell morphogenesis by actively directing the synthesis and maturation of PG. However, not all prokaryotic cells possess a cell wall. Mollicutes (*Acholeplasma*, *Mycoplasma*, and *Spiroplasma* spp.) are enveloped only by a cholesterol-containing cell membrane, and even so (despite lacking cell walls), these cells display different morphologies. How is shape determined in these organisms? Interestingly, cells of *Spiroplasma citri* encode five MreB homologues, and MreB has been detected in cells of *Spiroplasma melliferum*, too (91). Are MreB proteins also involved in the morphogenesis of the wall-less *Spiroplasma* cells? Structurally, *Spiroplasma* cells are unique in having a well-defined, dynamic, helical cell geometry (Fig. 4A) and a membrane-bound, cytoskeleton-like ribbon, which follows the inner, shortest helical path of the cell (Fig. 4B) (163). The cytoskeleton from *Spiroplasma* was isolated a long time ago, and it was thought to consist of a single protein, the 55-kDa fibril protein, currently exclusive to *Spiroplasma* (162). Recently, Kürner et al. (91) used cryo-electron tomography to study the 3D structure of the *S. melliferum* cytoskeleton in a close-to-native state. Tomograms showed that this cytoskeleton possesses two types of filaments (thick and thin) arranged in three parallel ribbons that are anchored to each other and to the cell membrane and that span the length of the cell (Fig. 4A to E). The two outer ribbons consist of thick filaments of fibril, as expected. The inner ribbon appears to be composed of nine thin filaments with a spacing of ~4 nm (the same width as that reported for *T. maritima* MreB protofilaments [164]). Kürner et al. suggested that these were composed of MreB, and they assumed that MreB filaments give the *Spiroplasma* cell a rod-like shape. Although no proof that these ribbons are composed of MreB protein or that MreB is involved in cell shape determination in *Spiroplasma* was provided, the approach is extremely challenging and promises a bright future for the investigation of the ultrastructure and spatial organization of the cytoskeleton in both prokaryotic and eukaryotic cells.

## ACTIN-LIKE PROTEINS AND DNA SEGREGATION

Evidence has emerged during the last few years for both a force-generating mechanism of chromosome segregation in bacteria and a centromere-like locus in the bacterial chromosome (63, 79, 97–99, 145). It has been shown that the complex of replication proteins (the replisome) is more or less stationary at midcell throughout most of the cell cycle (99), whereas the origin of replication, as a first step (followed by other

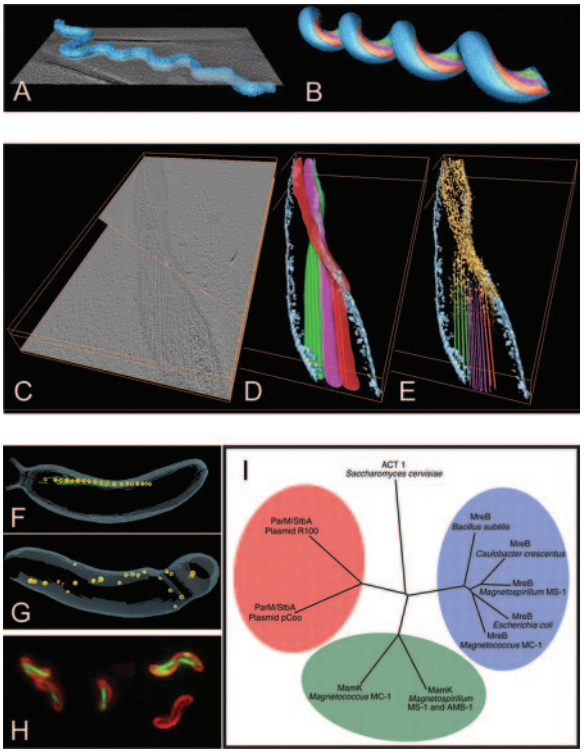


FIG. 4. Cytoskeleton structures organized by actin-like proteins in *Spiroplasma* and *Magnetospirillum* cells. (A to E) Cryo-electron tomography reveals the cytoskeleton structure of *Spiroplasma melliferum*. (A) Volume-rendering representation of a whole cell of *S. melliferum* with a tomogram slice cutting through it longitudinally. (B) 3D simulation of an *S. melliferum* cell indicating the localization of the three parallel ribbons of the cytoskeleton (red, purple, and green), which are anchored to the cell membrane (blue) and span the length of the cell. (Panels A and B were reprinted from reference 92 with permission from Elsevier.) (C to E) Superimposed slices of a tomogram (*z* sections are indicated by a bounding box) (C) and two corresponding 3D visualizations (D and E) of part of an *S. melliferum* cell showing the arrangement of the three ribbons. (C) The cytoskeleton comprises two outer ribbons of thicker filaments and a region of thinner filaments sandwiched between them. (D) Simplified 3D representation of the filament ribbons (green, purple, and red) that wind in parallel helically around the cell just underneath the cell membrane (blue). (E) Idealized visualization of the filaments, with a smooth transition to the original data (yellow). (Reprinted from reference 91 with permission of the publisher. Copyright 2005 American Association for the Advancement of Science.) (F to I) MamK, a homologue of MreB, organizes the magnetosome chain and forms filaments in vivo in *Magnetospirillum magneticum* sp. strain AMB-1. (F and G) 3D reconstructions of a wild-type AMB-1 cell (F) and a Δ*mamK* mutant cell (G). The cell membrane (gray), magnetosome membrane (yellow), magnetite (orange), and magnetosome-associated filaments (green) are rendered. (H) MamK fused to GFP (green) forms filaments that localize to the inner curvature of the cell (cell membrane is stained red with FM4-64). (I) Phylogenetic relationship between MamK and other bacterial actins, demonstrated by an unrooted tree. These proteins separate into three distinct groups, i.e., MamK (green), ParM/StbA (red), and MreB (blue). (Reprinted from reference 84 with permission of the publisher. Copyright 2006 American Association for the Advancement of Science.)

duplicated regions), rapidly moves toward opposite cell poles (177). Several genes involved in chromosome and plasmid segregation and their subcellular localization have been identified, but the mechanisms underlying prokaryotic DNA movement and positioning remained unknown. By analogy to the cytoskeleton-based eukaryotic spindle apparatus (83), the discovery of cytoskeletal filaments in bacteria made them good candidates for orchestrating chromosome segregation. The bacterial tubulin homologue FtsZ was an unlikely candidate; *ftsZ* mutants do not display segregation defects (109), and the localization of FtsZ at midcell is inconsistent with a role in segregating sister chromosomes toward opposite poles. However, an alternative and attractive possibility was put forward with the identification of bacterial actin homologues that form filamentous structures running the length of the cell.

