The cell-shape protein MreC interacts with extracytoplasmic proteins including cell wall assembly complexes in *Caulobacter crescentus*

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The bacterial actin homolog, MreB, forms helical cables within the cell that are required for maintenance of a rod shape. These helical structures are thought to be involved in the spatial organization of cell wall (peptidoglycan) synthesizing complexes of penicillin-binding proteins (PBPs). Here, we examined the role of the MreC cell shape protein in this process in *Caulobacter crescentus*. Subcellular fractionation experiments showed that MreC is a periplasmic protein and, as assayed by immunofluorescence microscopy, adopted helical or banded patterns along the cell length reminiscent of those formed by MreB and PBP2. The pattern of MreC and PBP2 localization remained when MreB cables were disrupted by treatment with the inhibitor A22. However, long-term absence of MreB led to cell shape changes and an eventual loss of MreC localization, suggesting that an independent structure, perhaps an intact peptidoglycan layer, contributes to the MreC localization pattern. Using affinity chromatography with MreC covalently bound to Sepharose, we isolated several PBPs from cell extracts that eluted from the column as heterogeneous complexes. In this same experiment, using mass spectrometry-based protein identification, we identified several outer membrane proteins, including TonB-dependent receptor transport proteins, that interacted with MreC. Imaging live cells containing fusions of these outer membrane proteins to green fluorescent protein showed that they adopted a subcellular localization pattern that was similar to that of MreC. These results suggest that MreC may function in the spatial organization of PBPs as well as other proteins that lie outside the cytoplasmic membrane.

MreB | penicillin binding proteins | peptidoglycan

Bacteria possess the remarkable capacity to generate progeny cells of almost identical size and morphology. For rod-shaped bacteria, the informational template for cell morphogenesis is thought to lie within the structure of the cell wall itself because isolated cell wall sacculi retain the overall shape of the intact cell (1). The bacterial cell wall, or peptidoglycan, consists of a glycan polymer of *N*-acetylglucosamine and *N*-acetyl muramic acid that wraps around the circumference of the cell, spanning the cell length. These glycan strands are cross-linked to each other via short peptides, thus forming a netlike structure that covers the entire cell surface (1). The peptidoglycan is assembled from intracellularly synthesized precursors by a family of enzymes known collectively as penicillin-binding proteins (PBPs) (1, 2). In general, the PBPs fall into two different classes; the high-molecular-weight PBPs (PBP1a, PBP1b, etc.) catalyze the synthesis of the glycan strands (transglycosylation) and peptide cross-links (transpeptidation), whereas the low molecular weight PBPs (PBP2, PBP3, etc.) perform only the transpeptidation reaction (1, 2). The nascent peptidoglycan is thought to be added to preexisting peptidoglycan at gaps in the cell wall that are created by the action of lytic transglycosylases.

For the morphology of rod-shaped bacteria to be maintained and propagated through successive generations, the activity of the PBPs and the lytic transglycosylases must be spatially coordinated with each other and temporally in step with the progression of the cell division cycle (1, 2). How this is accomplished is poorly understood; however, a number of mutants with defects in rod shape have been isolated and studied both in *Escherichia coli* and *Bacillus subtilis*. Several of these mutant strains contain mutations in PBPs (3–6), an observation consistent with the notion that the cell wall is a major determinant of cell morphology. Additional rod-shape mutants have defects in genes that do not encode enzymes directly responsible for peptidoglycan synthesis, such as *mreB*, *mreC*, *mreD*, and *rodA* (7–18). One of these, *mreB*, has been shown to encode a bacterial actin homolog (19) that, in a number of diverse bacterial species, forms helical filaments underneath the cytoplasmic membrane along the length of the cell (13, 15–17, 20–23). The mechanism by which MreB maintains the rod shape of the cell is not known, although experiments suggest that it may be responsible for spatially coordinating the activities of the cell wall synthesizing machinery. In *B. subtilis*, it has been shown that fluorescently labeled vancomycin, an inhibitor of cell wall synthesis, adopts a helical pattern of localization, suggesting that new peptidoglycan is assembled onto preexisting cell wall in a helical shape (24). This pattern of localization requires the MreB homolog, Mbl, which also localizes in a helical-like pattern across the length of the cell. Additionally, in *Caulobacter crescentus*, PBP2, which is thought to be responsible for the longitudinal growth of peptidoglycan, localizes in a helical pattern similar to that exhibited by MreB (15). MreB has also been demonstrated to fulfill other cytoskeletal functions, such as playing a role in the establishment of polarity (25) and chromosome partitioning (19, 25, 26).

