The cell shape proteins MreB and MreC control cell morphogenesis by positioning cell wall synthetic complexes

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Summary
MreB, the bacterial actin homologue, is thought to function in spatially co-ordinating cell morphogenesis in conjunction with MreC, a protein that wraps around the outside of the cell within the periplasmic space. In Caulobacter crescentus, MreC physically associates with penicillin-binding proteins (PBPs) which catalyse the insertion of intracellularly synthesized precursors into the peptidoglycan cell wall. Here we show that MreC is required for the spatial organization of components of the peptidoglycan-synthesizing holoenzyme in the periplasm and MreB directs the localization of a peptidoglycan precursor synthesis protein in the cytosol. Additionally, fluorescent vancomycin (Van-FL) labelling revealed that the bacterial cytoskeletal proteins MreB and FtsZ, as well as MreC and RodA, were required for peptidoglycan synthetic activity. MreB and FtsZ were found to be required for morphogenesis of the polar stalk. FtsZ was required for a cell cycle-regulated burst of peptidoglycan synthesis early in the cell cycle resulting in the synthesis of cross-band structures, whereas MreB was required for lengthening of the stalk. Thus, the bacterial cytoskeleton and cell shape-determining proteins such as MreC, function in concert to orchestrate the localization of cell wall synthetic complexes resulting in spatially co-ordinated and efficient peptidoglycan synthetic activity.

Introduction
The distinct morphology of most bacterial cells is dictated by the architecture of the peptidoglycan cell wall. In Gram-negative bacteria, the cell wall is thought to be a single large molecule that girdles the entire cell surface, consisting of a repeating glycan polymer of N-acetylglycosamine and N-acetylmuramic acid, the strands of which are connected by peptide cross-links (reviewed in Holtje, 1998; Vollmer and Holtje, 2001; Scheffers and Pinho, 2005). Peptidoglycan precursors are synthesized in the cytosol, then covalently bound to a lipid carrier and translocated to the outside of the cell where they are incorporated into the pre-existing cell wall. The enzymes catalysing the insertion of precursors into the growing cell wall are known as penicillin-binding proteins (PBPs). In Gram-negative bacteria, the PBPs are localized to the outer surface of the cytoplasmic membrane, such that their catalytic domains lie within the periplasmic space where they catalyse both transglycosylase and transpeptidase reactions. In Escherichia coli, peptidoglycan precursors are thought to be added to pre-existing peptidoglycan at gaps in the cell wall that are created by the action of peptidoglycan-degrading enzymes (reviewed in Holtje, 1998; Vollmer and Holtje, 2001). This group of enzymes, including the lytic transglycosylases and endopeptidases, are hypothesized to exist in complex with PBPs, forming a peptidoglycan-synthesizing holoenzyme.

In rod-shaped bacteria, the activity of PBPs must be spatially organized such that the insertion of new cell wall material occurs in a co-ordinated fashion along the long axis of the cylindrical cell. Recent experiments with MreB and MreC, two proteins originally shown to be required for normal rod cell shape in E. coli (Wachi et al., 1987), have provided important insights into how the spatial organization of PBP activity may be accomplished. Structural studies have demonstrated that MreB is a prokaryotic homologue of actin that can assemble into filamentous structures in a nucleotide-dependent manner (van den Ent et al., 2001; Esue et al., 2005). Subcellular localization experiments with Bacillus subtilis have shown that MreB forms helical cables that encircle and run along the length of the cell (Jones et al., 2001; Carballido-Lopez and Errington, 2003). This distinct pattern of MreB localization was also observed in Caulobacter crescentus (Fulge et al., 2004; Gitai et al., 2004), E. coli (Shih et al., 2003) and Rhodobacter sphaeroides (Slovak et al., 2005). In C. crescentus, the pattern of MreB localization is dynamic, with spiral structures running along the length of the cell that collapse to a midcell location prior to...
cytokinesis (Figge et al., 2004; Gitai et al., 2004). Cells with mutations in MreB (or its homologues) exhibit a loss of rod shape, gradually transforming into spherical or lemon-shaped cells (Jones et al., 2001; Carballido-Lopez and Errington, 2003; Figge et al., 2004; Gitai et al., 2004; Kruse et al., 2005) suggesting that the observed helical cables of MreB may have an important role in determining rod-shaped growth of the cell. In support of this idea, penicillin-binding protein 2 (PBP2) of C. crescentus also exhibits a helical-like or banded pattern of localization (Figge et al., 2004). Additionally, labelling of B. subtilis cells using fluorescent vancomycin (Van-FL) demonstrated that peptidoglycan precursors are incorporated into pre-existing cell wall material in a helical fashion that was dependent on the MreB homologue, Mbl (Daniel and Errington, 2003). These results suggest that MreB may be responsible for the spatial organization of PBPs and peptidoglycan synthesis.

The mreC gene located downstream of mreB in most rod-shaped bacteria has also been shown to be required for maintenance of cell shape (Dye et al., 2005; Kruse et al., 2005; Leaver and Errington, 2005). This gene encodes a protein that is localized to the outer surface of the cell, either containing a single membrane spanning region, in the case of B. subtilis (Leaver and Errington, 2005; van den Ent et al., 2006), or existing in the periplasm in C. crescentus (Divakaruni et al., 2005). Unlike mreB whose distribution is restricted to rod-shaped bacteria, mreC is present in the genomes of both rod- and coccoid-shaped cells suggesting that it may have a universal role in cell morphogenesis. MreC exhibits a banded or helical-like pattern of localization in both B. subtilis (Leaver and Errington, 2005; van den Ent et al., 2006) and C. crescentus (Divakaruni et al., 2005; Dye et al., 2005). Affinity chromatography experiments with C. crescentus cell extracts have demonstrated that a number of directly PBPs interact with MreC (Divakaruni et al., 2005). Additionally, two-hybrid experiments with B. subtilis protein showed that MreC was able to interact with several individual PBPs (van den Ent et al., 2006). These results suggest that MreC and PBPs form a complex in vivo and that the helical localization pattern of MreC may be ultimately responsible for the positioning of PBPs and the pattern of peptidoglycan synthesis.

