Bacterial intermediate filaments: in vivo assembly, organization, and dynamics of crescentin

Godefroid Charbon,^{1,4} Matthew T. Cabeen,¹ and Christine Jacobs-Wagner^{1,2,3,5}

¹Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520, USA;

²Section of Microbial Pathogenesis, Yale School of Medicine, New Haven, Connecticut 06510, USA; ³Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06520, USA

Crescentin, which is the founding member of a rapidly growing family of bacterial cytoskeletal proteins, was previously proposed to resemble eukaryotic intermediate filament (IF) proteins based on structural prediction and in vitro polymerization properties. Here, we demonstrate that crescentin also shares in vivo properties of assembly and dynamics with IF proteins by forming stable filamentous structures that continuously incorporate subunits along their length and that grow in a nonpolar fashion. De novo assembly of crescentin is biphasic and involves a cell size-dependent mechanism that controls the length of the structure by favoring lateral insertion of crescentin subunits over bipolar longitudinal extension when the structure ends reach the cell poles. The crescentin structure is stably anchored to the cell envelope, and this cellular organization requires MreB function, identifying a new function for MreB and providing a parallel to the role of actin in IF assembly and organization in metazoan cells. Additionally, analysis of an MreB localization mutant suggests that cell wall insertion during cell elongation normally occurs along two helices of opposite handedness, each counterbalancing the other's torque.

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The cytoskeleton is a central cellular component of eukaryotes and prokaryotes alike. Its various functions critically depend on its dynamics and cellular organization. Studies have revealed a remarkable number of sophisticated mechanisms governing the polymerization dynamics and the organization of actin filaments and microtubules in eukaryotes. Somewhat less is known about intermediate filaments (IFs)—the third major cytoskeletal system. There are multiple types of IFs, and they are involved in broad-ranging biological functions. IF proteins, while substantially diverging in sequence, are characterized by a structural organization of coiled-coil-rich regions flanked by N- and C-terminal sequences of varying length (Parry 2005). They also share the ability to spontaneously self-assemble in vitro (Herrmann and Aebersold 2004). In vivo, IF proteins assemble into biochemically stable structures onto which new IF subunits are continually added, not only at the filament ends but also laterally along the filaments (Kreis et al. 1983; Vikstrom

et al. 1989, 1992; Miller et al. 1991; Coleman and Lazarides 1992; Yoon et al. 2001). Interestingly, while being distinct in nature and behavior, all three cytoskeletal networks are interconnected inside cells, and evidence suggests that the proper assembly and organization of some IFs require intact microtubule and actin networks (Chang and Goldman 2004).

In recent years, analogous cytoskeletal structures have been shown to be widespread in prokaryotes (Graumann 2007; Pogliano 2008). The FtsZ tubulin homolog plays a critical role in cell division by assembling into a ring structure that constricts during cytokinesis (Margolin 2006; Pichoff and Lutkenhaus 2007). The FtsZ ring is highly dynamic (Ben-Yehuda and Losick 2002; Thanedar and Margolin 2004; Peters et al. 2007) and exchanges subunits with the cytoplasmic pool on time scales of seconds (Anderson et al. 2004). Another well-conserved cytoskeletal element is the actin homolog MreB. MreB assembles into helix-like structures that are thought to spatially restrict cell growth activities during cell elongation (Jones et al. 2001; Daniel and Errington 2003; Kruse et al. 2003; Figge et al. 2004; Gitai et al. 2004; Dye et al. 2005; Carballido-Lopez et al. 2006; Divakaruni et al. 2007; Mohammadi et al. 2007). These MreB structures are also dynamic, continuously exchanging subunits

⁴Present address: Department of Molecular and General Physiology, Roskilde University, DK-4000 Roskilde, Denmark.

⁵Corresponding author.

E-MAIL christine.jacobs-wagner@yale.edu; FAX (203) 432-5170.

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(Carballido-Lopez and Errington 2003b; Defeu Soufo and Graumann 2004; Kim et al. 2006) and, in several species, condensing into ring structures for part of the cell cycle (Figge et al. 2004; Slovak et al. 2005; Vats and Rothfield 2007). In addition to its well-known role in cell morphogenesis, MreB can play additional roles in chromosome segregation and cell polarity, and certain bacterial species contain additional actin or tubulin homologs involved in important functions such as plasmid segregation and organelle organization (Pogliano 2008).

IF proteins were long thought to be late inventions as they were primarily found in animal cells. But recent studies suggest that IF-like proteins might be widely represented across bacterial phyla (Izard 2006; Bagchi et al. 2008). Crescentin, a founding member of this new protein family, forms a filamentous structure along the inner cell curvature of the α -proteobacterium *Caulobacter crescentus* (Ausmees et al. 2003). In its absence, cells lose their curvature and adopt straight-rod morphology. Crescentin possesses IF-like domain organization, and similarly to eukaryotic IF proteins, it spontaneously assembles into IF-like filaments in vitro in the absence of additional factors (Ausmees et al. 2003). In cells, the crescentin structure is held at the cell membrane in a stretched configuration that must be maintained through a connection with the cell wall via unknown factors (Cabeen et al. 2009). The crescentin structure affects the cell wall incorporation rate such that less material is added on the crescentin-bearing side of the cell, leading to cell curvature during growth (Cabeen et al. 2009). Evidence suggests a mechanical model in which the crescentin structure provides some resistance to cell wall stretching during growth and thereby affects the kinetics of coordinated wall hydrolysis and insertion (Cabeen et al. 2009).

Many bacterial proteins share structural organization and polymerization properties similar to crescentin while having distinct cellular functions (Izard 2006; Bagchi et al. 2008). The discovery of this new family of bacterial cytoskeletal proteins emphasizes the importance of studying their dynamics and assembly mechanisms in vivo, for which virtually nothing is known. Here, we examine crescentin and its assembly in *C. crescentus* and show that crescentin shares striking similarities in cellular assembly properties and dynamics with metazoan IF proteins, providing further important support of their commonality. We further show that de novo assembly of crescentin is biphasic due to a mechanism that controls the length of the crescentin structure once it reaches the cell pole regions. We also uncover a novel function for MreB in the cellular organization of the crescentin structure.