The two genes that are almost invariably located downstream of *mreB*, *mreC* and *mreD*, encode proteins of unknown biochemical function. In *E. coli* and *B. subtilis*, both of these genes have been shown to be required for the maintenance of the rod shape of the cell (8–10, 14, 17, 18). In *B. subtilis*, MreC and MreD were found to localize in a helical or banded pattern similar to that exhibited by MreB (18). The localization pattern of MreB in *B. subtilis* and *E. coli* was found to depend on the presence of MreC and MreD (17, 18). Additionally, two-hybrid experiments with the *E. coli* proteins have shown that MreC interacts with both MreB and MreD (17), suggesting that these proteins may function in a complex. Depletion experiments in *B. subtilis* have shown that cells deficient in either MreC or MreD can survive and divide in the presence of high magnesium concentrations (18). Under these conditions, these sphere-like cells grow and divide exclusively by using the cell division machinery, indicating MreC and MreB function in coordinating lateral growth of the cell wall.

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Abbreviation: PBP, penicillin-binding protein.

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Here, we investigate the function of MreC in *C. crescentus.* We demonstrate that MreC is a periplasmic protein that localizes in helical patterns along the cell length. This pattern of localization, as well as that of PBP2, requires a normal cell shape, but is not disrupted in the absence of intracellular MreB cables. Affinity chromatography revealed that a heterogeneous complex of PBPs interacted with purified MreC. Furthermore, we also isolated several outer membrane proteins that bound to MreC. These proteins exhibited a banded subcellular localization pattern that was strikingly similar to that observed with MreC, MreB, and PBP2. These results suggest that MreC may function in the spatial organization of proteins that lie outside the cytoplasmic membrane.

Materials and Methods

Biochemical Techniques. Cells of *C. crescentus* LS107 (*syn-1000* Δ*bla6*) (wild-type, synchronizable strain, ampicillin resistant) (27) were grown to mid-logarithmic phase (OD_{600} = 0.7–1.0) and subjected to subcellular fractionation as described (15). For affinity chromatography, 6× His-tagged MreC was covalently linked to cyanogen bromide-activated Sepharose. MreC-interacting proteins were isolated from cell extracts solubilized with Triton X-100 (1% wt/vol) (28, 29) (for detailed procedures, see *Supporting Text*, which is published as supporting information on the PNAS web site).

Microscopy. To assay the localization of MreC, MreB, and PBP2 by immunofluorescence microscopy, cells were treated as described previously (30). MreC antibody was generated by a commercial source (Cocalico), using a 6× His-tagged N-terminal truncated portion of the *mreC* gene missing the predicted periplasmic targeting sequence. The MreC antibody was affinity purified by using an MreC-coupled Sepharose column. A22 was used at a concentration of 50 μg/ml. The localization of outer membrane proteins was accomplished by visualizing C-terminal GFP fusions in live cells. All samples were examined by using a Deltavision Spectris deconvolution microscopy system. Up to 15 0.1-μm optical sections were acquired and then deconvolved by using Applied Precision software.

Results

MreC Is a Periplasmic Protein that Localizes in a Helical Pattern. MreC homologs in many organisms are predicted to have a single transmembrane helix near the N terminus, with the majority of the polypeptide existing outside the cytoplasmic membrane. Analysis of the *C. crescentus* MreC sequence using a protein sorting algorithm (PSORT; http://psort.nibb.ac.jp; ref. 31) predicted that MreC was a periplasmic protein. We tested this prediction by fractionating *C. crescentus* cells and determining the subcellular location of MreC by immunoblot using anti-MreC antibodies (Fig. 1*A*). This experiment showed that the majority of the MreC was located in the periplasmic fraction, with a lesser amount, possibly as a consequence of cell lysis, present in the cytoplasmic fraction. As controls, we also assayed the location of ParB, a cytoplasmic protein, and PBP2, a cytoplasmic membrane protein (Fig. 1*A*). ParB was found almost exclusively in the cytosolic fraction. PBP2 was present only in the integral membrane protein fraction (Fig. 1*A*). Thus, this experiment indicates that MreC is a periplasmic protein in *C. crescentus*.