Here we examine the role of the C. crescentus cell shape-determining proteins, MreB and MreC, in organizing the subcellular localization of peptidoglycan synthetic proteins on both sides of the cytoplasmic membrane. We show that intact MreB cables are required for the positioning of a peptidoglycan precursor synthesis enzyme in the cytosol, while MreC is required for the positioning of the peptidoglycan synthesis holoenzyme in the periplasm. Additionally, we investigate the role of cytoskeletal proteins, MreB and FtsZ, in the cell cycle-controlled biogenesis of the C. crescentus polar stalk appendage. We find that MreB and FtsZ have distinct roles in directing stalk peptidoglycan synthesis, with MreB being required for stalk lengthening and FtsZ responsible for transverse peptidoglycan synthesis. Thus the function of these two proteins in stalk biogenesis recapitulates their roles in cell growth, where MreB is involved in lateral cell wall synthesis, and FtsZ is required for transverse septal peptidoglycan synthesis. Analysis of the role of cell shape-determining proteins in both stalk and cell growth suggests that these proteins are not only involved in the spatial positioning of cell wall assembly proteins but are also required for peptidoglycan synthetic activity.

Results

Localization of a peptidoglycan precursor synthesis enzyme requires intact MreB cables

The lipid-linked, disaccharide-pentapeptide peptidoglycan precursor subunit is synthesized in the cytosol or in association with the cytoplasmic membrane by a series of eight enzymatic reactions, the final two of which involve covalently bound undecaprenyl phosphate intermediates (Fig. 1). The final step in the synthesis of this precursor molecule is the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to undecaprenyl-P-P-N-acetylmuramyl (MurNAc)-pentapeptide forming undecaprenyl-P-P-(GlcNAc-β1–4)-MurNAc-pentapeptide (reviewed in van Heijenoort, 1998; Ha et al., 2001) (Fig. 1), and is catalyzed by the membrane-associated protein, MurG. Following this final step of synthesis, the lipid-bound precursor is delivered to the periplasm where it is incorporated into the expanding peptidoglycan by the PBPs. Previous experiments have suggested that the cell shape-determining proteins probably have a critical role in the positioning of PBPs in the periplasm (Figge et al., 2004; Divakaruni et al., 2005). We wanted to determine whether the cytoplasmic phase of peptidoglycan synthesis was also spatially organized by cell shape-determining proteins. In order to accomplish this, we constructed a fusion between the carboxyl-terminus of MurG and the red fluorescent protein, mCherry (Shaner et al., 2004). The observed pattern of MurG–mCherry localization was similar to that of the extracytoplasmic proteins (e.g. PBP2) involved in peptidoglycan synthesis with the fusion protein exhibiting a distinct banded pattern perpendicular to the long axis of the cell (Fig. 1A, D and E) or a punctate pattern (Fig. 1B, C and F). In most cells (greater than 90% out of at least 200 cells counted), abundant localization was also observed at one of the cell poles (see Fig. 1B). In order to ensure that the fusion to mCherry was not responsible for the observed localization pattern, we fused a seven-amino-acid residue epitope (FLAG tag) to the carboxyl
terminus of MurG and assayed localization using immunofluorescence microscopy (Fig. 1G, H and I). The localization pattern of epitope-tagged MurG was similar to that observed with the mCherry fusion. We then tested whether MurG–mCherry localization was dependent on an intact MreB cytoskeleton by assaying localization in cells that were treated with the MreB inhibitor, A22. Treatment with A22 for as little as 1 h had a dramatic effect on the subcellular localization pattern of MurG. The distinct banded pattern was never observed. Instead, the majority (> 85% of 200 cells counted) of A22-treated cells possessed a single focus of MurG–mCherry located either at the midcell or at a single pole (Fig. 1J and K). In other cases (c. 15%), there were two foci of MurG–mCherry, one located at the midcell and the other at the cell pole (Fig. 1L). Immunoblot of cell extracts using anti-dsRed antibody revealed that the A22 treatment did not alter the cellular levels of the MurG–mCherry fusion (Fig. S1A). In order to determine whether the effect of A22 on MurG–mCherry localization was reversible, cells that had been treated with A22 for 90 min were washed, incubated in medium lacking A22 for an additional 90 min, and then examined by fluorescent microscopy. In most of the cells (75% out of 200 counted), removal of A22 resulted in the restoration of a punctate pattern of MurG–mCherry localization along the cell length (Fig. 1M–O). In some cases (approximately 35%), the punctate pattern was also accompanied by strong localization at the pole and/or midcell. Thus, the re-establishment of the MreB cytoskeleton partially restores the MurG–mCherry pattern of localization. Next, we tested the effect of MreC depletion on MurG–mCherry localization. In contrast to the A22-treated cells, in MreC-depleted cells, MurG–mCherry retained a punctate pattern of localization, although in most cases the banded pattern was not observed (Fig. 1P–R). Under these depletion conditions, MreC was undetectable using immunoblot with anti-MreC antibody; however, MurG–mCherry levels remained constant (Fig. S1B). Taken together, these results indicate that the MreB cytoskeleton is critical for the localization pattern of MurG.

The subcellular pattern of MurG localization, as well as that shown previously for PBP2, suggests that new cell wall material might be incorporated into the peptidoglycan sacculus in a similar banded or helical pattern. Previous experiments using B. subtilis have shown that a fluorescently labelled derivative of the glycopeptide antibiotic, vancomycin (Van-FL) binds to regions of the cell that are actively synthesizing cell wall material (Daniel and Errington, 2003). Although Gram-negative bacteria are generally viewed as being resistant to vancomycin, an early previous report indicated that C. crescentus was sensitive to vancomycin (Johnson and Ely, 1977). We have now examined the labelling pattern of Van-FL in C. crescentus cells. As was found in B. subtilis and several other Gram-positive organisms (Daniel and Errington, 2003; Pinho and Errington, 2005), imaging of Van-FL-treated C. crescentus cells revealed either a punctate or helical pattern of fluorescence emanating from the cell periphery (Fig. 1S–X) resembling that of MreB, MreC, MreD (Fig. 1Z, AA and BB), and MurG. We also observed strong labelling at the midcell region of pre-divisional cells (Fig. 1W and X). Van-FL labelling required energized cells (1.5 mM sodium azide treatment; data not shown) and could be blocked by the presence of the cell wall synthesis inhibitor, cycloserine (Fig. 1Y). Previous experiments have indicated that active peptidoglycan synthesis does not occur at the poles of E. coli and B. subtilis cells (de Pedro et al., 1997; Daniel and Errington, 2003). We found