### The Actin-Like ParM Protein and Plasmid-DNA Segregation

The first evidence of actin-like filament-forming proteins required for DNA segregation was obtained from the partitioning system of the low-copy-number plasmid R1 of *E. coli*. The R1 partitioning locus (*par*) carries or encodes three elements, namely, *parC*, ParR, and ParM, which together form a nucleoprotein complex that secures the faithful distribution of plasmid copies to the daughter cells before cell division (for a review, see reference 58). Two plasmid molecules are paired by the binding of ParR to 10 direct repeats of *parC*, a *cis*-acting centromere-like DNA sequence (78). ParM (*par*titioning *m*otor) is an ATPase that interacts with the ParR-*parC* complex (77).

**ParM.** Immunofluorescence microscopy revealed that ParM formed highly dynamic filaments along the longitudinal axis of the cell (Fig. 5A), which were essential for the DNA partitioning process and displayed the properties expected for a force-generating or -directing cytoskeletal element (120). ParM (also called StbA) was one of the four bacterial proteins (together with Hsp70, FtsA, and MreB) reported to belong to the actin superfamily by Bork et al. (7; see above). Like the case for MreB, structural homology was uncovered behind the functional homology, and ParM was shown to have an atomic structure closely related to that of eukaryotic actin (and MreB) (Fig. 5C) and to undergo ATP-dependent polymerization/depolymerization into double-helical filaments similar to actin filaments (Fig. 5B) (helical repeat sizes are as follows: for F-actin, 55 Å; for MreB, 51 Å; and for ParM, 49 Å) (165). Importantly, the structure of ParM was solved in two states, i.e., in the absence of nucleotide and with a bound nucleotide (ADP) (165). The transition between these two states (unbound and ADP bound, called closed and open conformations, respectively) was shown to involve a conformational change in which the two major domains (I and II) undergo a rigid twist of ~25° with respect to each other upon nucleotide binding. This finding was extremely significant because actin (and any actin-like molecule) subunits are expected to undergo a similar domain rotation upon nucleotide binding and/or hydrolysis, but so far only the closed state (relative to the ParM conformations) of G-actin (and MreB) has been trapped in crystals and observed at a high resolution.

**Model for in vivo function of ParM filaments.** Cytological and biochemical studies revealed that segregating plasmids localize to the ends of the dynamic ParM filaments and that ParM interacts specifically with the plasmid DNA-bound protein ParR in an ATP-dependent manner (119). It was proposed that ParM nucleates via the interaction with the *parC*-

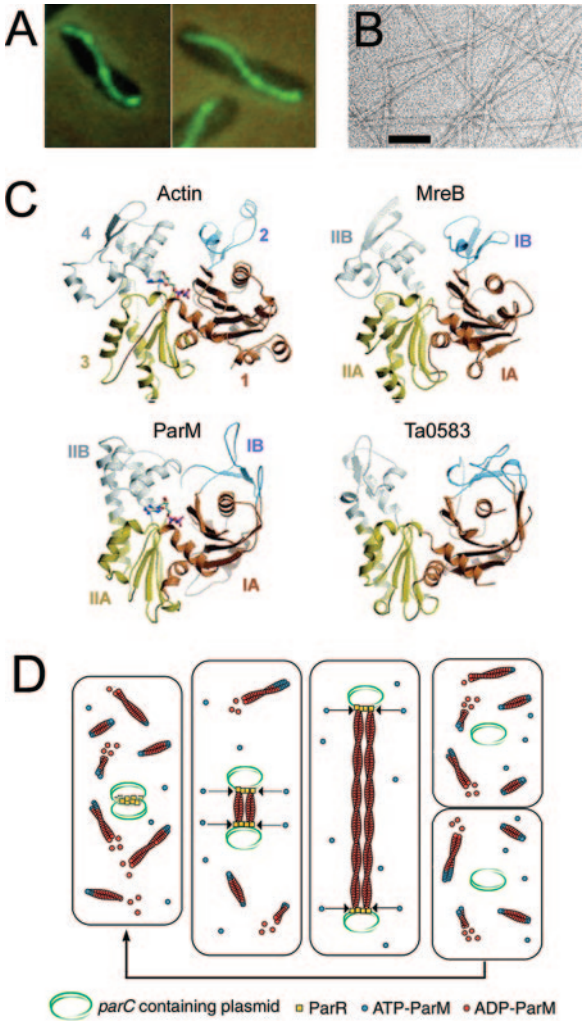


FIG. 5. Actin-like filaments formed by plasmid segregation protein ParM. (A) ParM axial filaments in *Escherichia coli* visualized by combined phase-contrast and immunofluorescence microscopy. (B) Electron micrograph of negatively stained ParM filaments. Polymers have a cross-sectional diameter of ~7 nm. Bar, 50 nm. (Panels A and B were reprinted from reference 120 with permission from Macmillan Publishers Ltd.) (C) Ribbon representation of the three-dimensional monomer structures of proteins of the actin superfamily, including eukaryotic actin and prokaryotic MreB, ParM, and Ta0583 (MreB–AMPPNP {adenosine 5′-[β-γ-imido]triphosphate}; ADP-bound form for the others) (PDB accession numbers are 1J6Z, 1JCG, 1MWM, and 2FSN, respectively). Subdomains IA, IB, IIA, and IIB correspond to subdomains 1, 2, 3, and 4 in actin. The ADP molecule bound to actin and to ParM is shown in ball-and-stick representation. (Reprinted from reference 137 with permission from Elsevier.) (D) Model for dynamic instability and in vivo function of ParM filaments during the cell cycle. ParM filaments (red) spontaneously assemble/disassemble, displaying dynamic instability in the cell. Upon plasmid (green) replication, plasmid pairs are held together by ParR-bound protein (yellow squares). Both ends of a set of ParM filaments are captured and stabilized by the ParR nucleoprotein complex. As more copies of ParM-ATP (blue) are added at the ParM-ParR interface, the filament/spindle elongates, pulling apart the plasmid copies toward either end of the cell before cell division occurs at midcell. (Reprinted from reference 57 with some modifications, with permission of the publisher. Copyright 2004 American Association for the Advancement of Science.)