Results

Regulation of crescentin levels and structure during the cell cycle

C. crescentus has a distinctive cell cycle that starts with a flagellated “swarmer cell.” After a certain period of

motility and growth, the swarmer cell ejects its polar flagellum and becomes a “stalked cell” by growing a polar stalk (a thin extension of the cell envelope). The stalked cell elongates into a predivisional cell that assembles a new flagellum at the pole opposite the stalk. Division produces a motile swarmer cell, which repeats the cycle, and a sessile stalked cell, which re-enters the cell cycle at a later stage, skipping the swarmer cell stage. Genome-wide gene expression array studies using synchronized cell populations revealed that the expression of the crescentin-encoding gene (*creS*) peaks at the stalked cell stage (Laub et al. 2002; McGrath et al. 2007). Western blot analysis, however, showed that the crescentin level does not change significantly during the cell cycle (MreB was a loading control [Fig. 1A; Figge et al. 2004], and the cell cycle regulator CtrA verified the synchrony [Domian et al. 1997]). Thus, despite transcriptional regulation, crescentin is present throughout the cell cycle. This has been reported for other *C. crescentus* proteins (Mohr et al. 1998; Jacobs et al. 2001).

Next, we examined whether crescentin localization changes during the cell cycle. We started with swarmer cells of a merodiploid strain (CJW815) that chromosomally expresses *creS-gfp* and *creS* from the native *creS* promoter. Similarly to GFP fusions to eukaryotic IF proteins (Ho et al. 1998; Yoon et al. 1998), crescentin-GFP is not functional alone but it associates with and labels the endogenous (untagged) crescentin structure when expressed in a wild-type background (Ausmees et al. 2003). The GFP-labeled structure can appear punctate along its entire length due to the mixture of crescentin and crescentin-GFP subunits. Time-lapse microscopy showed that in swarmer cells, the GFP-labeled crescentin structure extended along the entire inner curvature of the cells (Fig. 1B). The structure elongated during growth, although in a considerable fraction of the cell population (5% in swarmer cells and 40% in predivisional cells; $n = 329$ in both cases), it failed to fully extend into the old pole region (as shown by the crescentin-GFP-free region marked by an asterisk in Fig. 1B). Extension of the crescentin structure into the stalked pole region often resumed later, typically around or shortly after the time of cell separation (Fig. 1B). In all cases, the labeled crescentin structure was interrupted at the division site during cell constriction, presumably to accommodate cell division, so that each daughter cell inherited a crescentin structure at its inner curvature. In some cells, division was accompanied by the labeled crescentin structure “sliding” away from the newly formed pole (Supplemental Movie S1). The degree of the sliding movement varied from cell to cell.

Crescentin forms a stable, cohesive structure

Most known bacterial cytoskeletal elements form dynamic structures that continuously exchange subunits with the soluble pool (Stricker et al. 2002; Carballido-Lopez and Errington 2003a; Anderson et al. 2004; Garner et al. 2004; Becker et al. 2006; Larsen et al. 2007; Srinivasan et al. 2007). To examine whether the

Figure 1. Crescentin forms a stable and cohesive filamentous structure. (A) Western blot of crescentin protein levels during the cell cycle of wild-type CB15N cells. Samples from synchronized cell cycle populations were sonicated, and 20 μ g of total protein were loaded. Anti-CtrA and anti-MreB antibodies were used to assess synchrony quality and sample loading, respectively. (SW) Swarmer cell; (ST) stalked cell; (PD) predivisional cell. (B) Time-lapse series of the GFP-labeled crescentin structure (red, overlaid over phase images) during the course of the cell cycle, starting with synchronized CJW815 (CB15N *creS*::pBGST18creS-gfp::pBGENTcreS) swarmer cells. The cell shown represents a common example in which the expansion of the crescentin structure was delayed, resulting in crescentin-free regions (asterisk). Elongation of the crescentin structure toward the stalked pole later resumed (>>>). Bar, 1 μ m. (C) Photobleaching analysis of crescentin structure dynamics. After pretreatment with chloramphenicol (300 μ g/mL) for 30 min to block protein synthesis, CJW815 cells were placed on an agarose pad containing 300 μ g/mL chloramphenicol for 10 min before photobleaching. The panel shows a representative cell in which the central part of the GFP-labeled crescentin structure was bleached. Bar, 1 μ m. (D) Quantitative analysis of time-lapse recordings of 32 photobleached crescentin structures treated as described in C. Blue diamonds and green squares show the relative fluorescence intensities of bleached and unbleached regions of crescentin structures, respectively (as illustrated in the schematic). The data were corrected for background fluorescence and for photobleaching associated with image acquisition. (E) CJW2208 cells (CB15N *Pvan*::pBGENTPvancreS-tc) grown in the presence of 5 μ M vanillic acid to induce the synthesis of crescentin-TC. These cells were labeled with FAsH then washed and placed on an agarose-padded slide devoid of vanillic acid to stop further synthesis of crescentin-TC while endogenous crescentin was still being produced (from its native promoter).

crescentin structure exhibits dynamic exchange of subunits, we photobleached cells in the presence of a protein synthesis inhibitor (chloramphenicol) to prevent synthesis of new crescentin-GFP molecules and to look

specifically at any potential exchange of pre-existing molecules. A small defined region of the crescentin-GFP-labeled structure was photobleached in multiple cells ($n = 32$). Time-lapse imaging showed no discernable

signal recovery in the bleached regions for over 50 min following photobleaching (Fig. 1C,D), indicating that there was no observable exchange of subunits within the filamentous crescentin structure or between the filamentous structure and a cytoplasmic pool of crescentin subunits. Thus, in the absence of growth, crescentin forms a stable cohesive structure.

These photobleaching experiments did not, however, rule out the possibility that subunit exchange or axial sliding of protofilaments within the crescentin structure might occur during its growth. To test this possibility, we tracked the fate of the crescentin-TC (crescentin tagged with a tetracysteine motif) signal in a strain (CJW2208) that produced crescentin-TC from the vanillic acid-dependent promoter (*Pvan*) at the chromosomal *vanA* locus in addition to untagged crescentin from its native locus. Crescentin-TC is fully functional on its own (Cabeen et al. 2009; see also below) and can be visualized using the FAsH dye, which binds to the TC tag (Gaietta et al. 2002). Cells were first grown with vanillic acid, and the resultant crescentin-TC structures were FAsH-stained. The cells were then placed on a medium-containing agarose pad without vanillic acid to stop further synthesis of crescentin-TC at the start of time-lapse imaging (while maintaining expression of untagged crescentin from its native promoter). As expected for a stable structure, the FAsH signal did not spread out over time, even after two cell divisions (Fig. 1E). Thus, once crescentin subunits are incorporated into the crescentin structure, they remain in place relative to each other as the structure grows. This provides a clear biochemical distinction from other known bacterial cytoskeletal proteins but is consistent with the general stability of eukaryotic IFs (Yoon et al. 2001).