To determine whether MreC adopted a pattern of localization that was similar to that observed with MreB, we assayed MreC localization by using immunofluorescence microscopy with affinity-purified anti-MreC antibody (Fig. 1*B*). MreC localization appeared as two different patterns: the protein appeared as a helix that traversed the length of the cell, making approximately two to three complete turns from one end of the cell to the other (Fig. 1*B a–c*) or adopted a pattern that consisted of a series of bands perpendicular to the wide axis of the cell, often distributed along the cell length (Fig. 1*B d* and *e*). The number of bands in many of the smaller cells were suggestive of a helix that wrapped around the cell surface two to three times in smaller cells (Fig. 1*Bd*), and approximately five to six times in larger predivisional cells (Fig. 1*Be*). Both patterns of MreC localization were similar to those observed previously in *C. crescentus* with MreB (15, 16, 26) and PBP2 (16). We also tested whether the MreC localization pattern changed dynamically during the cell cycle. Interestingly, unlike MreB, the pattern of MreC localization was relatively static throughout the cell cycle (data not shown), similar to that observed with PBP2 spiral structures (15).

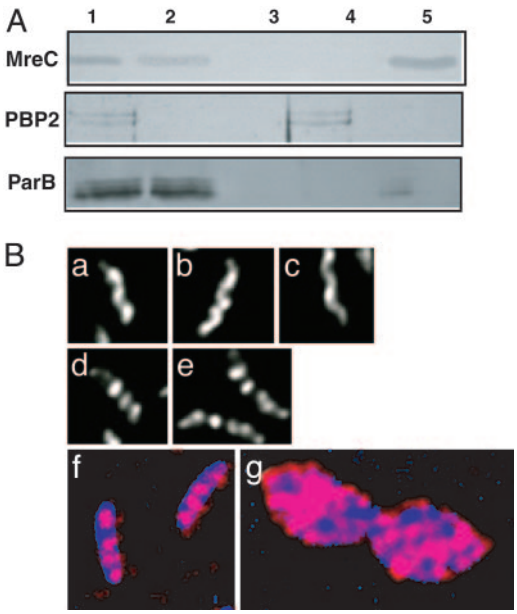

Fig. 1. MreC is a periplasmic protein that localizes in a helical pattern. (*A*) Subcellular fractionation of *C. crescentus* cell extracts was performed and the isolated fractions were subjected to immunoblot analysis using the indicated antibodies. Lanes 1, whole cell extract; 2, cytoplasmic fraction; 3, peripherally associated membrane-associated proteins; 4, integral membrane proteins; and 5, periplasmic fraction. PBP2 is an integral membrane protein, whereas ParB is a cytoplasmic protein. (*B*) Immunostaining of MreC with affinity purified rabbit antibodies and secondary antibody coupled to the fluorophore Cy-3 in wild-type (*a–e*) cells and MreB-depleted cells (*g*). MreC appears either as a fully connected helix (*a–c*) or as regularly spaced foci (*d* and *e*). Cells of strain JG5000, containing a xylose inducible *mreB* were grown in the presence of xylose, washed, and suspended in fresh medium lacking xylose. (*f*) MreC localization (pink) before the removal of xylose. (*g*) MreC localization after growth for 10 h without xylose. In *f* and *g*, DAPI (blue) was used as a counterstain for DNA.

We then tested whether the MreC localization pattern would be maintained in lemon-shaped MreB-depleted cells (Fig. 1*B f* and *g*). In this strain, *mreB* expression is under the control of a xylose inducible promoter (15). After 10 h of incubation in medium lacking xylose, the cells were devoid of detectable MreB (data not shown), had adopted a lemon-like shape, and lost the characteristic helical pattern of MreC localization (Fig. 1*Bg*). Instead, MreC appeared in disorganized patches of different sizes unevenly distributed throughout the cell. Thus, MreC requires a normal cell morphology to exhibit a helical localization pattern.

MreC and PBP2 Remain in Helical Subcellular Structures in the Absence of MreB Cables. The loss of MreC spirals after the depletion of MreB for an extended time period could indicate that this localization pattern either depended on MreB or required an intact peptidoglycan structure. To distinguish between these two

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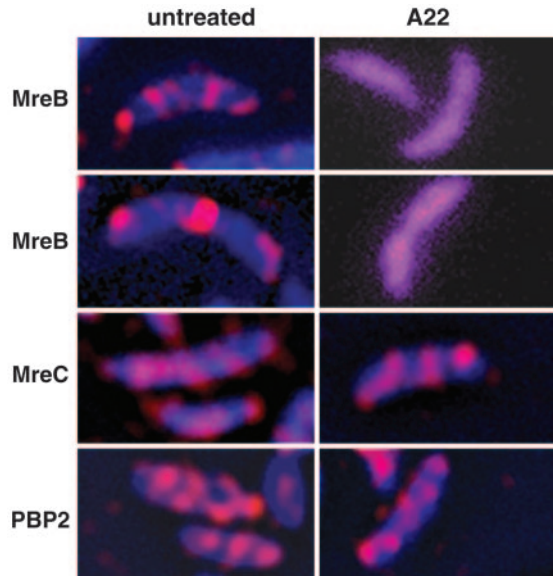

