Helical insertion of peptidoglycan produces chiral ordering of the bacterial cell wall

Siyuan Wang, Leon Furchtgott, Kerwyn Casey Huang, and Joshua W. Shaevitz

The regulation of cell shape is a common challenge faced by organisms across all biological kingdoms. In nearly all bacteria, cell shape is determined by the architecture of the peptidoglycan cell wall, a macromolecule consisting of glycan strands crosslinked by peptides. In addition to shape, cell growth must also maintain the wall structural integrity to prevent lysis due to large turgor pressures. Robustness can be accomplished by establishing a globally ordered cell-wall network, although how a bacterium generates and maintains peptidoglycan order on the micron scale using nanometer-sized proteins remains a mystery. Here, we demonstrate that left-handed chirality of the MreB cytoskeleton in the rod-shaped bacterium *Escherichia coli* gives rise to a global, right-handed chiral ordering of the cell wall. Local, MreB-guided insertion of material into the peptidoglycan network naturally orders the glycan strands and causes cells to twist left-handedly during elongational growth. Through comparison with the right-handed twisting of *Bacillus subtilis* cells, our work supports a common mechanism linking helical insertion and chiral cell-wall ordering in rod-shaped bacteria. These physical principles of cell growth link the molecular structure of the bacterial cytoskeleton, mechanisms of wall synthesis, and the coordination of cell-wall architecture.

Bacterial cells come in a wide variety of shapes and sizes, with morphology often linked to cellular behaviors that provide significant selective advantages (1, 2). In the vast majority of bacterial species, the peptidoglycan cell wall is the component of the cell envelope that mechanically dictates cell shape (3). Cell-wall structure and growth dynamics are critical to our understanding of bacterial physiology and cell biology, with cell-wall architecture relevant for mechanical interactions with other cells as well as the local microenvironment. To maintain a specific shape through growth and division, the cell wall must be synthesized under strict spatial and temporal controls (4–6). Synthesis must additionally insure the structural integrity of the wall material, preventing large holes from developing over time. Although previous efforts have identified and characterized a large fraction of the enzymes and biochemical reactions responsible for cell-wall synthesis (3, 7), the spatiotemporal mechanisms underlying the physical construction of the cell wall remain largely unknown.

The cytoskeletal protein MreB plays a key role in maintaining the rod-shaped morphology of bacteria such as *Escherichia coli*, *Bacillus subtilis*, and *Caulobacter crescentus* during elongation in which cells grow longer while maintaining a relatively constant cylindrical radius (1, 8). A homologue of eukaryotic actin, MreB has been reported to polymerize into approximately helical filaments inside cells, although the limits of conventional light microscopy have made accurate determination of the three-dimensional (3D) conformation of MreB difficult. Fluorescence experiments have indicated that the localization of peptidoglycan-synthesizing enzymes involved in elongation is guided by MreB, resulting in a helical pattern of incorporation during growth (6, 9). Although it was originally proposed more than a decade ago that helical insertion might be the fundamental driving force behind rod-shaped growth (10), only recently have we developed the computational tools to address mechanistic models of cell-wall dynamics and mechanics in a manner that provides experimentally testable hypotheses (11).

We have recently demonstrated that the contribution of the MreB cytoskeleton to cellular stiffness is comparable with that of the cell wall (12). Although the role played by polymer stiffness in cell growth is not fully understood, these results suggest that MreB structures can remain relatively unaffected by small, local perturbations in the cell-wall geometry and that MreB may apply a prestretching force during the synthesis and crosslinking of new peptidoglycan. Using a biophysical model of growth that includes cell-wall mechanics, turgor pressure, and patterning of peptidoglycan insertion by MreB, our simulations have provided further indication that the physical properties of MreB are critical to the maintenance of rod shape (11).