Growth of the crescentin structures is nonpolar, and subunit incorporation occurs along the filaments

How does assembly occur in vivo? New crescentin subunits must be incorporated at the ends of the structure to elongate it during cell growth. However, it was unclear whether new subunit addition occurred only at the structure ends or along its entire length as well. To distinguish between these possibilities, we photobleached the entire crescentin-GFP-labeled structure in merodiploid CJW815 cells ($n = 59$). Incorporation of newly synthesized crescentin-GFP molecules was then recorded by time-lapse microscopy during cell growth. We found that there were no preferential sites of incorporation as the crescentin-GFP signal reappeared along the entire photobleached structure at the inner cell curvature and not just at the structure ends near the poles (Fig. 2A). This was confirmed by three-dimensional representations of GFP signal intensity inside cells (Fig. 2B).

This demonstrated that crescentin can laterally associate with its filamentous form, another property shared with eukaryotic IFs (Kreis et al. 1983; Vikstrom et al. 1989, 1992; Miller et al. 1991; Coleman and Lazarides 1992; Yoon et al. 2001). What happens then when there is no crescentin structure and therefore no template to start

from? It has been shown that introducing a plasmid carrying the *creS* gene into a crescentin-null strain restores cell curvature (Ausmees et al. 2003), indicating that crescentin is able to form a functional structure de novo without the need of a pre-existing template (Margolin 2004). To characterize this de novo assembly, we placed a single copy of *creS-tc* under the xylose-inducible promoter (*PxyI*) in $\Delta creS$ cells (creating strain CJW1782). In the absence of xylose, crescentin-TC synthesis was repressed and the straight rod-shaped cells only displayed background staining of FAsH (Fig. 2C, panel i), similar in intensity to FAsH staining of cells lacking any TC-tagged protein (Fig. 2C, panel ii). After addition of xylose to liquid cultures, cell samples were stained with FAsH and collected at different times following induction of crescentin-TC synthesis (Fig. 2C, panels iii–vii). At $t = 25$ min, there were three distinct subpopulations of cells. One subpopulation revealed a seemingly full-length crescentin-TC structure spanning from pole-to-pole on one side of the cells (data not shown), whereas another subpopulation was still devoid of crescentin-TC structures as evidenced by background FAsH staining (data not shown). The third subpopulation consisted of cells with a single partial crescentin-TC structure, likely representing assembly intermediates (Fig. 2C, panel iii). The partial structure varied in length among cells and could be found, generally at the cell periphery, anywhere along one side of the cells. These observations suggest that (1) crescentin assembly initiates at or close to the membrane, (2) there is no preferential site of initiation, and (3) when assembly is initiated at one site, extension of this polymeric structure is favored over a second nucleation elsewhere. At $t = 60$ min, virtually all cells exhibited a full-length crescentin-TC structure and cells began to curve (Fig. 2C, panel iv). Occasionally, two crescentin-TC structures were found at opposite sides (Fig. 2C, panel v). This event was rare under these conditions (3%, $n = 547$ cells) but was more frequent when crescentin-TC synthesis was induced from a multicopy plasmid (data not shown), indicating that the occurrence of two structures increases with crescentin overproduction. Importantly, the crescentin-TC structures, irrespective of their size (i.e., even when they were partial structures as in early time points), were virtually always parallel to the long cell axis. Only ~1% of cells formed crescentin-TC structures that were at an angle (data not shown). At later time points, the cells and the associated crescentin structures, which are flexible (see also Fig. 6A,B [below]; Cabeen et al. 2009), became curved (Fig. 2C, panels vi,vii). Interestingly, even when the cells became hypercurved because of fivefold to sevenfold overproduction of crescentin-TC (Supplemental Fig. S1), the crescentin structure did not curve around the poles and extend around the cell periphery but instead ended at the poles (Fig. 2C, panel vii), indicating that the length of the crescentin structures is not dictated by expression level. Instead, a mechanism must exist to limit the elongation of the crescentin structure.

While the crescentin-TC fusion has the advantage of being fully functional, FAsH photobleaches rapidly

Figure 2. After initial pole-to-pole expansion along a straight line, the crescentin structure thickens through lateral association of crescentin anywhere along the existing structure. (A) CJW815 (CB15N *creS*::pBGST18*creS*-gfp::pBGENT*creS*) cells expressing both *creS* and *creS*-gfp from the native promoter were placed on an agarose pad and whole cells were photobleached. Accumulation of newly synthesized GFP-crescentin was followed over time by time-lapse fluorescence microscopy as cells grew and produced new crescentin-GFP molecules. (B) Three-dimensional fluorescence intensity plot of the cell shown in A. (C, left) CJW1782 cells (CB15N Δ *creS* *Pxyl*::pBGENT*PxylcreS*-tc) grown in the presence of glucose (instead of xylose) to repress crescentin-TC synthesis (panel i) and CJW1647 cells (CB15N Δ *creS* pMR10*PxylcreS*) producing untagged crescentin in xylose-containing medium (panel ii) were stained with FISH and used here as controls to show the background fluorescence due to FISH staining. (Right) Crescentin-TC synthesis in CJW1782 cells was induced by adding xylose in the culture medium. (Panels iii-vii) Cell samples were stained with FISH to visualize crescentin-TC at indicated times following xylose addition. (D) Synthesis of a GFP-labeled crescentin structure in CJW2207 cells (CB15N Δ *creS* *Pvan*::pBGENT*PvancreS*-tc::pHL32*PvancreS*-gfp) was induced on an agarose-padded slide containing vanillic acid (5 μ M). DIC image and corresponding crescentin-GFP signal were recorded by time-lapse microscopy. (E) Linescans of crescentin-GFP signal intensity across the length of the cell represented in D.

relative to GFP, preventing repeated image acquisition of the same cells in time-lapse experiments. Furthermore, variability in FISH staining efficiency prohibits reliable interpretation of signal intensities. Therefore, to gain additional information about crescentin assembly, we monitored the de novo assembly of crescentin using a crescentin-GFP fusion. To maintain similar levels between crescentin-GFP and functional crescentin (in this case crescentin-TC) and to induce their synthesis simultaneously, *creS*-gfp and *creS*-tc were placed independently under the same promoter (*Pvan*) on the chromosome in an otherwise Δ *creS* background (strain CJW2207). As expected, the initial steps of assembly (i.e., nucleation and extension of the filamentous structure) were missed because of the slow rate of GFP folding and chromophore formation (Tsien 1998), causing a notable delay between crescentin assembly and its visualization. However, time-lapse recording clearly showed that over time, the

crescentin structure increased in signal intensity along its entire length rather than extending around the cell poles (Fig. 2D). Measurements of signal intensities along the cell length over time confirmed the thickening of the crescentin structure (Fig. 2E). This indicates that when the crescentin levels are high, lateral association of crescentin subunits is favored over continued extension of the crescentin structure. This is consistent with a mechanism that slows down polymerization at the ends of the structure once they reach the polar regions.