Fig. 2. The MreC and PBP2 pattern of localization is maintained in the absence of MreB spirals. Localization of MreB, MreC, and PBP2 was assayed by immunofluorescence microscopy in wild-type cells that were either untreated and treated with A22 (50 μg/ml for 2 h), an inhibitor of MreB polymerization agent. Localization of indicated protein (pink) is shown overlaid with a DAPI-counterstain (blue).

possibilities, we assayed the localization of MreC in cells that were devoid of detectable MreB cables but had not undergone significant changes in shape (26). Previous experiments have demonstrated that the compound A22, when added to *C. crescentus* cultures, causes the relatively rapid disappearance of MreB helical structures well before there exist observable changes in cell shape. We treated cells with A22 and assayed the localization of MreC by using immunofluorescence microscopy. After a 2-h incubation period with the compound, the characteristic helical pattern of MreB localization was completely abolished (Fig. 2). This treatment also disrupted the localization of MreB at the midcell of predivisional cells (Fig. 2). In contrast, when these same cells were assayed for MreC localization (Fig. 2), there was no discernible alteration in the pattern of MreC observed after incubation in the presence of A22. PBP2 has also been shown to localize in a banded pattern similar to that exhibited by MreB (15). We also tested whether the localization pattern of PBP2 was perturbed after treatment with A22. We observed no significant change in the pattern of PBP2 localization in the presence of A22. PBP2 localization appeared in a banded or punctate pattern reminiscent of MreB and MreC localization in both A22-treated and untreated cultures (Fig. 2).

MreC Directly Interacts with Penicillin Binding Proteins. Depletion of MreB in *C. crescentus* leads to changes in cell shape and defects in the integrity of the cell wall (15, 16), prompting the hypothesis that the helical MreB cytoskeleton spatially coordinates the activity of the peptidoglycan synthesizing enzymes (PBPs). Because MreC resided in the periplasm where peptidoglycan synthesis occurs, we determined whether MreC and PBP2 interacted with each other. We covalently bound MreC to Sepharose beads and assayed for retention of penicillin binding proteins using column affinity chromatography. This strategy was similar to that used to show that PBPs of *E. coli* reside in complex with the lytic transglycosylase, MltA (29). The MreC–Sepharose was incubated with a Triton X-100 whole cell extracts, and after extensive washing, the retained proteins were eluted with a linear salt gradient (0.15–1.0 M NaCl), and fractions of the eluate were collected. A Tris-coupled column was run in parallel and retained none of the assayed proteins (see Fig. 6, which is published as supporting information on the PNAS web site). The fractions were subjected to immunoblot analysis using antibodies directed against the cytokinesis protein FtsZ, PBP2, MreB, and the chemotaxis receptor, McpA. The cytoplasmic FtsZ protein and McpA did not show any retention on the column (Fig. 3*A*). We were also only able to recover MreB in the flow-through and wash fractions (Fig. 3*A*). It is possible that detergent solubilization of the cell membrane material during extract preparation disrupted a potential MreB–MreC interaction. In contrast, PBP2 was present in almost all of the fractions, even those eluted with high concentrations of NaCl (Fig. 3*B*), suggesting that MreC interacts with PBP2 in cell extracts.

To examine whether other PBPs were also bound by the immobilized MreC, we labeled the eluted column fractions with a penicillin analog covalently bound to fluorescein (Bocillin FL). Analysis of the labeled polypeptides with a fluorescence gel scanner showed the retention of several penicillin binding pro-