Mutants in several species of rod-shaped bacteria form long, spiral cells many times the length of a wild-type cell. *B. subtilis* mutants have been shown to form right-handed helical cell shapes (13), whereas spiral mutants of *E. coli* are left-handed (14). These data have previously been used to postulate that insertion of cell-wall material takes place along a helical path. Helical insertion of one handedness was then thought to drive twisting of the cell during elongation with the opposite handedness. However, this simple interpretation does not take into account the effects of the underlying spatial pattern of peptidoglycan, which we demonstrate can fundamentally alter the interpretation of spiral morphologies.

Here, we show that rod-shaped cells twist during growth with the same handedness as insertion by imaging the MreB/MreC/MreD (MreB/C/D) proteins in 3D and tracking cell growth using cell-surface labeling techniques. Through comparison with a computational model of cell elongation and measurements of elastic anisotropy, we further conclude that helical insertion guided by MreB establishes an opposite-handed chiral order to the peptidoglycan lattice that indirectly leads to cell twisting with the same handedness as insertion. We demonstrate that this chirality is conserved across evolutionarily distant microorganisms, suggesting that its physical underpinnings can be realized in higher organisms and other biological contexts. Our results suggest a detailed mechanism of MreB-guided cell-wall synthesis, and demonstrate biophysical techniques for deconstructing the functional role of cytoskeletal structures in living cells.

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To whom correspondence may be addressed. E-mail: kchuang@stanford.edu or shaevitz@princeton.edu.

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Results

MreB/C/D Form Left-Handed, Helical Segments in Vivo in E. coli Cells. Because MreB is known to direct cell-wall synthesis, we first sought to determine the 3D conformation of MreB filaments in live E. coli cells. Previous studies of the localization of MreB in bacteria have been inconclusive. Both conventional and superresolution images of immunofluorescence labeling of native E. coli MreB in fixed cells and fluorescent fusions to YFP or GFP in live cells suggest a smooth and continuous helical pattern of organization that spans most of the cell length (6, 15, 16). These structures are sometimes presented using deconvolution image organization that spans most of the cell length (6, 15, 16). Recent images of a different fluorescent fusion in E. coli, MreB-RFP, appear to show a more spotty staining pattern (17, 18). However, despite some level of heterogeneity, this punctate pattern appears to lie on top of larger, low-intensity helical segments (17, 18).

Fluorescence imaging in B. subtilis from multiple groups has been equally as difficult to synthesize. Unprocessed immunofluorescence images of both MreB and its parologue Mbl indicate that these proteins form very long helical structures in fixed cells (10). However, more recent images of fluorescent fusions of these proteins to GFP in live cells display much smaller structures that often appear to be oriented helically (19, 20).

One major limitation in many of these experiments has been the lack of true 3D image information. Whereas comparing single-layer images, generated using total-internal reflection microscopy and/or a high numerical-aperture objective, to larger-depth-of-focus data can help in estimating the extent of 3D features, accurate measurement of the size and shape of fluorescent membrane-bound structures requires a full 3D dataset and analysis.

To determine the full extent of the 3D localization of MreB in E. coli cells, we induced expression of YFP-MreB from a plasmid in exponentially growing cells (see Materials and Methods) and acquired 3D image series of individual cells with an axial spacing of 75 nm (Fig. 1). In these experiments, MreB formed what appeared to be helical segments near the cell periphery, although we seldom observed full or double helices (Fig. S1). To quantify the orientation of MreB fluorescence, we took advantage of its membrane association and computationally mapped the cylindrical intensity distribution from a z-series (Fig. 1A) onto a single plane whose axes are circumferential and longitudinal position (see Materials and Methods, Fig. 1B). In this representation, a left-handed helix appears as parallel lines pointed up and to the right whereas a circumferential ring appears as a vertical line. To extract the dominant orientation from the two-dimensional unwrapped fluorescence pattern (Fig. 1B), we employed Radon transforms and spatial correlations to determine the likelihood of all possible helicities from each single-cell image (21).