Fig. 1. MreB actin directs the localization of the cytosolic peptidoglycan precursor-synthesizing enzyme MurG. At upper right is a schematic diagram depicting the final steps of the peptidoglycan precursor synthetic pathway. In stage one (cytosol) a UDP-N-acetylmuramic acid (MurNAc)-pentapeptide is synthesized. The amino acid residues of the pentapeptide are denoted by coloured polygons. The MurNAc-pentapeptide is then transferred to the undecaprenylphosphate lipid carrier through the action of integral membrane MraY protein. In the final step of precursor synthesis the N-acetylmuramoyl-moiety is added by the membrane-associated MurG protein. This now complete lipid-bound disaccharide-pentapeptide precursor is translocated to the cytoplasm (perhaps through the action of RodA) where it is incorporated into the pre-existing peptidoglycan via the action of PBPs.

A–F. Shown are fluorescence micrographs of C. crescentus cells expressing a MurG–mCherry fusion protein and observed with fluorescence deconvolution microscopy.

G–I. Fluorescence micrographs of C. crescentus cells expressing a carboxyl-terminal epitope-tagged (FLAG) MurG fusion protein. Both cases fluorescence proteins appear pink. DAPI (blue) was used to stain the DNA.

J–L. Effect of loss of MreB cables on the subcellular localization pattern of a MurG–mCherry fusion protein. C. crescentus cells expressing a MurG–mCherry fusion protein were treated with A22 for 90 min and subjected to fluorescent microscopy.

M–O. Effect of MreC depletion on the subcellular localization pattern of a MurG–mCherry fusion protein. Cells of the MreC depletion strain (JG5025) expressing a MurG–mCherry fusion were grown without inducer (xylose) for 6 h and subjected to fluorescent microscopy.

P–R. Effect of MreC depletion on the subcellular localization pattern of a MurG–mCherry fusion protein. Cells of the MreC depletion strain (JG5025) expressing a MurG–mCherry fusion were grown without inducer (xylose) for 6 h and subjected to fluorescent microscopy.

S–X. C. crescentus cells from mid-logarithmic cultures were labelled with a fluorescent derivative of vancomycin (Van-FL) for 20 min, fixed and subjected to fluorescent deconvolution microscopy. Van-FL labelling appears green. DAPI (blue) was used to stain the DNA. Midcell and polar labelling is indicated by arrows.

Y. Labelling was inhibited by the peptidoglycan synthesis inhibitor, cycloserine (500 µg ml⁻¹).

Z, AA and BB. Subcellular localization pattern of the cell shape-determining proteins fused to mCherry. Cultures of C. crescentus cells expressing mCherry–MreB (Z), MreC– (AA) and MreD–mCherry (BB) were subjected to fluorescent microscopy.

Scale bars correspond to 1 µm.

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that many *C. crescentus* cells had relatively strong Van-FL labelling at one or both poles (Fig. 1S and T), indicating that active cell wall synthesis occurs at the poles of *C. crescentus* cells. This may be a reflection of the requirement for peptidoglycan synthesis during biogenesis of the polar stalk appendage.

*MreC is required for the spatial organization of MltA, a lytic transglycosylase, and MipA, its interacting partner.*

Biochemical experiments in *E. coli* have shown that the membrane-bound lytic transglycosylase, MltA, can be isolated in complex with PBPs and another protein...
called MipA (Vollmer et al., 1999). The coupling of cell wall-degrading and -synthesizing activities is thought to permit the expansion of peptidoglycan without compromising cell wall integrity. As at least one PBP in C. crescentus (i.e. PBP2) is organized in a distinct banded pattern along the cell length (Figge et al., 2004), we wanted to examine whether the lytic transglycosylase MltA and its associated partner, MipA, exhibited a similar localization pattern. In order to accomplish this we created fusions of MltA and MipA with the red fluorescent protein, mCherry (Fig. 2). The MltA–mCherry pattern of localization (Fig. 2A–C) was strikingly similar to that observed with MreB, MreC, MreD (Fig. 1Z, AA and BB) and PBP2 (Figge et al., 2004; Divakaruni et al., 2005) appearing as a series of bands perpendicular to the long axis of the cell. MltA also was located at the cell pole in the majority of cells (greater than 90% out of 200 counted), and could be observed within the cell-proximal region of the stalk (Fig. 2A and B) and of the pole associated with the stalk (Fig. 2G and H). MltA fused to the FLAG epitope exhibited a strikingly similar subcellular pattern of localization when assayed using immunofluorescence microscopy (Fig. 1D–F). MipA–mCherry fusions also exhibited a similar banded pattern of subcellular localization (Fig. 2L–K) with localization also appearing at the cell pole (47% out of greater than 200 cell counted) (Fig. 2I) and/or the midcell region (38%) (Fig. 2J). Epitope-tagged MipA (MipA-FLAG) also adopted a banded-like pattern of localization (Fig. 2L and M). We then tested whether MreB was responsible for the observed subcellular localization patterns by assaying localization following treatment with the MreB inhibitor, A22. Like other extracytoplasmic proteins such as MreC and PBP2 (Divakaruni et al., 2005), the subcellular localization pattern of MltA– and MipA–mCherry was unaffected by a loss of MreB cables (Fig. 2O and P). We also assayed the localization of MltA and MipA under conditions of MreC depletion. The MreC depletion strain has the sole copy of mreC under control of an inducible xylose promoter. MreC depletion was accomplished by incubating these cells in the absence of inducer (xylose) and assaying localization after 3–5 h. The distinct banded and punctate pattern of MltA– and MipA–mCherry localization was not evident in cells that had been depleted of MreC (Fig. 2Q–T). These cells also did not exhibit any polar localization of MltA or MipA; instead very few foci were visible. Immunoblot of cell extracts revealed that MreC depletion had no significant effect on the cellular levels of either fusion protein (Fig. 2U and V), suggesting that both MltA and MipA complexes were disrupted in the absence of MreC. We hypothesize that MreC may have a critical role in maintaining the stability of the peptidoglycan-synthesizing holoenzyme.