ParR complex at midcell and that the subsequent filament polymerization provides the mechanical force to propel the associated molecules apart before cell division occurs (120). No other factors are known to be required for the partitioning process, making the *par* system the simplest and best-understood mechanism for DNA segregation in bacteria. Recently, Garner et al. (57) used total internal reflection fluorescence microscopy and fluorescence resonance energy transfer to provide spectacular evidence that the actin-like ParM filaments display dynamic instability and bidirectional polymerization (rather than polarized polymerization, like in F-actin [131, 132] and MreB [82; see above]). Dynamic instability (i.e., catastrophic decay) has usually been associated with microtubules (33) rather than with actin filaments and consists of periods of steady polymerization (elongation) followed by rapid disassembly (catastrophe) which are regulated by nucleotide hydrolysis. Interestingly, regulation of ParM filaments by dynamic instability appears to be an important component of the plasmid-DNA segregation process. These striking findings led to the current model, which is shown in Fig. 5D. According to this model, ParM spontaneously nucleates and polymerizes in the cell, and the filaments spontaneously depolymerize unless they are stabilized by interaction with ParR-*parC*-paired plasmids. Only ParM filaments with plasmid bound at both ends are stabilized against catastrophic disassembly, and bidirectional polymerization at the ParM-ParR interface drives plasmid segregation (57). Such an insertional polymerization merchanism has been proposed for elongating MT ends attached to kinetochores (8, 71, 116) and for various examples of actin-based motility (21, 34, 90, 128). Also, as mentioned above, MTs, but not F-actin, display dynamic instability. Thus, the function of the *par* system actually seems to be the direct equivalent of mitosis in eukaryotes. Do the bacterial actin-like ParM filaments use a force-generating strategy similar to that of the eukaryotic microtubule-based mitotic spindle? This apparent inversion of function is also extended to FtsZ, the tubulin homologue, which drives cytokinesis in bacteria, in a reversal of the actin-based contractile ring in eukaryotic cells. Is there a general inversion of actin and tubulin functions in the prokaryotic and eukaryotic lineages (59)? These findings are raising important questions regarding the evolution of the cytoskeleton. Furthermore, the identification of the ParM-dependent plasmid segregation mechanism raised the intriguing possibility of a similar, MreB-dependent, mitotic-like chromosome segregation mechanism in bacteria.

## The Actin-Like MreB Protein and Chromosomal DNA Segregation

Evidence from three divergent model organisms suggested that the morphogenetic MreB proteins might be involved in segregation of the bacterial chromosome. In *E. coli*, deletion of MreB resulted in segregation of the chromosomes in pairs (89), and overexpression of an ATPase mutant allele of *mreB* prevented nucleoid separation and simultaneously resulted in severe mislocalization of the origin region (*oriC*, the site of initiation of DNA replication) (Fig. 6A). Similarly, depletion of MreB in both *B. subtilis* and *C. crescentus* was reported to perturb DNA segregation before a defect in shape became visible, and MreB appeared to be required for polar localiza-

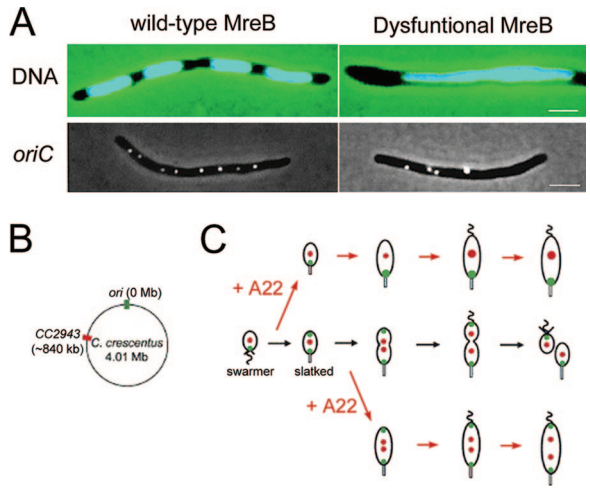


FIG. 6. MreB actin-mediated segregation of the bacterial chromosome. (A) Dysfunctional MreB inhibits chromosome segregation in *E. coli*. Cells ectopically overexpressed wild-type MreB or an MreB dysfunctional mutant (D165V), as indicated. The top row shows the nucleoid distribution (DNA stained with DAPI [4′,6′-diamidino-2-phenylindole]). The bottom row shows cells expressing a GFP-ParB fusion protein that binds to *parS* inserted near *oriC*. Bars, 2 μm. (Reprinted from reference 89 with permission from Macmillan Publishers Ltd.) (B and C) A22 blocks the segregation of origin-proximal, but not origin-distal, loci in *Caulobacter crescentus*. (B) Schematic of the genetic positions of the origin (green) and the CC2943 locus (red) in the *Caulobacter* genome. (C) Schematic illustration of the morphology and localization of the origin (green dots) and CC2943 (red dots) in untreated cells (middle row) and when A22 is added to swarmer cells (before segregation of the origins) (top row) and to stalked cells (after segregation of the origins) (bottom row). (Adapted from reference 62 with permission from Elsevier.)

tion of *oriC* (24, 61), although this role in *B. subtilis* is controversial (55; see below). The origin of replication is dynamically localized during the cell cycle, and it is the first region of the chromosome to be segregated in all bacterial species examined (104, 167, 175). The idea of a mitotic-like role of MreB in chromosome dynamics was therefore appealing, but demonstrating a direct role for MreB in chromosome segregation has proven difficult. MreB depletion is slow, pleiotropic, and ultimately lethal. Thus, it was difficult to assess if MreB's effect on chromosome dynamics was direct or a consequence of other functions or of general cellular deterioration. It is possible that the severe morphological defects of Δ*mreB* cells and/or polar effects of MreB depletion on downstream genes of the *mre* operon might affect chromosome segregation indirectly. The observation that rod-shaped (not perturbed in shape) *E. coli* cells expressing mutant MreBs do not segregate their chromosomes properly (Fig. 6A) (89) does support the idea of a direct role in segregation in this bacterium. However, this is less clear in the case of *B. subtilis*. Formstone and Errington (55) recently reported the construction of an in-frame deletion of *mreB* in *B. subtilis*. Remarkably, virtually normal growth and rod-shaped morphology were restored to the *mreB* null mutant in the presence of high concentrations of $Mg^{2+}$ and the osmoprotectant sucrose (55). Thus, under these conditions, it was possible to reassess a possible role of *B. subtilis* MreB in chromosome segregation, independent of shape defects and polar effects of downstream genes. Surprisingly, no detectable impairment of chromosome segregation or maintenance was evident in the complete absence of *mreB* (55). In contrast to other reports (24, 25), it was convincingly concluded that MreB does not have an essential role in chromosome segregation in *B. subtilis*. It remains possible, however, that a defect in chromosome dynamics in the *mreB* mutant is masked by more than one MreB homologue acting redundantly or by other factors under the conditions used in these experiments.