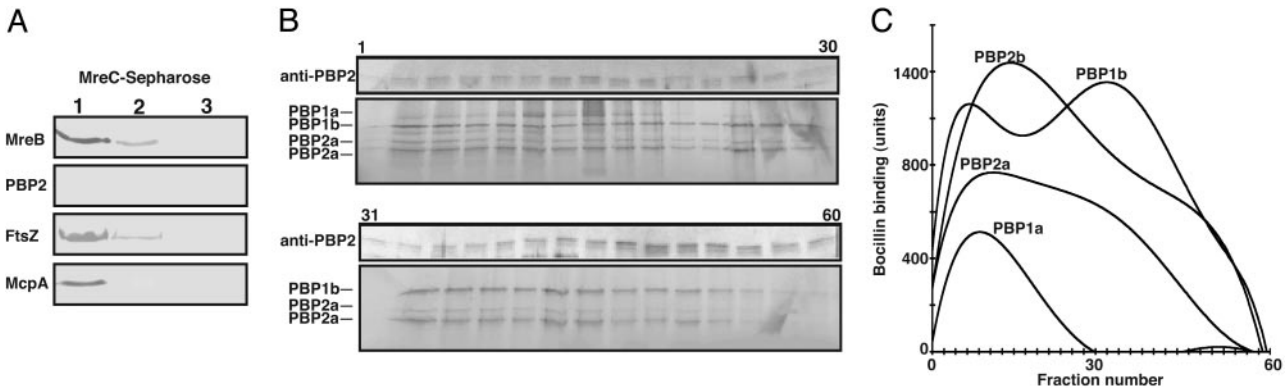

Fig. 3. MreC interacts with several different penicillin binding proteins *in vitro*. (*A*) Flow-through (lane 1), first wash (50 mM NaCl, lane 2), and second wash (150 mM NaCl, lane 3) fractions from an affinity chromatography experiment using detergent solubilized crude cell extracts applied to a MreC-Sepharose column were assayed for the presence of MreB, PBP2, FtsZ, and McpA by using immunoblot with specific antisera. (*B*) Fractions collected from a linear salt gradient elution following the initial washes of this affinity chromatography experiment. These isolated fractions (as indicated by numbers) were assayed for the presence of PBP2 by immunoblotting (anti-PBP2) and the presence of PBPs by affinity labeling with a fluorescently labeled penicillin derivative followed by SDS/PAGE and then transfer to a nitrocellulose membrane. The individual PBPs are indicated. [Note that the individual PBPs were assigned numbers according to previously documented profiles (15) and apparent molecular weight.] (*C*) Quantitation of fluorescently labeled penicillin binding to PBPs present in the column eluates as a function of fraction number.

teins by the MreC-coupled column (Fig. 3*A*). These included PBP2a and PBP2b as well as the high molecular weight PBPs, PBP1a and PBP1b. Like PBP2, some of these other PBPs eluted continuously over the entire span of the NaCl gradient. We were only able to detect PBPs using a labeled penicillin binding assay and not with a stain for proteins. Quantitative analysis of the fluorescence intensity of each labeled PBP showed that some species, such as PBP1a, exhibited a distinct peak in their elution profile, whereas others such as PBP2a and PBP2b were found distributed in most of the eluted fractions (Fig. 3B). Note that labeled PBP2 species are not detectable in the fractions containing higher concentrations of NaCl, but are detected by immunoblot analysis possibly attributable to decreased enzymatic activity at high NaCl concentrations. The wide elution profile of the PBPs observed here suggests that the PBPs bound to the MreC column in rather heterogeneous complexes.

Outer Membrane Proteins That Interact with MreC Exhibit a Similar Pattern of Subcellular Localization. Affinity chromatography using MltA of *E. coli* resulted not only in the recovery of penicillin binding proteins, but also several other proteins presumably derived from the periplasm (29). We wanted to determine what other proteins might bind to MreC and/or penicillin binding proteins in *C. crescentus*. Therefore, the fractions from the MreC–Sepharose column were subjected to SDS/PAGE and the gel was stained with Coomassie blue. The stained protein bands were then excised and subjected to mass spectrometry-based protein identification. The most abundant identified proteins included predicted outer membrane *C. crescentus* proteins (Fig. 7 and Table 1, which are published as supporting information on the PNAS web site). Among these were a number of the proteins belonging to the TonB-dependent receptor family of transport proteins (CC0991, CC0210, CC2820, CC3013, CC1750, CC2819). Other outer membrane proteins retained by the MreC–Sepharose were CC3494, a conserved OmpA family protein, and CC0163, a highly conserved outer-membrane protein of unknown function. Additionally, MreC was recovered in this experiment in relatively abundant quantities. Significantly lesser amounts of several cytosolic proteins including ribosome-associated proteins (EF-Tu, ribosomal proteins L6, L4, S4, S5) were retained by the MreC-Sepharose (Fig. 7 and Table 1). These are highly abundant proteins that are likely binding in a nonspecific fashion to the MreC–Sepharose, not an uncommon occurrence with protein affinity chromatography using crude cell extracts.