Next we wanted to determine whether the pattern of peptidoglycan synthesis as assayed by Van-FL labelling was influenced by the progression of the cell cycle. Synchronized populations of cells were obtained by isolating swarmer cells and suspending them in growth medium. After approximately 30 min, swarmer cells differentiate into stalked cells by shedding the polar flagellum initiating DNA replication, and growing a polar stalk at the former site of the flagellum (see Fig. 3E) (reviewed in England and Gober, 2001). We observed a burst of polar Van-FL labelling occurring at a single pole in cells that were differentiating into stalked cells (Fig. 3A, 20 and 40 min time points, Fig. 3D). Van-FL labelling appeared to be weaker along the length of these early stalked cells. As the labelled polar region may be in a different focal plane than the bulk of the cell, we collected images from both above and below the apparent medial section (Fig. 3C). These images also revealed relatively weaker Van-FL labelling throughout the cell suggesting that peptidoglycan synthetic activity may be reduced early in the C. crescentus cell cycle. As the cell cycle progressed, strong polar labelling diminished, and lateral labelling increased. Strong midcell labelling then appeared in pre-divisional cells as a prelude to cytokinesis (Fig. 3A, 60 and 80 min time points, Fig. 3D). Cell division in C. crescentus results in the formation of differing progeny cells, a motile swarmer cell and a stalked cell (Fig. 3E). Interestingly, immediately following cell division, one-half of the progeny cells exhibited another burst of strong polar Van-FL labelling (Fig. 3A and D). Staining the membrane with FM4-64 cells that were labelled with Van-FL revealed that the strong burst of Van-FL labelling occurred at the stalked pole of the cell (Fig. 3B). The polar stalk of C. crescentus is comprised of cell envelope material including peptidoglycan. As the stalk is thought to lengthen throughout the lifetime of the cell (Smit et al., 1981; Poindexter and Staley, 1996), it is possible that stalk biogenesis may require a round of new peptidoglycan synthesis once every cell division cycle. Subcellular localization experiments have shown that MreB cables dynamically localize to the midcell preceding the onset of cytokinesis (Figge et al., 2004; Gitai et al., 2004). One possibility is that MreB may be required for midcell, cell division-related, peptidoglycan synthesis. We tested whether MreB had a role in either stalked pole or midcell peptidoglycan synthesis by performing a cell cycle Van-FL labelling experiment in the presence of the MreB inhibitor, A22. We found that A22-treated cells exhibited the burst of polar peptidoglycan synthesis early in the cell cycle; however, the fraction cells exhibiting the polar burst of Van-FL labelling was considerably less (Fig. 4B) than that observed in untreated cells (Fig. 3D). Additionally, in
Fig. 2. The localization of MltA, a lytic transglycosylase, and its conserved interacting protein, MipA, is dependent on MreC.

A–H. Shown are fluorescence micrographs of *C. crescentus* cells expressing an (A–C) MltA–mCherry or (D–F) MltA–FLAG fusion protein. The fusion proteins appear pink. DAPI (blue) was used to stain the DNA. Scale bars correspond to 1 μm. Stalked pole localization is indicated with arrows. Localization pattern of the (G) MltA–mCherry fusion protein shown with an accompanying (H) DIC image. The stalked poles are marked with arrows.

I–N. The subcellular localization pattern of the periplasmic, MltA-associated protein, MipA, fused to (I–K) mCherry or the (L–N) FLAG epitope tag. Polar and midcell localization is indicated with arrows.

O–P. Effect of loss of MreB cables on the subcellular localization pattern of MltA– and MipA–mCherry fusion proteins. *C. crescentus* cells expressing either MltA– or a MipA–mCherry fusion proteins were treated with A22 for 90 min and observed by fluorescence microscopy.

Q–T. Effect of MreC depletion on the subcellular localization pattern of MltA– and MipA–mCherry fusion proteins. Cells of the MreC depletion strain (JG5025) expressing either MltA– or a MipA–mCherry fusion proteins were grown without inducer (xylose) for 6 h.

U. Steady-state levels of the MltA–mCherry fusion protein and MreC during the course of MreC depletion compared with the fusion in the wild-type background measured by immunoblot. The time in hours following the removal of inducer is indicated by the numbers above each lane.

V. Steady state levels of the MipA–mCherry fusion protein and MreC during the course of MreC depletion compared with the fusion in the wild-type background measured by immunoblot.
In some cases, strong labelling was evident at both poles of the cell (Fig. 4A, 60 min). Unlike untreated cells, strong polar labelling in a fraction of A22-treated cells did not diminish as the cell cycle progressed (Fig. 4A and B). Importantly, very few A22-treated cells exhibited Van-FL labelling at midcell later in the cell cycle (Fig. 4A and B). This observation suggests that the localization of MreB to the midcell is likely to be required for midcell (septal) peptidoglycan synthesis.

The formation of a ring-like structure at the midcell by FtsZ, a prokaryotic tubulin homologue, is the earliest known cytological event in the initiation of cytokinesis (reviewed in Bramhill, 1997). If Van-FL labelling is an indicator of active peptidoglycan synthesis, then cell cycle-regulated midcell labelling should depend on the cytokinetic protein FtsZ. We tested this idea by performing a cell cycle Van-FL labelling experiment on synchronized cells that were depleted of FtsZ (see Experimental procedures). Previous experiments have demonstrated that isolated swarmer cells have undetectable levels of FtsZ (Wang et al., 2001). Thus under conditions of depletion (media without inducer), the cell cycle begins and continues without FtsZ. As anticipated, synchronized cells depleted of FtsZ did not label with Van-FL at the midcell (Fig. 4C). Surprisingly, although FtsZ depleted cells did exhibit Van-FL labelling along the length of the cell extending to the poles, the strong burst of Van-FL labelling early in the cell cycle was never...
observed. As a control, a portion of these cells were suspended in medium containing xylose to induce FtsZ expression and then labelled at 40 and 60 min following suspension in fresh medium. In this case, strong polar and then robust midcell labelling was evident (Fig. 4E). These results suggest that FtsZ is required for a cell cycle-regulated burst of peptidoglycan synthesis both at the pole of the cell, and later in the cell cycle, at the site of cell division.