The first conclusive evidence of a role for MreB proteins in chromosome segregation, in particular, of a specific (centromere-like) region of the chromosome, was finally provided last year. In an attempt to rapidly disrupt MreB function and dissect the cellular activities of MreB in *C. crescentus*, Gitai et al. (62) explored the use of small molecules, an approach that has proven highly successful for dissecting the functions of the eukaryotic cytoskeleton (50). A22 is a small molecule previously identified through a random screen for inhibitors of chromosome partitioning in *E. coli* and found to induce spherical cells (73). A screen for *C. crescentus* A22-resistant mutants identified MreB (probably its nucleotide-binding pocket) as the cellular target of A22 (62). The addition of A22 affected *Caulobacter* growth and morphology in a dose-dependent fashion, and the progression of cell shape deformation was similar (albeit faster) to that caused by *mreB* depletion (62). Consistently, A22 specifically, rapidly (<1 min), and reversibly perturbed MreB localization. By labeling both the origin and a midcell-positioned chromosomal locus (Fig. 6B) in the same living cell, it was shown that A22 reversibly blocked chromosome segregation. This effect was not due to impairment of DNA replication or replisome formation, and it was observed in wild-type cells but not in one of the A22-resistant *mreB* mutants, indicating that A22 acts specifically through MreB. Thus, A22 allowed the acute role of MreB in chromosome segregation to be assessed for the first time, excluding any secondary effect caused by the change in morphology resulting from the long-term absence of MreB. The administration of A22 at different stages of the *Caulobacter* cell cycle demonstrated that the MreB-perturbing compound completely blocks the movement of newly replicated loci near the origin of replication but has no effect on the segregation of other foci if it is added after origin segregation (Fig. 6C). Finally, chromatin immunoprecipitation assays were used to demonstrate a specific physical association between MreB and origin-proximal foci. It was concluded that the origin-proximal regions of the chromosome directly or indirectly bind to MreB and segregate through an MreB-dependent mechanism and that the rest of the chromosome follows the origin, using an MreB-independent mechanism.

Earlier this year, Kruse et al. (87) showed that MreB is also the target of A22 in *E. coli* and that it interacts with RNA polymerase (RNAP) in vivo and in vitro. RNAP had previously been proposed as a driving force for chromosome segregation in bacteria (39, 101). Kruse and colleagues presented convincing evidence that both MreB and RNAP are required for chromosome segregation in *E. coli*. By investigating the effects of A22 (thus excluding indirect effects of morphological distortions observed in MreB-depleted cells), MreB was shown to be required for segregation of *oriC* and bulk DNA (i.e., the nucleoid and the terminus region). Inactivation of RNAP, either by the addition of rifampin (which blocks transcription

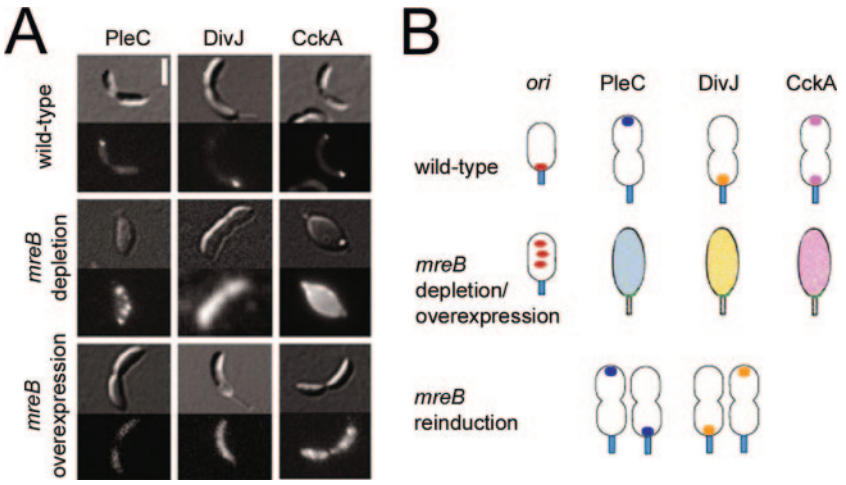


FIG. 7. MreB-directed polarity in *Caulobacter crescentus*. (A) MreB affects the localization of the PleC, DivJ, and CckA developmental regulators. Representative images of cells expressing PleC-GFP, DivJ-GFP, and CckA-GFP are shown. Shown are the localization in wild-type cells, in cells with *mreB* depleted for 24 h, and in cells overexpressing *mreB* for 6 h. For each cell, images shown were taken with differential interference contrast light microscopy (top image) and GFP fluorescence (bottom image). Bar, 1 μm. (Reprinted from reference 61 with permission of the publisher. Copyright 2004 National Academy of Sciences, U.S.A.) (B) Schematic representing the MreB-dependent localization of molecular markers of cell polarity. In the wild type (top row), the origin of replication (red ovals) and the histidine kinases PleC (blue ovals), DivJ (orange ovals), and CckA (purple ovals) localize to specific poles during the cell cycle. Depletion or overexpression of MreB (middle row) causes the origin to localize to multiple foci and PleC, DivJ, and CckA to delocalize and diffuse throughout the cell. Reinduction of MreB expression (bottom row) repolarizes PleC and DivJ, but the localization of the polar markers is randomized to either pole. (Reprinted, with some modifications, from reference 103 with permission from Elsevier.)

initiation in bacteria) or by using temperature-sensitive RNAP alleles, prevented nucleoid and terminus segregation (87). Finally, flow cytometry analyses showed that MreB depletion and inactivation of RNAP conferred virtually identical and unusual chromosome segregation defects. These findings raised the obvious question of the function of the MreB-RNAP interaction. Kruse et al. (87) suggested that MreB and RNAP function together in chromosome segregation, and they provided two possible interpretations of their data. One possibility is that the MreB cytoskeleton immobilizes the transcription machinery in such a way that the motor power of RNAP would drive chromosome segregation. Alternatively, and consistent with other findings that make the dynamic MreB cytoskeleton another possible candidate for the generation of the force needed for chromosome segregation (24, 26, 62, 89), chromosomal DNA bound by RNAP could be actively driven toward opposite cell poles by interaction with the MreB cables (87).