Extension of the crescentin structure is bidirectional and regulated by cell length

Our findings suggested that crescentin structure extension might depend on cell length. We examined this possibility by monitoring crescentin assembly in elongated cells. To do this, we used a Δ *creS* strain

(CJW2209) in which the cell division protein FtsZ is under xylose-inducible synthesis, whereas crescentin-TC and crescentin-GFP synthesis are vanillic acid-inducible. Cells were first grown in the presence of xylose (to allow *ftsZ* expression) and in the absence of vanillic acid (to repress *creS-tc* and *creS-gfp* expression) to obtain straight-rod cells. Then, after being washed, these cells were placed on a slide that lacked xylose but contained vanillic acid to induce synthesis and coassembly of crescentin-TC and crescentin-GFP while blocking FtsZ synthesis and cell division. Time-lapse imaging and measurements of fluorescence intensities along the cell length revealed that the crescentin structure, after an initial extension phase, gets progressively thicker while extending slowly (note that cell coiling is prevented when filamentation occurs on a solid surface) (Fig. 3A,B). This resulted in filamentous cells with a thick internal crescentin structure flanked with large regions devoid of crescentin. It should be noted that elongation of the crescentin structure, even if slow, occurred at both ends (Fig. 3B), indicating that crescentin assembly is bidirectional under these conditions.

By plotting the length of the crescentin structure and the cell length as a function of time, we found that the elongation of the crescentin structure follows a biphasic curve (Fig. 3C). The first phase consists of a rapid elongation of the crescentin structure to a length slightly shorter than the cell length. This is followed by a second, slower phase of elongation. This suggests that once the structure reaches the vicinity of the poles, the ends of the structure get “capped” or modified, resulting in a considerable reduction in elongation rate. From then on, most

crescentin subunits are added laterally, thickening the crescentin structure. This effect was not due to crescentin overproduction or crescentin-GFP, as depletion of FtsZ in a wild-type background (strain YB1585) produced filamentous cells that were coiled only in their central region and had straight ends (Fig. 3D), consistent with crescentin-free ends. This was verified by depleting FtsZ in CJW2281 cells that produced crescentin-TC labeled with FLAsH (Fig. 3E).

Our data suggested that the switch of growth mode occurs when the crescentin structure reaches the polar regions. Thus, initiating crescentin assembly in longer cells should result in accordingly longer crescentin structures. To test this prediction, CJW2209 cells of varying lengths were prepared by depleting FtsZ in liquid culture for different amounts of time. Synthesis and coassembly of crescentin-TC and crescentin-GFP were then induced in these cells on agarose pads and monitored while continuing FtsZ depletion. Measurements from these time-lapse recordings were in full agreement with our hypothesis as there was a linear correlation between initial cell length and the crescentin structure length after a defined time (e.g., 300 min) (Fig. 4A). The crescentin structure exhibited biphasic elongation even in cells of widely varying length (Fig. 4B). Initial cell length at $t = 0$ (crescentin induction) had a clear effect both on the timing of the capping event (defined as the switch between rapid and slow elongation) and on the final length of the crescentin structure (Fig. 4C). Cells that were shorter at crescentin induction produced shorter crescentin structures than long cells after the same amount of time (300 min) (see Fig. 4D for examples).

Figure 3. Crescentin assembly is biphasic. (A) CJW2209 cells (CB15N *ftsZ*::pBJM1 $\Delta creS$ *Pvan*::pBGENTPvancreS-tc::pHL32PvancreS-gfp) from a PYE xylose culture (allowing *ftsZ* expression and cell division) were placed on an agarose-padded slide with vanillic acid but without xylose to induce crescentin-TC and crescentin-GFP while depleting FtsZ and blocking cell division. Time-lapse imaging followed the assembly of the GFP-labeled crescentin structure during cell filamentation. Selected images are shown. Colored dashed lines represent regions used for the linescans shown in B. (B) Linescans of crescentin-GFP signal intensity along the length of the cell shown in A. (C) The length of the cell (blue) shown in A and the length of its GFP-labeled crescentin structure (red) were plotted over time. (D) YB1585 cells (CB15N *ftsZ*::pBJM1) carrying *ftsZ* under the xylose-inducible promoter were grown without xylose for 6 h to deplete FtsZ, then imaged in liquid (i.e., without cell immobilization) using phase contrast microscopy and 1 msec exposure time. (E) After growth in PYE xylose medium, CJW2281 cells (CB15N *ftsZ*::pBJM1 $\Delta creS$ *Pvan*::pBGENTPvancreS-tc) were transferred to liquid PYE medium containing 50 μ M vanillic acid but no xylose in order to simultaneously induce crescentin-TC synthesis while depleting FtsZ. After 4 h of growth, the cells were stained with FLAsH and mounted on an agarose-padded slide to visualize the crescentin-TC structure.

Figure 4. Elongation of the crescentin structure is determined by cell length. (A) Cells of different length were generated by depleting CJW2209 cells (CB15N *ftsZ*::pBJM1 $\Delta creS$ *Pvan*::pBGENTPvancreS-*tc*::pHL32PvancreS-gfp) of FtsZ for 0–150 min. Synthesis and assembly of a GFP-labeled crescentin structure was then induced on agarose-padded slides containing vanillic acid (to induce *creS-tc* and *creS-gfp*) and lacking xylose (to maintain repression of *ftsZ* expression). Ensuing cell growth and extension of the crescentin structure were then immediately recorded by time-lapse microscopy. The length of the crescentin structure after 300 min of growth was then plotted as a function of cell length at $t = 0$ ($n = 66$). (B) Same as A except that the growth and extension of the crescentin structure recorded during time-lapse microscopy was plotted over time. The *top* and *bottom* plots show examples of a “short” cell ($\sim 3 \mu\text{m}$) and a “long” cell ($\sim 10 \mu\text{m}$), respectively, at the start of the time lapse ($t = 0$). (C) Same as B except that the length of the crescentin structure was plotted as a function of cell elongation (i.e., cell length increment) rather than time. Shown are examples of cells ranging from 2.7 μm to 11.5 μm at the time of initiation of crescentin-GFP synthesis. (D) Same as A except that shown are DIC and fluorescence micrographs of a short cell (*left*) and long cell (*right*) at $t = 0$ and after $t = 300$ min. The long cell was generated by depletion of FtsZ in PYE glucose liquid medium for 150 min prior to induction of GFP-crescentin synthesis on the agarose-padded slide containing vanillic acid and lacking xylose.