Morphogenesis of the stalk requires two different cytoskeletal proteins

Previous experiments have found that cells depleted of MreB (Fig. S2) (Wagner et al., 2005) or those treated with A22 (data not shown) possessed short stalks, indicative of a defect in stalk elongation. Likewise cells depleted of the cell shape-determining proteins, RodA (Wagner et al., 2005), whose gene is located within the same operon as

Fig. 4. Cytoskeletal FtsZ and MreB proteins are required for the temporal and spatial regulation of peptidoglycan synthesis.

A. MreB cables are required for midcell peptidoglycan synthesis and cell division. A synchronized population of *C. crescentus* cells were treated with A22 and allowed to progress through the cell cycle. As described in Fig. 3, samples were labelled for 20 min with Van-FL (green) and subjected to fluorescence microscopy. Time in minutes when each sample was processed for microscopy is indicated. A22-treated cells fail to exhibit midcell Van-FL labelling and do not divide. Arrows indicate strong polar labelling.

B. Shown is a graph depicting the proportion of cells with strong polar and midcell Van-FL labelling during the course of the cell cycle in the presence of A22 (A). At least 200 cells were scored for polar and/or midcell localization at each indicated time point.

C. The cytokinetic FtsZ protein is required for temporally regulated polar and midcell peptidoglycan synthesis. Isolated swarmer cells of the *C. crescentus* FtsZ depletion strain YB1585 (Wang et al., 2001) were permitted to progress through the cell cycle in the absence of inducer (xylose). Samples were labelled with Van-FL (green) and subjected to fluorescence microscopy.

D. Shown is a graph depicting the proportion of cells with strong polar (solid line) and midcell (dashed line) Van-FL labelling during the course of FtsZ depletion (C). At least 200 cells were scored for polar and/or midcell localization at each indicated time point.

E. A portion of *C. crescentus* YB1585 cells from (C) were suspended in medium containing xylose to induce FtsZ expression and then labelled at 40 and 60 min. In this case, polar and then septal labelling is evident (indicated with arrows).

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mreB, mreC, mreD and pbp2 in C. crescentus, and MreC have defects in stalk growth (Fig. S2). As FtsZ was found to be required for the cell cycle-regulated burst of polar peptidoglycan synthesis, it may also have a role in stalk biogenesis. C. crescentus stalks contain cross-bands that traverse the width of the stalk (Poindexter and Hagenzieker, 1982) (Fig. 5A and B). Stalks of synchronized, FtsZ-depleted cells appeared of relatively normal length, but lacked the characteristic cross-bands (Fig. 5C and D) present in wild-type stalks (Fig. 5A and B). Additionally, the stalks from FtsZ-depleted cells appeared relatively smooth and homogeneous, compared with the dark granular and wrinkled-appearing stalks from wild-type cells. Consistent with observations using light microscopy, the stalks from synchronized, A22-treated cells appeared as short stubs at the cell poles indicative of a defect in elongation (Fig. 5E and F). Thus stalk morphogenesis requires two different cytoskeletal proteins during the cell cycle. This dual requirement is apparently necessary to direct two topologically different modes of stalk peptidoglycan synthesis. One being an FtsZ-dependent mode in the transverse direction resulting in the formation of cross-bands that are perpendicular to the long axis of the stalk. The other, which is MreB dependent, is apparently required for lateral peptidoglycan synthesis and stalk elongation. Interestingly, the differing roles of FtsZ and MreB in stalk peptidoglycan synthesis are strikingly analogous to their prescribed roles in cell growth and division, where FtsZ is required for transverse septal growth and MreB is required for the co-ordination of lateral cell wall growth.

Cytoskeletal and cell shape-determining proteins are required for peptidoglycan synthesis

What function do MreB and FtsZ serve during stalk morphogenesis? One possibility is that these proteins may recruit other morphogenetic proteins, perhaps, in the case of FtsZ, other cytokinetic proteins (FtsA, FtsQ, FtsW) that are required for activating peptidoglycan synthesis (reviewed in Bramhill, 1997). This scenario is analogous to the role FtsZ plays in septal peptidoglycan synthesis. Evidence in favour of MreB and FtsZ being required for active peptidoglycan synthesis stems from the observation that MreB-depleted C. crescentus cells do not exhibit an expansion in cell width and fail to grow if FtsZ is also absent (Dye et al., 2005). The lack of cell expansion in these cells might be attributable to a reduction in peptidoglycan synthesis. In this regard, when compared with A22-treated cells or FtsZ-depleted cells, we found that
cells deficient in both MreB and FtsZ exhibited a marked reduction in the intensity of Van-FL labelling (Fig. S3). This observation suggests that peptidoglycan synthetic activity requires the presence of at least one of these two cytoskeletal proteins.

Interestingly, *C. crescentus* cells deficient in MreC or RodA have a different phenotype than MreB-depleted cells (compare Figs 1 and 2 with Fig. S2). When MreC or RodA are depleted, the mutant cells expand relative to wild-type cells, but never adopt the large, swollen, lemon-like appearance of MreB-depleted cells. MreC and RodA mutant cells appear to arrest in expansion along the long axis of the cell suggesting, with analogy to FtsZ-, MreB-deficient cells, that they may possess reduced peptidoglycan synthetic activity. In order to test this idea, we performed Van-FL labelling on cells that had been depleted of either MreC or RodA (Fig. 6). MreC depletion had a relatively rapid effect on the intensity of Van-FL labelling (Fig. 6A–F). Depletion for as little as 2 h led to a marked reduction in the intensity of labelling and the dis-
appearance of the characteristic punctate and banded pattern. A small fraction (less than 5% out of 200 cells counted) of cells exhibited intense Van-FL labelling at the midcell with diffuse weak fluorescence being evident throughout the rest of the cell (Fig. 6C). The majority of cells (approximately 95%) possessed only the weaker diffuse labelling pattern (Fig. 6D). Following incubation for 10 h under depletion conditions, the cells were expanded, but did not appear lemon-shaped like MreB-depleted cells, and possessed very weak, almost undetectable, Van-FL fluorescence (Fig. 6E and F). Depletion of RodA had a similar, if not as rapid effect on the intensity of Van-FL labelling (Fig. 6G–L). Cells that had undergone depletion of RodA for 2 h exhibited weaker labelling than wild-type cells but still retained a punctate or banded pattern of fluorescence (Fig. 6I and J). Following 10 h of depletion, the cells exhibited still weaker labelling accompanied by a marked loss of banded or helical fluorescence (Fig. 6K and L).