In summary, in light of the above stunning findings and the work with ParM, it is now believed that MreB filaments, in *C. crescentus* (and likely in *E. coli*) at least, actively direct the migration of the origins toward the poles, probably in coordination with lateral cell wall growth. However, the mechanism to achieve this remains to be elucidated. Whether the helical MreB filaments are used as "rails" for the *ori* regions to travel along (propelled by motor proteins that remain undiscovered), whether the treadmilling behavior of the MreB monomers through polarized MreB filaments is the driving force that generates movement (26, 82), or whether MreB filaments act as a ParM-type mitotic apparatus that pushes apart the *ori* regions awaits experimental demonstration. Another major challenge is to identify the putative centromere region of the bacterial chromosome and the putative protein complex (kinetochore complex) that interacts with this region to drive chromosome segregation.

## MreB-LIKE PROTEINS AND CELL POLARITY

The establishment and maintenance of a polarity axis and differentiation of the cell poles are fundamental aspects of the development of most cells. Indeed, essential processes such as growth, divison site placement, and movement rely on a defined axis of cell polarity. In eukaryotic cells, cytoskeletal systems provide the structural basis for cell polarization (38, 49, 134, 174), but the origin and the mechanisms responsible for cell polarization and asymmetry in prokaryotic cells remain poorly understood. Recently, a new function of the actin-like cytoskeleton was uncovered when MreB-like proteins were shown to be involved in generating cell polarity in *C. crescentus* and *E. coli* (61, 123, 152, 173). Gitai et al. (61) described the requirement for the MreB filaments in the establishment of global *C. crescentus* cell polarity, through MreB-directed asymmetric localization of developmental regulators at the cell poles. *C. crescentus* divides asymmetrically to generate two functionally different progeny cells, known as the swarmer cell (with a flagellum at one pole) and the stalked cell (with a stalk structure at one pole) (Fig. 2D). Prior to cell division, proteins that specify cell fate, such as the integral membrane histidine kinases DivJ, PleC, and CckA, are segregated asymmetrically to opposite poles of the cell (Fig. 7, wild type) (74, 103). A second molecular marker of cell polarity is the origin of replication, which dynamically localizes to one or both poles during the cell cycle (untreated cells [Fig. 6C]) (79). By examining MreB depletion and overexpression strains, Gitai et al. (61) demonstrated that MreB at the appropriate concentration is required for both the polar localization of the *oriC* sequence

and the dynamic localization of regulatory proteins to the correct cell pole (Fig. 7). The mechanism by which MreB mediates polar localization, however, remains to be elucidated. Analogous to the cytoskeleton-directed establishment of polarity in eukaryotic cells (38, 134, 135, 174), polarized MreB filaments could directly traffic the movement of polarity markers (75). Alternatively (or simultaneously), given the role of MreB in cell wall biosynthesis, it could affect the positioning of landmarks necessary for protein localization. The rapid reestablishment of polar colocalization of mislocalized polar proteins when *mreB* expression was reinduced in an MreB-depleted strain (Fig. 7B) favored the former possibility (61). Furthermore, the polar localization of asymmetric polar markers was restablished but randomized when MreB helices were reconstituted after depletion (Fig. 7B), suggesting a loss of memory and a general randomization of the cell's polarity. It has been proposed that MreB contains polarity information to determine subcellular localization, translated either from the molecular polarity inherent in an actin-like filament (61) or from signaling complexes recruited by landmark proteins at cell polarity sites (93). Indeed, TipN, a cell polarity determinant (landmark protein) recently identified in *C. crescentus*, has been suggested to be upstream of MreB in regulating cell polarity (93). TipN was shown to localize to the new pole through most of the cell cycle and to be essential for orienting the polarity axis of the cell. The MreB cytoskeleton was not responsible for the polar localization of TipN. However, TipN affected the dynamic organization of MreB during the cell cycle (93). In light of these findings, it was proposed that TipN recruits or organizes signaling proteins at the new pole and that this polarized signaling complex might polarize and transduce the positional information to the MreB filaments, which, in turn, might propagate polarization inside the cell and regulate the localization of polar markers (93).

Another report, however, favors the idea of a role for MreB in defining cell polarity through its ability to direct deposition of cell wall material. Wagner et al. (173) showed that *C. crescentus* requires MreB for stalk synthesis (suggested to be a specialized form of cell elongation) and also for stalk placement and prevention of ectopic pole formation, therefore defining polarity. MreB depletion led to a stalk elongation defect, and cells recovering from MreB depletion were unable to recover proper polarity and developed bumps and branches, often tipped by stalks, a strong indication that these sites were behaving as ectopic poles (173). Similar budding and branching phenotypes have been observed in *E. coli* mutants lacking multiple PBPs (29, 32, 179). It was concluded that MreB is critical to the maintenance of cell polarity and stalk placement in *C. crescentus*, probably through its role in PG synthesis (173). Interestingly, overproduction of Mbl in *B. subtilis* also led to branching and shape abnormalities: cells were branched at different angles and displayed Y shapes and deformed polar caps (13). Branches are unrelated to the division process and are generally understood as the consequence of local asymmetries or disturbances in the metabolism of the sacculus (66). The shape abnormalities resulting from the overproduction of Mbl were associated with an altered helical pitch of the filaments, and Mbl was consistently clustered at the bases of bumps and bud-like morphologies but not at the tips of developed branches (12, 13). For *E. coli* cells, it has been shown that PG (and outer membrane proteins) is stable at the poles and that the tips of branches behave as polar caps and are correlated with areas of inert PG (30, 32, 122). Thus, a strong correlation between the occurrence of cell wall active growth and Mbl clustering was suggested. Taken together, these observations support the idea that MreB proteins play a role in general cell polarity through their ability to direct the localization of proteins and deposition of new cell wall material.

Recently, two independent studies showed that the MreB cytoskeleton is required for polar targeting of several polar components in *E. coli* as well. Proteins displaying MreB-dependent polar localization in *E. coli* included the aspartate chemoreceptor Tar (inner membrane), the chemotaxis protein CheY (cytoplasmic), the *Shigella* sp. virulence factor IcsA (outer membrane), and the *Vibrio cholerae* type II secretion protein EpsM (inner membrane) (123, 152). Although IcsA and EpsM are polar constituents exclusive to other species, when introduced into *E. coli* they also localized to the poles (16, 144, 151), indicating that their mechanism of polar localization is conserved. In one of these two studies, *mreB* was identified in a screen of a transposon mutant library for factors required for the polar localization of IcsA in *E. coli* (123). In cells lacking *mreB*, IcsA localized to multiple faint patches over the entire surfaces of the cells. Similarly, EpsM and two distinct cytoplasmic domains of IcsA that are independently sufficient for its polar targeting (16) (co)localized in multiple peripheral foci within the cytoplasm of *mreB* mutant cells (123). It was concluded that MreB is required, either directly or indirectly, for the restriction of certain polar material containing positional information to defined sites in the cytoplasmic membrane and that, in the absence of MreB, multiple ectopic pole-like sites that contain such material (and that are at least partially competent for the secretion and insertion of polar outer membrane proteins) are formed (123).