Because MreC is localized to the periplasm, we focused on an analysis of the outer membrane proteins that bound to the MreC–Sepharose column. In particular, we wanted to determine whether any of these proteins localized in a pattern that was similar to MreC and PBP2. To accomplish this, we constructed GFP fusions with the C terminus of five different MreC-interacting outer membrane proteins: three predicted TonB-dependent receptors, CC0991, CC1750, and CC0210, as well as the OmpA family protein, CC3494, and CC0163, a protein of unknown function. These fusions were integrated into the chromosome by single-crossover homologous recombination such that the sole copy in the cell was fused to GFP. When observed by fluorescence microscopy in living cells, these fusions exhibited a distinguishing punctate (CC1750, CC0210, CC0163) or banded (CC0991, CC3494) pattern of localization that was similar to the MreC and PBP2 localization pattern (Fig. 4). We then tested whether the observed patterns of localization were dependent on MreB by treating the cells for two hours with the MreB depolymerizing compound, A22. As was the case with MreC and PBP2, the localization pattern of all of these outer membrane protein-GFP fusions was unperturbed in the absence of MreB spirals (Fig. 4). However, depletion of MreB for 10 h resulted in lemon-shaped cells that had each of these GFP fusions distrib-

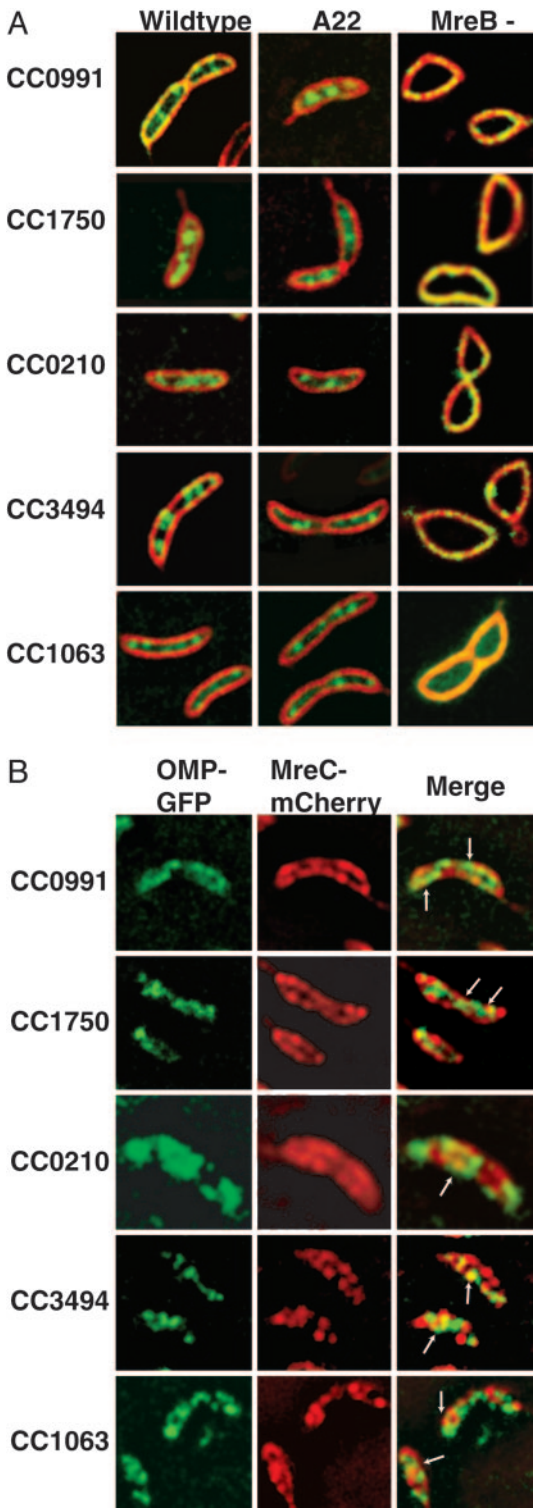

Fig. 4. The localization pattern of MreC-interacting outer membrane proteins is not dependent on MreB cables. (*A*) C-terminal GFP fusions to outer membrane proteins retained on the MreC affinity column form banded or punctate patterns in the outer membrane as visualized in live cell images. GFP fusions to the indicated proteins were visualized by deconvolution microscopy in wild-type, A22-treated (50 μg/ml for 2 h), and MreB-depleted (growth of JG5000 for 10 h without xylose). The fusion protein is green and the cytoplasmic membrane is stained red with the fluorescent lipophilic compound FM4–64. (*B*) Visualization of an MreC–mCherry and outer membrane protein-GFP fusions within the same cell. Images of localized protein fusions are shown individually as indicated and merged together. Arrows indicate regions of colocalization.