The apparent reduction of peptidoglycan synthetic activity in MreC- and RodA-deficient cells could be a consequence of a loss of MreB localization or PBP2 localization. Accordingly, we assayed the localization of MreB and PBP2 by immunofluorescence microscopy in cells that had been depleted of MreC or RodA. Depletion of MreC or RodA for an extended period of time (10 h) had no effect on the localization of MreB (Fig. 6M–P). In both cases, intact cables of MreB that wrapped around the cell circumference were clearly visible. In contrast, depletion of MreC resulted in a loss of the characteristic banded PBP2 localization pattern (Fig. 6Q–T) with most (95% out of 200 counted) of the cells exhibiting either a diffuse foci (77%) or strong midcell localization (18%). Interestingly, RodA depletion had little effect on the localization pattern of PBP2 (Fig. 6U–X). These results suggest that MreC and RodA are both required for peptidoglycan synthetic activity. The fact that PBP2 localization is perturbed in MreC-deficient cells but not under conditions of RodA depletion suggests that MreC and RodA have two distinct effects on peptidoglycan synthetic activity.

Discussion

Morphogenesis of bacterial cells necessitates that the cell wall is assembled in a spatially co-ordinated fashion such that, in the case of many bacteria, cell division results in progeny cells that are almost identical in size and appearance. In order to accomplish this, the enzymatic activities assembling the cell wall must be properly positioned in three-dimensional space along a substrate molecule (the pre-existing cell wall) that is of vast physical proportions. The experiments presented here show that the bacterial actin homologue, MreB, is required for the positioning of peptidoglycan precursor synthesis in the cytoplasmic compartment. In the periplasm, the cell shape-determining protein, MreC, is required for the spatial organization of components of a peptidoglycan assembly holoenzyme. Our experiments indicate that MreB- and MreC-directed positioning of cell wall assembly complexes not only has a crucial role in maintaining proper cell shape, but also may be essential for peptidoglycan synthetic activity.

Role of MreB and FtsZ in directing stalk and midcell peptidoglycan synthesis

The labelling of cells with a fluorescent derivative of vancomycin (Van-FL) has become a powerful method to visualize regions of the cell involved in nascent peptidoglycan synthesis in Gram-positive bacteria (Daniel and Errington, 2003; Leaver and Errington, 2005; Pinho and Errington, 2005). In most cases, the outer membrane of Gram-negative bacteria provides a permeability barrier for the entry of vancomycin into the periplasmic space; however, C. crescentus cells are sensitive to vancomycin, and thus we were able to observe the pattern of Van-FL labelling and nascent cell wall synthesis in this Gram-negative organism. Surprisingly, cell division-associated peptidoglycan synthesis was abolished in synchronized, A22-treated cells, indicating that MreB is required for midcell peptidoglycan synthesis and cell division in C. crescentus. This differs from E. coli and B. subtilis cells where MreB and its homologues are thought to be involved in lateral extension of the cell wall, but not cytokinesis. Unlike these two organisms, previous subcellular localization experiments have demonstrated that C. crescentus MreB localizes to the midcell before the onset of cytokinesis. Cell division in C. crescentus does not involve the formation of a septum that is morphologically similar to that of B. subtilis and E. coli. Instead, the cell envelope gradually grows inwards at the midcell eventually forming a barrier between the two nascent daughter cell compartments of the pre-divisional cell. This different mode of cytokinetic peptidoglycan growth apparently requires two different bacterial cytoskeletal proteins, MreB, as well as FtsZ.

Cells making the transition from a swarmer cell to a stalked cell type exhibited a marked increase in peptidoglycan synthesis at one pole of the cell that was dependent on the cytokinetic FtsZ protein. Subcellular localization experiments using immunogold electron microscopy have shown that C. crescentus cells possess a small pool of FtsZ at the stalked pole (Quardokus et al., 2001). Previous experiments, as well as those presented here, have shown that several cell shape-determining proteins including MreB, MreC and RodA are required for stalk growth (Wagner et al., 2005). We have found that cells lacking polymerized MreB produce short stub-like
projections at the stalked pole whereas FtsZ-depleted cells synthesize stalks of apparently normal length, but lack the characteristic cross-bands. Thus the role of MreB and FtsZ in stalk biogenesis recapitulates their function in cell growth, where FtsZ is required for transverse septal peptidoglycan synthesis and MreB for lateral cell wall synthesis. As the stalk does not contain FtsZ, synthesis of the cross-band likely occurs at the polar region of the cell, and MreB-dependent lengthening of the stalk results in its eventual outward migration away from the cell pole. Our results suggest that FtsZ-dependent cross-band synthesis occurs once every cell cycle. Interestingly, earlier experiments examining populations of Caulobacter cells in natural environments and in continuous culture indicated that the number of cross-bands present in stalks could serve as an indicator of the age of the stalked cell (Poindexter and Staley, 1996).

**Role of cell shape-determining proteins in spatially organizing peptidoglycan synthesis**

One model for peptidoglycan expansion in Gram-negative bacteria proposes that the removal of an old strand of peptidoglycan through the activity of the peptidoglycan-degrading enzymes is coupled to the concerted synthesis of three new strands via associated PBPs (reviewed in Vollmer and Holtje, 2001). Our experiments have demonstrated that the lytic transglycosylase, MltA, and its interacting protein, MipA, exhibit a subcellular pattern of localization that is similar to that of PBP2, and is dependent on MreC. We hypothesize that MreC is required for maintaining the spatial localization of multiprotein complexes containing PBPs, MltA and MipA. Biochemical evidence for the existence of such complexes was provided by affinity chromatography experiments demonstrating a physical interaction between the lytic transglycosylase, MltA, and its interacting protein, MipA, and PBP1b in *E. coli* cell extracts (Vollmer *et al*., 1999). Additionally, affinity chromatography experiments with *C. crescentus* have shown that MreC interacts with several different complexes of PBPs (Divakaruni *et al*., 2005).