MreB fluorescence formed either one or a few dominant helical segments in all cells imaged. When multiple segments were present, they were often disjoint and did not appear to be part of one superhelical structure. MreB fluorescence segments were oriented predominantly in a left-handed fashion with a mean helix angle of $\theta_{MreB} = -74 \pm 3^\circ$ (Fig. 1C and F). (All statistical errors in this paper are standard errors unless otherwise mentioned.) Herein, we quote geometric angles as “helix angles” that are defined as the angle between a helix and the axis of a surrounding cylinder (Fig. 2A). Throughout this work, we consider four distinct helix angles defined by (i) the orientation of MreB fluorescence, which dictates the pattern of insertion sites (MreB helix angle); (ii) the orientation of the glycan strands within the cell wall (glycan angle); (iii) the trajectory of fiducial markers attached to the cell envelope during elongation (growth angle); and (iv) the trajectory of fiducial markers attached to the cell envelope during the loss of turgor pressure (stiffness angle). We also
adopt the convention that negative values represent left-handedness. Interestingly, we occasionally observed cells with individual helical segments that were right-handed, although these cells often contained other left-handed MreB segments that made up a majority of the MreB distribution (e.g., Fig 1C, viii). We applied this analysis methodology to a z-series of fluorescence from the uniformly distributed membrane dye FM4-64 to verify that our unwrapping algorithm does not artificially generate diagonal patterns. This control experiment yields the expected uniform density around the cell cylinder (Fig. S2).

MreC and MreD are encoded in the same operon as MreB and are thought to colocalize with MreB. These proteins interact with cell-wall synthesis enzymes in the periplasm, connecting the cell-wall spatial information from MreB to this outer cellular compartment. We imaged YFP-MreC and YFP-MreD and found that their fluorescence also formed left-handed helical patterns.

![Image: E. coli cells twist in a left-handed direction during elongation.](Fig. 2)

Fig. 2. E. coli cells twist in a left-handed direction during elongation. (A) Schematic illustration of the different helix angles used in this paper. (B) A cell expressing cytoplasmic mCherry (red) with two Dragon Green-labeled beads (yellow) bound to its top surface imaged at two instances in time. (i) DIC microscopy, (ii) fluorescence microscopy, and (iii) computational extraction of the cell outline and bead centers all indicate a left-handed twist (arrow). Scale bars represent 2 μm. (C, D) Circumferential and longitudinal distances between the two beads are linearly related for both cephalaxin-treated (C) and untreated (D) cells. (Inset) DIC and fluorescence images of a representative cell, scale bar represents 1 μm. Line fits (solid lines) and 95% confidence intervals of the fits (dotted lines) are shown. The growth angle in (D), deduced from the slope of the fit line, is $-6.4 \pm 0.7^\circ$ (95% confidence interval). (E–G) Strain, MC4100 araR/pSW1 ($P_{BAD} \colon mCherry$). (E) Circumferential and longitudinal distances between two fluorescently labeled flagellar hooks are also linearly related. (Inset) Fluorescence and analyzed images of a representative cell expressing cytoplasmic EGFP (scale bar represents 5 μm). The growth angle for this cell is $-5.2 \pm 0.5^\circ$ (95% confidence interval). Strain, MTB9/pWR20 (EGFP). (F) Mean and standard error of the growth angle of various E. coli strains. N = 10 for YS34, N = 5 for MC1000, MC4100 araR/pSW1, MCA1000 araR/pSW1—no cephalaxin, and WA220. N = 3 for MTB9/pWR20. The YS34 and MC1000 cells are labeled with membrane dye FM4-64. (G) Ensemble distribution of the growth angles in F. The magenta line indicates the ensemble average.
Similar to the values calculated from MreB images, YFP-MreC and YFP-MreD segments had average helix angles of $-69 \pm 5^\circ$ and $-75 \pm 4^\circ$, respectively (Fig. 1D, E, G, and H). The agreement between the overall fluorescence distributions and the helix angles in particular for YFP-MreB/CD supports the notion of colocalization of these proteins, as well as the participation of MreB in cell-wall growth.