uted around the cell periphery. Loss of localization in cells with shape defects is similar to that observed with MreC and PBP2, indicating that the localization pattern of these outer membrane proteins may require an intact peptidoglycan layer and/or an organized localization pattern of MreC/PBP2. We next tested for colocalization of these outer membrane proteins with an MreC fused to a red-fluorescent protein (MreC–mCherry) (32). In many cases, the localization outer membrane–GFP fusion proteins overlapped with MreC–mCherry fusion protein or appeared to alternate with the MreC pattern of localization (Fig. 4B). This latter pattern was especially evident in cells expressing the CC3494-GFP fusion. Additionally, there were large areas in the cell in which only the MreC–mCherry fusion was present suggesting that MreC may be more abundant than many of these outer membrane proteins. These experiments suggest that some proteins in the periplasm and outer membrane may be localized in highly organized, interdependent patterns.

Discussion

The bacterial actin homolog MreB localizes in a helical pattern and is required for the maintenance of a rod cell shape. Here, we show that MreC, encoded by the gene downstream of *mreB*, is located within the periplasmic space in *C. crescentus* and localizes in a helical or banded pattern in a fashion similar to that of MreB and PBP2. The localization pattern of MreC, observed here with immunofluorescence microscopy, is similar to that seen in *B. subtilis*, *E. coli*, and *C. crescentus* when GFP fusions are used. In the case of the *C. crescentus* MreC homolog, it is an example of a periplasmic protein localizing in an organized subcellular pattern of localization.

It has been proposed that MreB filaments function in maintaining cell shape by spatially organizing PBPs in the bacterial cytoplasmic membrane. Immunoprecipitation experiments with *C. crescentus* and affinity chromatography with *E. coli* proteins have demonstrated that many of the PBPs exist as a multienzyme complex (28, 29). These latter experiments with *E. coli* have shown that the PBPs exist in complex with lytic transglycosylases, a reflection of the requirement for coordinating the hydrolysis of preexisting peptidoglycan and the insertion of new cell wall material (29). This multienzyme complex has also been shown to be associated with proteins that are not involved in peptidoglycan metabolism (29). We performed affinity chromatography to determine whether MreC formed an association with the peptidoglycan synthesizing complex of *C. crescentus*. PBPs bound to the MreC–Sepharose column over a wide range of salt concentrations suggesting that they were present in heterogeneous complexes, an observation similar to that found when *E. coli* extracts were used in comparable experiments (28, 29). This finding demonstrates a physical interaction between a shape-determining protein and the PBP complex, suggesting that a major function of these proteins lies in coordinating the activities of the peptidoglycan synthesizing factories in three dimensional space. In support of this idea, genetic experiments in *B. subtilis* have indicated that MreC and MreD have a crucial role in mediating lateral cell wall growth (18). Additionally, growth of the peptidoglycan along the long axis of the cell is proposed to occur in a helical fashion (24), a pattern similar to that adopted by MreC and PBP2. The precise role of MreC in this process is still largely unclear. It may function as a scaffolding protein that connects the subunits of the peptidoglycan synthesizing complex, as proposed for the periplasmic *E. coli* protein, MipA (29). Because genes encoding MreC are found in both rod and coccoid-shaped bacteria, we propose that MreC–PBP interactions are likely to be required for some as yet unknown, universal aspect of peptidoglycan assembly. In rod shaped organisms, MreC may also likely function in linking, through some intermediary membrane protein, the penicillin binding proteins to the internal MreB cytoskeleton (Fig. 5).