The second study showed that targeting of Tar and IcsA to the poles is dependent on MreB and independent of the Min system (152). The Min system (MinCDE) is also involved in cell polarization in *E. coli* and other organisms, as it is required for the establishment of the division site at midcell (reviewed in references 141 and 143). The Min proteins oscillate repeatedly between polar zones by redistribution within a cytoskeleton-like helical structure that coils around the cell from pole to pole and that is similar to that formed by MreB (153). However, the two membrane-associated helical structures have been shown to be independent (152, 153; see below). Thus, the MreB and Min cytoskeleton-like systems appear to act independently in the establishment of an axis of polarity and in the differentiation of the cell poles in *E. coli* (152). Interestingly, it was recently reported that besides its (MreB-dependent) polar localization, Tar (and the histidine kinase CheA) also displays a helical arrangement in the lateral membrane (154). This was deduced to reflect the sites of membrane insertion of Tar via the general protein secretory (Sec) machinery, which was also shown to be arranged into a helical array in *E. coli* (154; see below). Since the Tar coils did not colocalize with those of MreB, it was concluded that the Sec/Tar helices are distinct from those of MreB and that Tar employs an indirect mechanism for polar localization: it is randomly inserted into lateral cytoplasmic membrane re-

gions, which are organized in a helical array independently of MreB, and then it migrates to the cell poles (154).

## MreB-LIKE PROTEINS AND CELL DIVISION

As described previously, the subcellular localization of MreB changes dynamically throughout the cell cycle, and MreB helices condense into a tight ring at the future plane of cell division in *E. coli*, *C. crescentus*, and *R. sphaeroides* cells (52, 61, 153, 156). In *B. subtilis*, MreBH at least also localizes in ring-like structures at future cell separation sites (15; see above). Such midcell localization patterns are reminiscent of those of several division proteins, which assemble in a ring-like structure at sites of imminent division (for a review, see reference 44). In dividing eukaryotic cells, F-actin localizes to midcell to form the actin-myosin contractile ring that drives cytokinesis (102). In bacteria, this role is performed by the tubulin-like FtsZ protein, which assembles into the filamentous Z ring that mediates cell division (109). The helical localization of MreB-like proteins was not affected in the absence of FtsZ (13, 15). However, the formation of division-plane MreB rings depended on FtsZ in *C. crescentus* (52, 61), suggesting that MreB proteins might also play a role in cell division. This idea is supported by several observations. First, cells of *C. crescentus* treated with A22 failed to divide, suggesting either the existence of a DNA segregation defect-induced cell cycle checkpoint or an independent role of MreBs in cell division (62). Second, cell division and the placement of the Z ring were perturbed in *B. subtilis mreB* and *mbl* mutants (55, 80). These defects were thought to be secondary to the propagation of the Z ring around the wider and irregular sidewalls of the *mreB* and *mbl* mutant cells. However, they could also result from a cooperative role of MreBs with FtsZ during cell division. MreB proteins could be directly responsible for orienting the FtsZ ring or could be part of a mechanism to localize the proper division plane. It has also been suggested that MreB could act discontinuously at the division site to reset the correct diameter each time the cell divides (55).

Alternatively, MreB may coordinate the cell division-elongation on-and-off control system believed to control growth in rod-shaped bacteria. According to the two-competing-sites model (105), there are two morphogenetic, spatially specialized, and presumably mutually exclusive pathways for PG synthesis, one for elongation (along the long axis) and the other for division (septum specific). The shape of the cell is determined by the balance between these two competing reactions (sites) (105). Indeed, an attractive hypothesis is that MreB controls the switch between the division and elongation CW growth modes. This is consistent with early studies of *E. coli* that suggested that MreB functions as a negative regulator of the septum-specific division protein FtsI (172) and with the observation that, also in *E. coli*, overexpression of wild-type *mreB* inhibits cell division (but has no segregation or shape defects) (Fig. 6A) (89). Furthermore, in *C. crescentus*, in the absence of MreB (or MreC), the elongation-specific PBP2 lost its helical pattern over the lateral wall and mislocalized to the division plane in an FtsZ-dependent manner (40). In wild-type cells, MreB transitioned between a spiral and a ring, whereas PBP2 (and MreC) always remained helical (40). Thus, it was proposed that MreC promotes helical PBP2 localization along the lateral wall (see above), whereas MreB inhibits division plane localization of PBP2 (40). The midcell (mis)localization of PBP2 in *C. crescentus* cells lacking MreB is consistent with the observation that, in *E. coli*, a GFP-PBP2 fusion localized in the bacterial envelope in a spot-like pattern and also at midcell during cell division (28). The unrelated PBP2 of *S. aureus* (coccoid, lacking MreB homologues) also localizes to midcell (where all CW synthesis occurs in this organism [see above]), and this targeting has been suggested to depend upon substrate recognition (130, 147).

It remains unclear whether MreB proteins play a primary role in cell division or if the effects on division are a secondary consequence of other functions. Whatever the mechanism, the function of MreB in this process remains an important question for future work.

## OTHER PROKARYOTIC ACTIN-LIKE PROTEINS

### MamK

Recently, homologues of MreB have been identified in magnetotactic bacteria. Magnetosomes are intracellular membranous organelles that contain magnetite (iron) crystals and are highly organized in linear chains to orient magnetotactic bacteria in geomagnetic fields, thereby causing the cells to align and to swim with respect to local or induced magnetic field lines. Using electron cryotomography, Komeili et al. (84) imaged *M. magneticum* sp. strain AMB-1 cells and found that the magnetosomes were positioned and organized by a network of cytoskeletal filaments (Fig. 4F). These ran parallel to four or five individual magnetosomes along a chain, with up to seven filaments flanking each magnetosome. The filaments, which were 200 to 250 nm in length and had a thickness of ~6 nm (just as expected for an F-actin filament), appeared to be composed of MamK, a homologue of MreB (84). A functional MamK-GFP fusion localized to straight filaments extending across most of the cell along its inner curvature (Fig. 4H), consistent with the magnetosome-associated filaments observed by electron cryotomography. (It is interesting that this localization is similar to that of the membrane-bound ribbon-like cytoskeleton of *Spiroplasma*, which is presumably composed of MreB filaments and also follows the inner curvature of the cell [see above and Fig. 4B].) In a *mamK* mutant, magnetosomes appeared to be dispersed throughout the cell, and no cytoskeletal filaments were associated with them (Fig. 4G). Although the possibility that MamK is required for the filaments to form without being part of their structure cannot be excluded, it was concluded that the MreB-like MamK protein is the most likely candidate for the cytoskeletal network that organizes magnetosome membranes into a chain roughly parallel to the long axis of the cell. MamK proteins of different species are more similar to each other than to their respective MreB paralogues. Thus, MreB, ParM, and MamK were predicted to form three phylogenically and functionally distinct groups of prokaryotic actin-like proteins (Fig. 4I).