**Cell Twisting is a General Property of Elongation in *E. coli*:** To observe the dynamics of the cell wall during growth, we developed a protocol that uses polymer beads as landmarks on a growing bacterial cell. First, to enable observation over long periods of elongation, *E. coli* cells were induced to grow filamentously with the drug cephaloxin, a beta-lactam antibiotic that inhibits FtsI and prevents constriction of the division-promoting FtsZ-ring (22). We then bound the bottom surfaces of the cells to a coverslip and attached polylysine-coated polystyrene beads to the top surface using an optical trap (12, 23). Finally, we tracked the motion of the beads during cell elongation using time-lapse microscopy. This motion reflected the dynamics of the outer membrane, which is linked to the cell wall through hydrophobic interactions with the wall-bound lipoprotein Lpp (24).

Fig. 2B shows a typical cell with two beads attached near the poles. To easily distinguish between the cell body and the beads, we induced expression of cytoplasmic mCherry from a plasmid and used beads that were labeled with Dragon Green fluorescent dye. In addition to an increase in the longitudinal distance between the two beads over time that represents cell elongation, we observed rotation of one bead relative to the other around the longitudinal axis. The determination of twist handedness is facilitated by placement of the beads on top of the cell relative to the coverslip surface. In the example shown, the lower bead rotates in a counterclockwise fashion when looking down the axis of the cell from the top, representing a left-handed twist of the cell surface. This twist occurs with the same handedness as the MreB-guided insertion.

We parameterized the cell geometry and bead positions in a cylindrical coordinate system using a high-resolution detection algorithm (see Materials and Methods) that allowed us to accurately determine the circumferential distance between the two beads as a function of their longitudinal distance along the cell. The circumferential distance changed linearly with the longitudinal distance in this cell (Fig. 2C), with a highly conserved slope of magnitude $-0.115 \pm 0.003$ (95% confidence interval). We define the arc tangent of the slope as the growth angle of the cell ($\theta_{growth}$), i.e., the helix angle of the bead motion relative to the cell's long axis (Fig. 2A). The growth angle of this cell is $-6.5 \pm 0.2^\circ$ (95% confidence interval). Note that both the MreB helix angle and growth angle are left-handed.

The growth angle and handedness were also statistically equivalent across cells and strain backgrounds (Fig. 2F), with an average growth angle of $-6.7 \pm 0.2^\circ$ measured for 33 cells (Fig. 2G). Importantly, our measurements were in agreement between unlabeled wild-type *E. coli* cells and cells expressing the functionally complementing mreB::fp fusion as the sole copy of mreB (growth angle of $-9.5 \pm 0.4^\circ$ calculated from $N = 7$ cells). Twist always occurred in a left-handed manner; we never observed an elongating *E. coli* that twisted right-handedly or not at all under normal growth conditions. Cell twisting also occurred in non-cephalexin-treated cells with a similar growth angle during the limited observation time available before cell division occurred (Fig. 2D and F).

To demonstrate that cell twisting reflects the dynamics of the cell wall and not just the outer membrane, we took advantage of *E. coli*’s locomotory mechanism. The *E. coli* flagellar motor is anchored to the cell wall (25), and hence motion of the motor corresponds to local cell-wall dynamics. We tracked fluorescently labeled flagellar hooks in elongating cells and observed left-handed cell-wall twisting during elongation with a growth angle similar to that derived from bead rotation ($-6.0 \pm 0.5^\circ$ in $N = 3$ cells, Fig. 2E and F).

**Growth Twist is Established by Helical, MreB-Mediated Cell-Wall Inversion:** To determine whether twisting during growth is dependent on MreB, we treated cells with 10 μg/mL A22, an antibiotic that causes the rapid disassembly of MreB polymers within 1–2 min (26). On short timescales (<10 min), cell shape is largely unaffected by the depolymerization of MreB and cells continue to elongate (11). Instead, cells grown in the presence of A22 gradually lose their rod shape over several doubling times (11, 27), which corresponds to several hours in our experimental conditions (1, 5).