Collectively, these findings strongly support the presence of a mechanism that is triggered by the crescentin structure reaching the polar regions of the cell. This mechanism reduces the rate of incorporation of crescentin subunits at the ends of the structure, resulting in significant lateral association of crescentin molecules with the existing structure.

Crescentin can assemble and mediate cell curvature on any side of the cell independently of pre-existing curvature

As described above, *de novo* assembly of crescentin primarily occurred along one cell side. This raised the question of whether crescentin preferentially nucleates at one particular side of the cell or whether side selection is random. Since, in the absence of crescentin and cell curvature, lateral sides are indistinguishable, we took advantage of the large regions devoid of crescentin in filamentous FtsZ-depleted cells (Figs. 3, 4). Restoration of FtsZ synthesis in these cells can result in division in crescentin-free regions, generating cells devoid of crescentin. By using the existing crescentin structure as a spatial reference, we found that *de novo* assembly of crescentin could occur at either side of the cells (Supplemental Movies S2, S3). This suggests that there is no predetermined cell side for crescentin assembly.

While in most cases, extension of a pre-existing crescentin structure occurred, we observed on rare occasions the formation of a second structure within the same cell compartment on the opposite side (Fig. 5A, arrow), demonstrating that crescentin can also assemble on the outer curvature.

Cell curvature was sometimes artificially created as the flexible filamentous cells lay down on the slide. When this happened, crescentin was able to assemble along artificially created outer cell curvatures and was able to change the curvature morphology from convex to concave over time during growth (Fig. 5B). Hence, the assembly and function of crescentin appear insensitive to cell geometric constraints such as curvature.

MreB plays a critical role in crescentin-mediated cell curvature

In order to provide any mechanical resistance to affect the kinetics of cell wall growth and generate curvature, the crescentin structure must be connected to the cell wall (Cabeen et al. 2009). We reasoned that MreB might mediate this connection, as MreB interacts with cell wall enzymes (Carballido-Lopez et al. 2006; Kawai et al. 2009). This would be analogous to the interaction between IFs and actin filaments in eukaryotic cells (Hubbard and Lazarides 1979; Green et al. 1987; Yoon et al. 1998; Weber

Figure 5. De novo assembly of the crescentin structure can occur and generate cell curvature on any side of the cell regardless of pre-existing cell curvature constraints. (A) CJW2046 cells (CB15N *ftsZ*::pBJM1 *creS*::pBGST18creS-gfp::pBGENTcreS) producing crescentin and crescentin-GFP from the endogenous promoter were depleted of FtsZ for 4 h, then placed on an agarose pad containing xylose to replete FtsZ and subjected to time-lapse microscopy. Selected images at indicated times (minutes) are shown. Fluorescent images (top row) show the GFP-labeled crescentin signal, which was overlaid (red) with the corresponding DIC images (bottom row). The arrow indicates a crescentin structure assembling on the outer curvature of the cell. Red dotted lines show cell outlines obtained from the DIC images. (B) Same as in A with top images showing DIC and fluorescent images at $t = 0$ min and bottom images showing ensuing time-lapse images of the cell region defined by the white box in the top DIC image. Arrows indicate a division site.

and Bement 2002; Godsel et al. 2005; Windoffer et al. 2006).

A22 is a drug that disrupts the MreB cytoskeleton (Gitai et al. 2005), though this disruption is partial (Aaron et al. 2007; Karczmarek et al. 2007). Nevertheless, treatment of cells with A22 for 3 h resulted in cell widening and detachment of crescentin structures (Fig. 6A). The percentage of double crescentin structures (Fig. 6A, asterisk) also increased from 1% to 2% ($n = 362$) to ~7% ($n = 383$) under these conditions. Crescentin structure detachment could be more easily visualized in A22-treated filamentous cells because the crescentin structures are longer. Detachment of these long structures caused them to relax and contract from an immobile extended configuration to a mobile, helical form (Fig. 6B; Supplemental Movie S4). Furthermore, when normalized cells (CJW2207) were pretreated with A22 before inducing crescentin synthesis, crescentin retained the ability to polymerize but failed to stably attach along the cell envelope as evidenced by the motion of curved or helical crescentin structures (Fig. 6C).

Although A22 treatment caused cell widening, release of crescentin structures could occur independently of cell widening. This was shown using an *mreB*_{G165D} point mutant, which was isolated on plates containing 50 μ M of A22. As with other A22-resistant mutants (Gitai et al. 2005), the mutation (G165D) lies within the ATP-binding pocket of MreB. In wild-type cells, MreB changes from

a helical distribution to a central ring-like localization after FtsZ ring formation (Supplemental Movie S5; Figge et al. 2004; Gitai et al. 2004). It has been shown that a T167A mutation in the ATP-binding pocket affects the ability of the protein to form rings near mid-cell (Gitai et al. 2005). The G165D mutation had a similar effect, as *MreB*_{G165D} rarely assembled into rings at mid-cell but instead formed “patches” (Fig. 6D; Supplemental Movie S6). The *mreB*_{G165D} mutant cell population was heterogeneous, comprising slightly elongated cells with abnormal curvature as reported for other *mreB* mutants (Gitai et al. 2005). These cells either were sigmoid or had little to no curvature (Fig. 6E,F). In all cases, cells were slightly narrower ($0.52 \pm 0.06 \mu\text{m}$, $n = 321$) than normal ($0.63 \pm 0.06 \mu\text{m}$, $n = 295$). This is similar to the slight cell width decrease observed in *Escherichia coli* carrying MreB mutants impaired in ATPase activity (Kruse et al. 2003). Importantly, in ~6% of *mreB*_{G165D} cells ($n = 322$), the crescentin structure appeared detached from the membrane (Fig. 6G) and exhibited motion within the straight cells (Supplemental Movie S7). This is an ~10-fold increase relative to wild-type *mreB* cells, which under similar conditions displayed ~0.6% of detached crescentin structures ($n = 362$). We obtained similar results with two other A22-resistant *mreB* mutants (with mutations resulting in T167A and V324A substitutions) (data not shown). It should be noted that the deficiency in MreB ring formation is unlikely to be responsible for the