MltA is an outer membrane-anchored lipoprotein with its catalytic domain within the periplasmic space, distantly separated from the cytoplasmically localized MreB cytoskeleton. Localization of both MltA and the periplasmic MipA was not perturbed by the MreB inhibitor, A22. This result is similar to previous findings in which the localization of MreC, PBP2 and MreC-interacting outer membrane proteins was not disrupted by treatment of the cells with A22. Therefore, these extracytoplasmic proteins are maintained in helical complexes that are unaffected by transient alterations in MreB localization. The role of MreB cytoskeleton in organizing cell wall assembly in Gram-positive organisms, which lack an outer membrane, may differ significantly. For example, recent experiments with *B. subtilis* have shown that a cell wall endopeptidase, LytE, localizes to the cell wall in a helical pattern that is dependent on the MreB isoform, MreBH (Carballido-Lopez *et al*., 2006). It is hypothesized that MreBH positions LytE so that it is exported to regions of the cell wall that are actively engaged in incorporating new cell wall material.

The experiments presented here show that MurG, the enzyme catalysing the final step in synthesis of the lipid-bound disaccharide-pentapeptide peptidoglycan precursor, exhibited a subcellular pattern of localization that was dependent on intact MreB cables. Peptidoglycan synthesis is generally regarded to consist of three distinct phases reflecting the subcellular location of the synthetic activity (reviewed in van Heijenoort, 1998; Ha *et al*., 2001). The first cytoplasmic phase involves the stepwise synthesis of a UDP-MurNAc-pentapeptide intermediate. In the following, cytoplasmic membrane-associated phase, this intermediate is transferred to the undecaprenylphosphate lipid carrier through the action of integral membrane MraY protein (see Fig. 1), followed by the addition of the N-acetylmuramic acid moiety by the membrane-associated MurG protein. This now complete, lipid-bound disaccharide-pentapeptide precursor is flipped out to the periplasm by an unknown mechanism where it is incorporated into the pre-existing peptidoglycan via the action of PBPs. The undecaprenylphosphate lipid carrier is then recycled back to cytoplasmic face of the lipid bilayer for the next round of synthesis. Experiments with *E. coli* have suggested that the availability of the undecaprenylphosphate lipid-carrier imposes limits on the rate of precursor synthesis (Kohlrausch *et al*., 1989). Free undecaprenylphosphate released following the incorporation of the GlcNAC-MurNAc-pentapeptide into mature peptidoglycan would have to be efficiently recycled back to the inner surface of the cytoplasmic membrane in order to maintain an optimal rate of precursor synthesis. One possibility is that the localization of MurG serves to spatially connect precursor synthesis to peptidoglycan synthesis permitting efficient transfer of intermediates and recycling of lipid substrate from one biosynthetic complex in the cytosol to the other in the periplasmic space and vice versa. As the cell wall is essentially an enormous single substrate molecule, the co-ordination of spatially organized complexes on either side of the cytoplasmic membrane may be required for efficient catalytic activity.

**Activation of peptidoglycan synthesis by the bacterial cytoskeleton and cell shape-determining proteins**

Disruption of MreB cables resulted in a localization of MurG to the midcell and/or polar region of the cell. We speculate that interactions with FtsZ or perhaps other
components of the cytokinetic machinery may recruit MurG to these regions of the cell in the absence of intact MreB cables. Interestingly, recent experiments with \textit{C. crescentus} have demonstrated that MurG accumulates at the midcell in wild-type cells in a cell cycle- and FtsZ-dependent fashion (Aaron \textit{et al}., 2007) where it is presumably required for efficient midcell peptidoglycan synthesis. It is interesting to note that treatment of cells with A22, and the resulting accumulation of MurG to the pole and midcell, did not decrease the intensity of Van-FL labelling (see Fig. 4). We hypothesize that cell wall synthesis under these conditions occurs in an FtsZ-dependent manner. Indeed, using Van-FL labelling, we found that new peptidoglycan synthesis in A22-treated cells was markedly reduced in the absence of FtsZ, suggesting that FtsZ is required to activate cell wall synthesis in MreB-depleted cells. In MreB-depleted \textit{C. crescentus} cells, polymerized FtsZ is disorganized and randomly distributed throughout the cell (data not shown). Thus, FtsZ-activated cell wall synthesis would likely result in the spatially random insertion of new peptidoglycan, eventually generating an expanded, rounded cell. FtsZ-dependent peptidoglycan synthesis is likely to function in lateral cell growth in organisms that do not possess MreB. For example, in \textit{Corynebacterium}, a rod-shaped bacterium that does not possess MreB, cell elongation is achieved by the lateral expansion of the cell wall near the poles (Daniel and Errington, 2003). As the poles of rod-shaped cells are derived from former cell division sites, polar peptidoglycan synthesis in \textit{Corynebacterium} may be directed by the cell division machinery. Likewise, in \textit{E. coli} cells with mutations in some small PBPs, FtsZ is required for peptidoglycan elongation (Varma \textit{et al}., 2007).