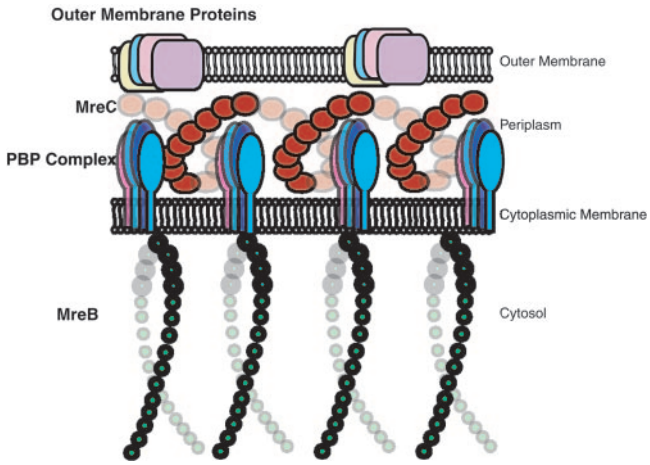

Fig. 5. Model depicting MreC interactions with cytoplasmic and outer membrane proteins. Internal MreB helices (green) lying underneath the cytoplasmic membrane are proposed to be the master organizer of the spatial distribution of proteins that lie outside the cytoplasm. These include PBP complexes (multicolored; inner membrane) that are anchored in the cytoplasmic membrane but have the bulk of their polypeptide in the periplasm, the periplasmic MreC protein (red), and a subset of outer membrane proteins (multicolored; outer membrane). The experiments presented here demonstrate that MreC interacts with both PBPs and several outer membrane proteins. We propose that MreC plays a critical role in helping establish or maintain these proteins in their patterns of localization, perhaps linking, through some intermediary membrane protein, the PBPs and outer membrane proteins to the internal MreB cytoskeleton.

Using affinity chromatography coupled with mass spectrometry-based protein identification, we have isolated and identified several additional MreC-interacting proteins. These included several outer membrane, TonB-dependent receptors and two other conserved outer membrane proteins. Subcellular localization experiments with GFP fusions to each of these proteins have demonstrated that they localize in spiral, punctate or banding patterns that are similar to those adopted by MreC and PBP2. This pattern of localization may be a property that is shared by many proteins that lie outside the cytoplasmic membrane. For example, experiments have shown that the outer membrane LamB protein of *E. coli* can adopt a spiral pattern of localization (33). Other experiments with *E. coli* have indicated that protein export occurs at discrete sites at the cytoplasmic membrane (34) and also that the lipopolysaccharide in the cell envelope forms a helical pattern (35). Our results indicate that the internal MreB helix may, perhaps interacting through MreC, be responsible for organizing these extracytoplasmic proteins (Fig. 5). What purpose would be served by maintaining a spatial organization of proteins outside the cell? Peptidoglycan assembly enzymes would be required to have their activities spatially coordinated to maintain the shape and integrity of the cell wall. For other proteins, the advantage for the cell in adopting a pattern of organization is less obvious. Perhaps organizing proteins involved in transport such as outer membrane porins or TonB-dependent receptors permits interaction with localized cytoplasmic partners. In the latter case, it has been demonstrated that TonB-dependent receptors in the outer membrane must interact with TonB and energy transducing proteins (ExbB and ExbD) in the cytoplasmic membrane (36). It is possible that a fixed spatial location facilitates such interactions across the periplasmic space.

Previous experiments have shown that the MreB pattern of localization changes dynamically during the *C. crescentus* cell cycle, with the longitudinally distributed spirals collapsing to the midcell at the time when cytokinesis initiates. We found that the

MreC pattern of localization was relatively static during the cell cycle, with the number of spirals, or bands in a single cell increasing with the cell length (data not shown). Similar to that observed with PBP2 localization, MreC did not concentrate at a midcell location in predivisional cells. This result suggests that the MreC and PBP2 localization patterns may not depend on an intact MreB higher-order structure. We assayed whether MreC localization required MreB helical cables in two different ways. Treatment with the compound A22, which rapidly depolymerizes and prevents the polymerization of MreB helical structures (26, 37), did not perturb the localization pattern of MreC, PBP2, or the GFP–outer membrane protein fusions. Depletion of MreB from the cell for an extended period did lead to a loss of MreC, outer membrane protein, and, as shown previously, PBP2 localization (15). These depleted cells have acquired gross changes in shape and defects in the integrity of the peptidoglycan layer. Taken together, these results suggest that MreC, PBP2, and these outer membrane proteins may require an intact, morphologically normal cell wall to adopt a helical pattern of localization. We propose that the subcellular localization pattern of these extracytoplasmic proteins is fastened in place by interaction with a helical structure present in the peptidoglycan layer.

We envision that this cell-wide helical pattern may be associated with regions of active peptidoglycan assembly. MreB cables may be ultimately responsible for the accurate tracking of MreC and the peptidoglycan synthesizing complexes in a helical pattern (Fig. 5). However, because rapid loss of MreB helical structures with A22 treatment or transient changes in the localization pattern during the cycle did not result in immediate corresponding alterations in MreC and PBP2 localization or cell shape, we speculate that an intrinsic helical pattern in the peptidoglycan layer, once established, can persist and propagate for short periods of time in the absence of an MreB cytoskeletal structure.

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