and CWJ1788 cells (CB15N *mreB*_{G165D} *creS*::Tn5). (F) Scanning electron micrographs of CWJ1789 cells (CB15N *mreB*_{G165D}). (G) Straight CWJ1791 cells (CB15N *mreB*_{G165D} *creS*::pBGST18*creS*-gfp::pBENTcreS) with a detached GFP-labeled crescentin structure. (H) Crescentin-Flag coimmunoprecipitation with MreB using anti-Flag antibodies. Protein extracts from wild-type CB15N *C. crescentus* cells were prepared (negative control as they lack Flag-tagged proteins) along with extracts from CWJ932 cells (CB15N *creS*::pJM21*creS*-flag) producing crescentin-Flag at endogenous levels in place of crescentin. After immunoprecipitation (using anti-Flag antibody) and elution with Flag peptide, each eluate was loaded on a SDS-PAGE gel and analyzed by Western blotting using anti-Flag and anti-MreB antibodies. Also shown is a corresponding Western blot of cell lysates (input; 20 μg of total protein).

crescentin delocalization phenotype since the MreB_{Q26P} mutant also fails to form a mid-cell ring, yet the cells are curved (Aaron et al. 2007) and crescentin localization is normal (data not shown). Regardless, the fact that multiple MreB point mutations (G165D, T167A, and V324A) cause a partial crescentin delocalization without causing cell widening is consistent with MreB, and not just cell enlargement, affecting crescentin attachment to the cell envelope. In support of this, we showed that a functional crescentin-Flag fusion pulls down MreB (Fig. 6H), but not FtsZ (data not shown), from *C. crescentus* extracts; both *creS*-flag and *mreB* were expressed at endogenous levels.

Recently, we showed that when crescentin is ectopically synthesized in the γ -proteobacterium *E. coli*, it can form functional filamentous structures along the cell membrane, producing cell curvature during growth (Cabeen et al. 2009). This remarkable transfer of function despite the large evolutionary divergence between *E. coli* and *C. crescentus* indicates that the connection between crescentin and the cell wall is mediated by

Figure 6. Proper organization of the crescentin structure in the cell requires MreB function. (A) Images of CWJ815 cells (CB15N *creS*::pBGST18*creS*-gfp::pBENTcreS) producing crescentin and crescentin-GFP from the native promoter and treated with 50 μM A22 for 3 h prior to imaging. Asterisk shows an example of two crescentin structures within a single cell. (B) Time-lapse images of CWJ2046 cells (CB15N *ftsZ*::pBJM1 *creS*::pBGST18*creS*-gfp::pBENTcreS) producing crescentin and crescentin-GFP from the native promoter. Cells were grown in absence of xylose to deplete FtsZ for 2 h and then placed on an agarose pad with xylose and 25 μM A22 to disrupt MreB localization while restoring *ftsZ* expression. Restoring FtsZ synthesis was necessary to prevent growth arrest, which occurs when both FtsZ and MreB functions are disrupted. Selected images are shown; see Supplemental Movie S4 for the whole sequence. (C) Time-lapse sequence of CWJ2207 cells (CB15N Δ *creS* *Pvan*::pBENTPvan*creS*-tc::pHL32Pvan*creS*-gfp) grown in PYE liquid medium containing 50 μM A22 for 330 min prior to simultaneous induction of crescentin-TC and crescentin-GFP synthesis on an agarose-padded slide containing 50 μM vanillic acid and 25 μM A22 to disrupt MreB localization. Imaging shows the localization of the GFP-labeled crescentin structure. (D) Fluorescence and DIC micrographs of CWJ1790 cells (CB15N *mreB*_{G165D} *Pxyl*::pXGFP4-C1*mreB*_{G165D}) producing GFP-MreB_{G165D}. (E) DIC images of CB15N cells (wt), CWJ763 cells (CB15N *creS*::Tn5), CWJ1789 cells (CB15N *mreB*_{G165D}), and CWJ1788 cells (CB15N *mreB*_{G165D} *creS*::Tn5). (F) Scanning electron micrographs of CWJ1789 cells (CB15N *mreB*_{G165D}). (G) Straight CWJ1791 cells (CB15N *mreB*_{G165D} *creS*::pBGST18*creS*-gfp::pBENTcreS) with a detached GFP-labeled crescentin structure. (H) Crescentin-Flag coimmunoprecipitation with MreB using anti-Flag antibodies. Protein extracts from wild-type CB15N *C. crescentus* cells were prepared (negative control as they lack Flag-tagged proteins) along with extracts from CWJ932 cells (CB15N *creS*::pJM21*creS*-flag) producing crescentin-Flag at endogenous levels in place of crescentin. After immunoprecipitation (using anti-Flag antibody) and elution with Flag peptide, each eluate was loaded on a SDS-PAGE gel and analyzed by Western blotting using anti-Flag and anti-MreB antibodies. Also shown is a corresponding Western blot of cell lysates (input; 20 μg of total protein).

generic components, such as MreB. In support of this, a functional Flag-tagged version of crescentin pulled down *E. coli* MreB from cell extracts of curved *E. coli* MC1000 cells (Supplemental Fig. S2A). This predicted that crescentin structures would fail to achieve membrane attachment and to produce cell curvature in rod-shaped bacteria that lack MreB even if these bacteria were more closely related to *C. crescentus* than *E. coli* is. We confirmed this prediction using *Agrobacterium tumefaciens*, a rod-shaped α -proteobacterium that is a much closer relative of *C. crescentus* but that, unlike *C. crescentus* and *E. coli*, is devoid of MreB. Crescentin was able to form filamentous structures in *A. tumefaciens*, but these structures were detached, mobile, and unable to cause cell curvature (Supplemental Fig. S2B; Supplemental Movie S8).

Collectively, these findings strongly argue that MreB function is required for cell curvature generation by governing the proper organization and localization of the crescentin structure within the cell.