In \textit{E. coli}, depletion of MreB, MreC or RodA produces almost identically appearing spherical cells exhibiting an apparent loss of cell wall integrity (Begg and Donachie, 1985; Wachi \textit{et al}., 1987; Doi \textit{et al}., 1988; Kruse \textit{et al}., 2005), possibly attributable to the uncontrolled action of lytic enzymes. In contrast, in \textit{C. crescentus}, depletion of these proteins results in different morphological phenotypes. For example, under MreB depletion, the cells appear as relatively large lemon-shaped cells, whereas MreC- or RodA-depleted cells appear swollen relative to wild-type cells but do not achieve the large rounded appearance of MreB mutants. Therefore, MreB-depleted mutants continue to undergo cell wall expansion, with MreC- and RodA-depleted cells appearing to exhibit an arrest in cell wall growth, suggesting that MreC and RodA may also be required for peptidoglycan synthetic activity. Consistent with this idea, we observed a marked reduction of Van-FL labelling in RodA-depleted cells, and a complete absence of labelling in MreC-depleted cells. As MreC is required for the lateral localization pattern of PBP2, MlaA and MipA, it is possible that the loss of lytic enzyme/PBP complexes in the absence of MreC abolishes peptidoglycan synthesis. A different mechanism may lead to the cessation of peptidoglycan synthesis in RodA-depleted cells as PBP2 localization remained relatively intact in the absence of RodA. RodA is an integral membrane protein and has been suggested to function in the translocation of the lipid-linked disaccharide-peptidoglycan precursor peptidoglycan precursor from the cytoplasmic face of the membrane to the periplasm (Ehler and Holjte, 1996), where, through the action of PBPs, it is incorporated into the cell wall. Therefore, the decrease in peptidoglycan synthetic activity under RodA depletion may possibly reflect a reduction in the availability of precursor molecules in the periplasmic space. MreB and FtsZ are likely to be required for yet a different stage of peptidoglycan synthesis, possibly functioning to position cytosolic peptidoglycan precursor synthetic activities or other cell shape-determining proteins. Thus, the bacterial cytoskeleton and cell shape-determining proteins such as MreC and RodA function in concert to orchestrate the localization of cell wall synthetic complexes resulting in spatially co-ordinated and efficient peptidoglycan synthetic activity.

**Experimental procedures**

**Bacterial strains, media and growth conditions**

\textit{Caulobacter crescentus} strains, LS107 (syn-1000 ΔblaΔ) (synchronizable strain, ampicillin sensitive) (Stephens \textit{et al}., 1997), and their derivatives were grown in peptone yeast extract medium (PYE), M2-glucose (M2G), M2G-Nitrate (M2N) medium. Cultures were supplemented with the required combinations of: carbohydrate [glucose and/or xylose (0.2%)], nitrogen source (ammonium chloride or sodium nitrate), and supplemented with appropriate antibiotics when required. A22 was used at a concentration of 50 µg ml⁻¹. Synchronized swarmer cell populations were obtained by the method described in Evinger and Agabian (1979). In order to deplete essential proteins [FtsZ YB1585 (Wang \textit{et al}., 2001), RodA (YB363) (Wagner \textit{et al}., 2005) and MreC (JG5025)], cells were grown in PYE medium containing inducer (xylose) to mid-logarithmic phase (OD₆₀₀ = 0.7–1.0), washed three times with media lacking xylose and incubated in PYE without inducer. Cells containing inducible fusion proteins (MreB−, MreC−, MreD−mCherry) were grown in media lacking inducer (xylose or nitrate) and shifted to media containing inducer. Cells were grown for at least 6 h in inducer-containing media and examined by fluorescent microscopy while in mid-logarithmic phase.

**Bacterial strain construction**

The MurG–mCherry fusion was created by using polymerase chain reaction (PCR) to amplify the entire \textit{ftsW-murG} operon including the 5' upstream promoter region from \textit{C. crescentus} genomic DNA. This was subcloned upstream of an mCherry coding region (Shaner \textit{et al}., 2004) lacking its translational
start sequences, resulting in a fusion of mCherry to the carboxyl-terminus of MurG. This plasmid was introduced into \textit{C. crescentus} cells by electroporation creating an integration of the fusion and entire plasmid through single-cross-over homologous recombination. The resulting strain (JG5022) contained both wild-type \textit{murG} and a \textit{murG–mCherry} fusion. A similar strategy was used to construct MipA– (JG5023) and MitA–mCherry (JG5024) expressing strains. MurG, MitA and MipA were fused to a carboxyl-terminal FLAG epitope using a parallel strategy by cloning the respective DNA fragments into pJM21 and introduced into \textit{C. crescentus} by electroporation-creating strains JG5027, JG5028 and JG5029 respectively. The xylose-inducible MreB–mCherry fusion (LS4289) (Dye \textit{et al.}, 2005) and the nitrate-inducible MreC–mCherry fusion (N. Dye, unpublished) were introduced in \textit{C. crescentus} on a replicating plasmid. An inducible MreD–mCherry fusion was constructed by replacing the \textit{mreC} DNA from the inducible \textit{mreC–mCherry} plasmid with that of the \textit{mreD} coding region, creating a fusion of mCherry to the carboxyl-terminus of MreD. The MreC depletion strain (JG5021) was created by first placing the \textit{mreC} coding region downstream of the xylose-inducible promoter (Meisenzahl \textit{et al.}, 1997) in pRKxyl (Figge \textit{et al.}, 2004) and then introducing the resulting plasmid into \textit{C. crescentus} cells containing a deletion in \textit{leuA} by conjugation. Next a deleted \textit{mreC} allele from LS4275 (Dye \textit{et al.}, 2005) was introduced into this strain by bacteriophage \phi Cr30 transduction with selection for leucine prototrophy.

Van-FL labelling

Both synchronous and non-synchronous cell populations were incubated with BodipyFL-conjugated vancomycin (Van-FL, Molecular Probes) at a final concentration of 3 \mu M for 20 min and fixed in 2.5% formaldehyde, 30 mM sodium phosphate (pH 7.4) for 15 min at 25°C and 45 min on ice. The cells were washed three times with PBS (140 mM NaCl, 3 mM KCl, 8 mM NaH2PO4, and 1.5 mM KH2PO4) and once with GTE (50 mM glucose, 10 mM EDTA, 20 mM Tris-HCl at pH 7.5) and suspended in GTE. The cells were mounted to slides with GTE (50 mM glucose, 10 mM EDTA, 20 mM Tris-HCl at pH 7.5) and formaldehyde in 50 mM cacodylate–HCl buffer pH 7.4. After 10 min at 25°C the cells were washed with 1 ml of PYE and adsorbed on glow discharged carbon coated grids, stained with 0.5–1.0% uranyl acetate for 30 s and imaged on a Hitachi H7000 Electron Microscope at 10 000x and 250 000x magnification.

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References


**Supplementary material**

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