### Ta0583

*mreB* genes are found in archaeal genomes, too (80). However, in contrast to their bacterial counterparts, almost nothing

is known about the archaeal actins. Very recently, the crystal structure of a predicted actin homologue, Ta0583, of the thermophilic bacterium *Thermoplasma acidophilum* was determined at a 2.1-Å resolution (137). The structure of Ta0583 showed that it clearly belongs to the actin superfamily. It contains the core structure of actin and subdomains IA, IB, IIA, and IIB of actin homologues (Fig. 5C). Ta0583 appears approximately equidistant from actin and MreB on the structural level and combines features of both bacterial actin homologues, i.e., MreB and the plasmid-encoded ParM protein. However, on both the sequence level and the structural level, Ta0583 is slightly closer to ParM than to MreB and actin, and the conformation of the nucleotide (ADP) cocrystallized with Ta0583 closely resembles that of ParM-bound ADP (i.e., in the open conformation of ParM) (165; see above). In vitro, Ta0583 displayed ATPase activity that was inhibited by A22 and formed crystalline sheets with a longitudinal repeat of 51 Å (F-actin repeat, 55 Å; MreB repeat, 51 Å; and ParM repeat, 49 Å). Although polymerization of Ta0583 was not induced by ATP (or GTP or ADP), its crystal packing indicated an inherent propensity to form filamentous structures (probably nucleated by as yet unknown factors). Sequence analysis showed that archaeal actin homologues are very divergent, and homologues of Ta0583 were found only in the order *Thermoplamatales*. On the basis of these findings, it was proposed that Ta0583 orthologues derive from a ParM-like actin homologue that was once encoded by a plasmid and was transferred into a common ancestor of the *Thermoplasmatales* (137). A peculiarity of the members of this order is that the cells are pleiomorphic and lack a cell wall. It was suggested that Ta0583 might be involved in cell organization (137). Establishing the function of Ta0583 is an important topic for future work.

## MreB-ASSOCIATED PROTEINS: TOWARD AN UNDERSTANDING OF THE FUNCTIONS OF THE BACTERIAL ACTIN-LIKE CYTOSKELETON

### Trafficking of Proteins: Going Helical

The subcellular localization of an increasing number of proteins, as detected by fluorescence microscopy, has been reported in the last few years. Since the discovery of the MreB helical filaments in 2001 (80), other proteins have been shown to localize in a helical pattern. Dynamic spiral-like structures are formed by FtsZ (and FtsZ-associated proteins) in *B. subtilis* and *S. coelicolor* cells undergoing sporulation (5, 64) and, under certain conditions, in *E. coli* cells (110, 159). Similarly, spirals that coil along the long axis of the cell are formed in *E. coli* by the MinC and MinD cell division inhibitors (152, 153; see above); the LamB outer membrane protein, which is involved in nutrient uptake (60); the Tar aspartate chemoreceptor and the CheA histidine kinase, which are members of the chemotaxis family (152); and the SetC sugar efflux transporter (46). They are also formed by some components of the highly conserved general secretory (Sec) machinery in both *B. subtilis* (11) and *E. coli* (154). However, all these helix-like structures seem to be independent of MreB proteins and their determinants remain unclear, indicating that there is no simple relationship between a spiral or helix-like organization and the actin-like cytoskeleton. The most striking example is probably that of the Sec machinery. In *B. subtilis*, the helix-like localization patterns of Sec components were also observed in either *mbl* or *mreBH* mutants and in MreB-depleted cells (11, 15). In *E. coli*, the helical arrays of Tar (presumably reflecting Sec-dependent sites of membrane insertion) and of MreB did not overlap (154; see above). It was concluded that the helical distribution of the Sec machinery is independent of the MreB cytoskeleton in these two organisms (11, 15, 154). However, this point has not been determined unambiguously and remains particularly intriguing. Indeed, the presence of multiple sites of protein export distributed in helical arrays in the rod-shaped *B. subtilis* and *E. coli* organisms is in marked contrast with the presence of a unique export site (namely, the ExPortal) in the coccoid organism *Streptococcus pyogenes*. In *S. pyogenes*, the Sec translocons are clustered in a single microdomain, which is in an asymmetric hemispherical location in the cellular membrane (140). Since the Sec machinery localizes to a single site in a spherical organism, which lack an MreB system, and localizes in a helical pattern in rod-shaped organisms that contain an MreB system, it is tempting to envisage a relationship between the two, which the data available at present cannot completely rule out. In *B. subtilis*, positioning of the essential Sec components might depend on, or communicate with, more than one MreB homologue (acting redundantly). For *E. coli*, it remains to be confirmed (i) that Tar is inserted in the membrane via a Sec-dependent process and (ii) that the noncolocalization of Tar and MreB helices does not result from the different dynamics of membrane proteins versus that of MreB cables in the cytosol. It is also possible that the MreB-directed helical PG (i.e., the rigid CW) anchors the Sec machinery (or certain kinds of membrane lipids) to form a helical array. Again, any perfect colocalization would be lost during growth because of the different dynamics of MreB cables versus expansion and rotation of the external CW.

SetB, an integral membrane protein of *E. coli* that is closely related to SetC (i.e., most likely part of a sugar transporter complex), is one of the three proteins (together with MreC and LytE [see above]) known so far to localize in a helix and to directly interact with an MreB homologue. SetB-GFP forms regular helical structures similar to the MreB helices, and SetB interacted with MreB in a two-hybrid assay (46). Interestingly, SetB was identified through a genetic screen for suppressors of the chromosome segregation defect exhibited by mutants of ParC, a subunit of topoisomerase IV responsible for topological unlinking of the chromosomes during segregation (45, 46). Deletion of SetB caused a delay in chromosome segregation, whereas its overproduction caused chromosome disintegration and stretching (46). It was suggested, although not proved, that the SetB and MreB helices are associated and that they work together in chromosome segregation in *E. coli*. It should be noted, though, that the helical structures formed by SetC and MreB did not colocalize. The SetB and MreB helices seemed spatially coordinated, but rather than overlapping, they seemed to be interwound and to describe parallel paths (similar to the MreB and MreC helices in *C. crescentus*) (40). Thus, the precise role of SetB in segregation, the determinant of its localization, and whether its two-hybrid interaction with MreB is biologically significant remain to be elucidated.