Aberrant cell curvature is mediated by distortion of the crescentin structure during growth

A connection defect between the crescentin structure and the cell wall would explain the presence of straight-shaped cells in the population of the *mreB_{G165D}* mutant. However, this defect was partial as some *mreB_{G165D}* cells displayed a sigmoid cell morphology (Fig. 6E), which was verified by scanning EM (Fig. 6F). This abnormal curvature was dependent on crescentin since inactivation of *creS* caused all *mreB_{G165D}* cells to become straight (Fig. 6E). This indicates that MreB_{G165D} creates sigmoid morphology by affecting crescentin. Accordingly, crescentin structures appeared to stretch across the sigmoid *mreB_{G165D}* cells from one curved side to the other (Fig. 7A). This prompted us to examine how crescentin assembles de novo in this *mreB_{G165D}* background, using inducible crescentin-TC or crescentin-GFP strains (CJW1788 or CJW2883, respectively). We expected that crescentin would assemble into S-shaped structures along the cell membrane (as opposed to straight structures as in the wild-type *mreB* background; Figure 2C), and that this abnormal assembly pattern would be responsible for sigmoid cell morphology. Instead, to our surprise, crescentin assembled into normal straight structures parallel to the long cell axis. It was only upon growth that the crescentin-TC structures and the cells became S-shaped (Fig. 7B). This was observed in liquid cultures of CJW1788 cells (Fig. 7B) as well as on agarose pads with CJW2883 cells blocked for cell division (by FtsZ depletion) to better show the twisting of crescentin structures during cell elongation (Fig. 7C; Supplemental Movie S9). Thus, the abnormal localization of the crescentin structures (which caused the sigmoid cell shape in both liquid and solid media) was not generated during crescentin assembly, but during cell growth. This indicated that something about cell growth in this mutant distorted the crescentin structures.

A major clue came from recording the growth of *creS::Tn5 mreB_{G165D}* cells depleted of FtsZ to allow cell filamentation (and lacking crescentin to remove any crescentin-induced cell growth effect). When growth and filamentation occurred in liquid media, the cells exhibited the expected morphology (Fig. 7D). By contrast, growth on an agarose-padded slide resulted in dramatic and sudden cell twisting, producing double-stranded helices that progressively added helical turns (Fig. 7D; Supplemental Movie S10). The twisting occurred in all *mreB_{G165D}* cell filaments, with a strong bias for the formation of right-handed helices (91%, $n = 52$). No twisting was ever observed in wild-type *mreB* cells filamenting under similar conditions (Supplemental Movie S10). This strongly suggests that, during elongation in *mreB_{G165D}* cells, the two cell poles rotate in opposite directions relative to each other (Fig. 7E). The resistance to rotation provided by a solid surface would generate strain that would be relieved by cell twisting. This would not occur in liquid cultures where there is no resistance (Fig. 7D). Such torsion appears absent or minimal in wild-type *mreB* cells such that no cell filament twisting could

be observed (Supplemental Movie S10). These observations have important implications about MreB function and cell growth pattern (see Discussion). They are also consistent with the crescentin structure being connected to the cell wall. Twisting of the cell wall during growth in the *mreB_{G165D}* mutant causes the crescentin structure to twist as well (Fig. 7E; Supplemental Movie S11). As it twists, the crescentin structure would provide an internal resistance to the torsion in *mreB_{G165D}* cells (Fig. 7E; Supplemental Movie S11), much as the solid surface of the pad provides an external resistance. This in turn would cause the cells to grow with sigmoid morphology. Such growth-induced torsion may also aid detachment of crescentin structures from the cell wall, although detachment and motion of the crescentin structures was also observed when growth was inhibited by the addition of chloramphenicol (Supplemental Movie S12), suggesting that crescentin attachment is at least partially impaired in the *mreB_{G165D}* mutant.

Discussion

We show that crescentin displays dynamics and in vivo assembly properties of eukaryotic IF proteins. These data bolster the other IF-like characteristics of crescentin, such as its structural organization and in vitro polymerization properties (Ausmees et al. 2003). Given the widespread presence of crescentin-like proteins in bacteria (Izard 2006; Bagchi et al. 2008), this strongly suggests that bacteria, like eukaryotes, widely exploit the properties of IF proteins for diverse biological functions.

We also characterized the de novo assembly of crescentin structures and uncovered a cell length-dependent mechanism that slows down the elongation rate of the crescentin structure and causes lateral expansion (i.e., thickening). Evidence indicates that this occurs when the ends of the structure reach the polar regions. This may involve bipolar proteins and/or physical contact with the poles. Either way, this switch from elongation to predominantly lateral growth provides a way to control the length of the crescentin structure and spatially restricts the structure along only one lateral side of the cell. How uncontrolled elongation of IFs is prevented in eukaryotic cells remains unclear. It is conceivable that a similar switch in growth mode is at play.

Our study also uncovers a novel function for MreB in organizing the crescentin structure within the cell, reminiscent of the organizing role of actin in IF formation and organization (Chang and Goldman 2004). In eukaryotic cells, connectivity between actin filaments and IFs can be mediated by different protein linkers. The interaction between MreB and crescentin may also be indirect. The known interaction of MreB with cell wall enzymes may provide attachment points to the cell wall to stably affix the crescentin structure to the membrane. This attachment maintains the crescentin structure in a stretched conformation, as release from the membrane results in contraction into a helical structure (Fig. 6B; Supplemental Movie S4; Cabeen et al. 2009). Insertion of cell wall material around the cell circumference during cell

Figure 7. Aberrant growth of the *mreB_{G165D}* mutant causes cell twisting on solid medium or when an internal crescentin structure is present. (A) Localization of the GFP-labeled crescentin structure in a sigmoid *mreB_{G165D}* CJW1791 cell (CB15N *mreB_{G165D}* *creS*::pBGST18*creS*-gfp::pBGENT*creS*). (B, left) FIASH-stained CJW1785 cells (CB15N *mreB_{G165D}* *creS*::Tn5 *Pxyl*::pBGENT*PxylcreS*-tc) grown in PYE glucose liquid medium to show background fluorescence due to FIASH staining. (Right) Induction of crescentin-TC synthesis in CJW1785 cells was achieved by adding xylose to the liquid culture. Cells were sampled at indicated times and stained with FIASH to visualize crescentin-TC. (C) Time-lapse images of CJW2883 cells (CB15N *mreB_{G165D}* *creS*::Tn5 *Pvan*::pBGENT*PvancreS*-tc::pHL32*PvancreS*-gfp *ftsZ*::pXMCS7*ftsZ*) carrying the *mreB_{G165D}* mutation. Cells were first grown in absence of xylose to deplete FtsZ for 1 h, after which synthesis of a GFP-labeled crescentin structure was induced on an agarose-padded slide containing vanillic acid. Selected images of the GFP-labeled crescentin structure (in red in the overlays) at indicated times (minutes) are shown. (D) DIC micrographs of FtsZ-depleted CJW2882 cells (CB15N *mreB_{G165D}* *creS*::Tn5 *ftsZ*::pXMCS7*ftsZ*) after growth and filamentation in PYE liquid culture (left) or on an agarose pad (right). Arrows indicate regions of cell twisting. (E) Cartoons illustrating how cells carrying the *mreB_{G165D}* mutation grow in the absence (left) or presence (right) of a crescentin structure. Cell elongation causes the two poles to rotate in opposite directions relative to each other. Thus, growth causes fixed points on the cell wall to move relative to each other according to the pole rotation pattern. A crescentin structure that is connected to the cell wall will be twisted by growth, thereby modifying the curvature of the cell.

elongation must stretch the biochemically cohesive and stably attached crescentin structure to a breaking point, suggesting that its connections to the cell wall are modulated. Such modulation may involve the dynamics of the MreB cytoskeleton.

The cell twisting of the *mreB_{D165G}* mutant during filamentation on solid surfaces but not in liquid media could be explained by a torque created by a helical pattern of peptidoglycan insertion (such that poles rotate in opposite directions). This is consistent with the torsion

of the internal crescentin structure during *mreB*_{D165G} cell growth (in both solid and liquid media). Helical growth has been suggested in several bacterial species (Daniel and Errington 2003; Divakaruni et al. 2007; Varma et al. 2007), but the wild-type *mreB* strain shows no sign of crescentin structure torsion or cell twisting during filamentation. This may be explained by the observation of double MreB helices crisscrossing each other in various bacteria (Jones et al. 2001; Shih et al. 2003; Figge et al. 2004). This suggests that in the wild-type situation, cell wall insertion occurs along two helices of opposite handedness, each canceling the torque created by the other. This counterbalance would be disrupted in the *mreB*_{D165G} mutant in which MreB organization is impaired.

Materials and methods

Strains, plasmids, and growth conditions

Strains and plasmids are listed in Supplemental Table S1, and their construction is in the Supplemental Material. Transformations, conjugations, and phage transductions were carried out as described (Ely 1991). *C. crescentus* strains were grown at 30°C in PYE medium or M2G minimal medium (Ely 1991). Exponential-phase cultures were used for all experiments. When required, gene expression was induced by adding 0.3% xylose or 500 μ M vanillic acid (unless indicated otherwise) for the time indicated. When needed, cultures were synchronized as described (Evinger and Agabian 1977). A22 drug treatments were performed in PYE or M2G supplemented with 2% PYE. A22-resistant mutants were selected on PYE plates containing 50 μ M A22.

Light microscopy

Microscopy was performed with a Nikon E1000 microscope with a DIC 100 $\times$ or Phase 100 $\times$ objective and a Hamamatsu Orca-ER LCD camera, or a Nikon E80i microscope with a DIC 100 $\times$ objective and an Andor iXon+ camera. The latter setup was used for photobleaching experiments with a Photonic Instruments Micropoint Laser system with a 481-nm Laser Dye, set at 15 pulses with a power of 2 at the attenuator plate and 15 for the internal attenuator, laser intensity cut by 70% through a beam splitter, and attenuated 16 $\times$ by neutral density filters. Images were taken and processed with Metamorph (6.1r0 or 7.1.4) and ImageJ software.

Crescentin-GFP fluorescence intensity measurements shown in Figures 2E and 3B were obtained using line scans spanning the length of the cell and by measuring the maximum intensity across the cell width.

For time-course experiments, cells were grown in liquid PYE. At the times indicated, cells were sampled, washed in M2G medium, and spotted on an M2G agarose pad for immediate imaging. For time-lapse experiments, cells were spotted on 1%–2% agarose-padded slides containing M2G supplemented with PYE (1% normally or 2% in the cases of A22 treatments or with *mreB*_{G165D} cells), vanillic acid, and/or xylose when appropriate. To label crescentin-TC, the cells were grown in liquid cultures containing 5 μ M FLAsH (Invitrogen) for at least 20 min and washed twice with M2G medium before imaging.

Scanning electron microscopy (SEM)

Cells were harvested, washed twice in M2G, and resuspended in fixative (5% glutaraldehyde, 4% formaldehyde in 0.08M

sodium phosphate buffer at pH 7.2) for 30 min at room temperature. Fixed cells were washed twice in PBS and mounted onto poly-L-lysine-coated coverslips. Cells were dehydrated through an ascending series of ethanol baths ending in 100% ethanol, and were critical-point-dried and gold-coated. Samples were examined by using a FEI XL-30 ESEM FEG microscope (acceleration voltage, 10.0 kV; spot size, 3; working distance, 7.5 mm or 10 mm).

Immunoprecipitation and Western blotting

Coimmunoprecipitation was performed essentially as described (Ebersbach et al. 2008), except that lysozyme was not used before sonication. Cleared lysates corresponding to 7 mg of total protein were incubated with 50 μ L anti-Flag M2-agarose beads (FlagIPT-1 kit, Sigma) and adjusted to a volume of 800 μ L. Beads were preincubated for 1 h with IP1 buffer containing 2.5% BSA and washed in IP1 buffer. Samples were incubated overnight and washed four times with wash buffer (IP1 buffer without glycerol) using a Hamilton pipette to remove all liquid between each wash. Beads were then resuspended in 50 μ L of wash buffer containing 3XFlag peptide (FlagIPT-1 kit, Sigma) to elute crescentin-Flag. After 1 h of incubation at 4°C, the samples were centrifuged and the supernatant fractions were immunoblotted using anti-MreB and anti-crescentin antibodies (both 1:10,000) for 1 h and 2 h, respectively.

For immunoprecipitation of *E. coli* CJW3007 (MC1000/pBAD18[Cm]creS-Flag) cells, we began with a 340-mL M9-glycerol culture (containing 0.2% glucose or arabinose to repress or induce crescentin-Flag expression, respectively, and 40 μ g/mL cephalaxin when appropriate). The remainder of the experiment was performed as described above, except that 100 U of DNase I (Roche) was added after sonication and cleared lysates (7 mg of total protein) were added to 100 μ L of anti-Flag M2-agarose beads that had been preincubated with IP1 buffer containing 2% BSA. Samples were eluted with 250 μ L of wash buffer containing 0.6 M NaCl and 3XFlag peptide. After 1 h of incubation at 4°C, the samples were centrifuged and the supernatant fractions were immunoblotted using *E. coli* α -MreB (kind gift of K. Gerdes; 1:10,000) and α -Flag M2 (Sigma) antibodies (1:1500) for 1.5 h and 1 h, respectively.

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Godefroid Charbon, Matthew T. Cabeen and Christine Jacobs-Wagner

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