MreC (and MreD in *B. subtilis*) has also been shown to localize in a helical configuration that resembles that of MreB

(35, 40, 95; see above). In *C. crescentus*, GFP fusions to outer membrane proteins found to interact with MreC in vitro (by affinity chromatography coupled to mass spectrometry-based protein identification) displayed spiral, punctate, or banded patterns of localization similar to those adopted by MreC (and PBP2) (35). As in the case of MreC (35, 40), the localization of these outer membrane protein-GFP fusions was unperturbed by rapid disruption of MreB spirals by A22, but it was lost in cells with shape defects resulting from MreB depletion for an extended time period (35). It was proposed that a helical spatial organization might be a property shared by many periplasmic proteins and associated with regions of active PG assembly. MreB cables might be ultimately responsible for the accurate tracking of MreC and the PG-synthesizing complexes in a helical pattern. By these means, an intrinsic helical pattern in the PG layer is established, which can persist and propagate for a certain period of time in the absence of an internal MreB structure (35).

### Are There MreB-Associated Proteins That Modulate Filament Organization?

By analogy to eukaryotic actins, it is anticipated that a myriad of MreB-associated proteins exist in the cell (at least 170 distinct actin-binding proteins have been identified in eukaryotic cells) and that some of them modulate filament dynamics. Several proteins that interact with the tubulin-like FtsZ protein and play a role in the dynamics of FtsZ assembly in vivo have been identified. ZapA is an FtsZ-polymerizing factor (65), while EzrA, MinC, and SulA inhibit FtsZ assembly in *B. subtilis* (44, 100). The actin-like DNA-segregating ParM protein is stabilized via the interaction with the ParR-*parC* nucleoprotein complex (119; see above). However, no interacting proteins are yet known for MreB-like proteins that spatially or temporally regulate their assembly/disassembly and/or that are involved in monomer or protofilament interactions. EM showed that MreB, unlike F-actin and ParM, spontaneously forms rings without auxiliary proteins (47). Nevertheless, the diameter of the rings formed by MreB in vitro was smaller (approximately one-fifth) than that observed in vivo (80). Cross-linking factors and/or associated proteins may be required to anchor the MreB bundles to the membrane and to generate a larger spiral diameter around the periphery of the cell. Clearly, future research will prompt the identification of binding partners and interacting modulators that would help us to understand how MreB proteins function and are organized in the bacterial cell.

### The MreB Hub: a Central Organizing Role for the Actin-Like Cytoskeleton

Like the case for MreB/ParM and actin (or FtsZ and tubulin), no homologues of actin-binding proteins can be identified in bacteria by amino acid sequence comparison. A combination of both genetic and biochemical screening methods is therefore needed to uncover MreB-interacting proteins. In a pioneer work, Butland et al. (10a) recently reported the first large-scale analysis of protein complexes in *E. coli*. In this study, 648 affinity-tagged proteins (about 16% of the *E. coli* proteome), expressed at endogenous levels, were purified to homogeneity, and their interacting protein partners were identified by mass spectrometry. An interaction network containing conserved and essential protein complexes involved in diverse biological processes was uncovered. Most of the proteins had few interacting partners, whereas a subset of hubs formed a great number of connections. These hubs are predicted to be highly conserved. MreB appeared to be one of the 20 most connected nodes (>15 interactions), and it was included in the set of 71 proteins that potentially fulfill critical roles across all bacteria.

Similarly, an actin cytoskeleton protein interaction network, composed at present of >65 interactions, has been established in *B. subtilis* by genome-wide yeast two-hybrid screens (R. Carballido-López, unpublished data). The proteins shown to specifically interact with MreBCD, Mbl, and MreBH belong to very different functional categories, anticipating that MreB-like proteins might be involved in a variety of cellular processes. Furthermore, many of these interactions link the actin cytoskeleton network to the replication network (126), the division network, and the preliminary stress response network (P. Noirot, unpublished data) of *B. subtilis*, further suggesting the centrality of the cytoskeleton in coordinating key cellular functions.

In summary, large-scale approaches have identified putative MreB-interacting partners and provided insights into the functions of previously uncharacterized bacterial proteins while outlining the overall topology of a microbial interactome whose core components (such as MreB) are broadly conserved across prokaryotes. The biological significance of each protein interaction identified by these approaches, however, needs to be demonstrated.

## CONCLUSIONS

The discovery of the prokaryotic homologues of actin (as well as of other cytoskeletal elements in bacteria) has forced a radical rethink about the origin of the cytoskeleton and revolutionized our view of bacterial cell architecture. The current view is that of a complex, highly structured, and dynamic bacterial subcellular organization, where the actin cytoskeleton, like the case in eukaryotic cells, plays a central organizing role. Although the mechanisms by which MreB-like proteins mediate insertion/degradation of CW material, segregation, and polarity remain to be elucidated, it has been shown that they are involved in these (and probably other) essential cellular processes. It is thought that MreB filaments serve as an organizer or tracking device for the movement of chromosomes and for the targeting of many proteins to their sites of biological function, thus playing a role analogous to the eukaryotic cytoskeleton in macromolecular trafficking. A major challenge now is to identify the targets that might move along and/or that are positioned by the actin-like filaments. The mechanistic details of how MreB proteins function and how they are spatially and temporally organized using other cellular factors are also main directions for future work.

Although we now know that actin filaments have almost certainly existed for more than 2 billion years, eukaryotic and prokaryotic actins seem to have developed into genuine cytoskeletal systems with very different and highly extended evolutionary histories (37). However, despite different functionalities and probably uniquely associated proteins, new insights

into our understanding of eukaryotic actin might be obtained by studying its bacterial orthologues. Despite decades of effort, the atomic structure of F-actin has not yet been resolved and was deduced only from the atomic structure of the actin monomer by modeling (67). MreB crystals (164) have provided the first ultrastructural view of actin-like strands at atomic resolution. ParM crystals (165), on the other hand, have provided the first approach at atomic resolution to the multiple conformational states presumed to exist in F-actin and all actin homologues (42, 56).

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