If MreB drives both elongation and cell twisting through the localization of cell-wall synthesis, cells should be unable to maintain a left-handed growth angle in the presence of A22. Consistent with this expectation, the growth angle of the cells slowly decreased to zero over roughly a doubling time following the addition of A22 (Fig. 3), during which time rod shape was maintained. Cells continued to elongate after twisting halted with a linear increase in the axial distance, indicating that the decrease in growth angle is not caused by a decrease in growth rate (Fig. 3B). These observations suggest that MreB is responsible for cell twisting during normal elongation, but that some helical order persists transiently after the disassembly of MreB, either in proteins downstream from MreB in the wall assembly process or within the cell wall itself (Fig. 3C).

**Fig. 3.** The growth twist of *E. coli* depends on the MreB cytoskeleton. (A) DIC images of an FtsZ-depleted cell grown at room temperature with A22. Scale bar represents 5 μm. (B) The circumferential and longitudinal distance between the beads are linearly related during a phase of twisting elongation until twisting ceases and circumferential distance remains constant as the cell elongates during straight elongation. (A, B) Strain, WX7 (ΔftsZlgl100 (Plac::ftsZ2)). (C) The growth angle (mean and standard error) of *E. coli* cells with and without A22 treatment. A22 treatment, which causes rapid depolymerization of MreB, results in a slow decrease in the growth angle over a timescale of one doubling time. YS34: without A22 (N = 10). A22 (short): cells were incubated with A22 for 3 min before observation, and imaged for 45 min at room temperature in the presence of A22 (N = 12). A22 (long): cells were incubated with A22 for 30 min at 37°C before observation, and then imaged at room temperature in the presence of A22 (N = 10). WA220 (parental strain for A22-resistant strain WA221): without A22 (N = 5) and with A22 (long) (N = 6). WA221 (A22-resistant strain): without A22 (N = 5) and with A22 (long) (N = 5). Double asterisks (**) indicate significant difference from zero growth angle in t test, with p < 0.01.
using an A22-resistant strain of *E. coli* (WA221) that contains a single point mutation in the *mreB* gene, we found no significant defect in growth twisting upon the addition of A22 (Fig. 3C) (28). Interestingly, this strain had a much larger growth angle magnitude than the parental wild type strain, WA220, both with and without A22, further implicating MreB as the defining factor of the growth angle.

**Helical Cell-Wall Insertion Leads to a Global Chiral Self-Ordering of the Peptidoglycan Network.** Although we currently do not have the experimental capability to monitor the dynamics of individual synthesis enzymes and glycan strands, we are able to use computational modeling to explore these experimentally inaccessible microscopic phenomena. We have recently developed a biophysical model for the growth dynamics of rod-shaped Gram-negative bacteria that captures the physical properties of the cell wall by representing the glycans and peptide crosslinks as mechanical springs (29). In our model, the cell wall is represented as a network composed of crosslinked glycan strands with a physiological distribution of glycan strand lengths (29). Elongation of the cell wall is achieved by repeatedly inserting strands with lengths between 10 and 20 disaccharide subunits (11). Using our experimental measurements of MreB fluorescence patterns to generate simulated insertion patterns, new strands are initiated from peptide crosslinks proximal to helical MreB segments positioned interior to the cell wall and extending the length of the cell. Because the bending stiffness of MreB is significant relative to the cell wall (12), we do not directly incorporate the mechanics or dynamics of MreB into our model, instead assuming that the MreB pattern remains unaffected by local cell growth. Each strand is inserted in a prestretched state, a growth mechanism that has been demonstrated to help maintain cell radius (11, 30). After each insertion step, the cell wall is relaxed to an equilibrium state in which the restoring forces of the peptidoglycan balance the outward force due to a turgor pressure of 1 atm. Using this model, we previously demonstrated that a simple helical pattern of insertion, mimicking an MreB-guided mode of growth, is sufficient to maintain cell shape during extensive elongation, as long as the pattern of insertion is insensitive to fluctuations in the cell-wall density (11).

The initial conditions for our simulations are a peptidoglycan network in which the glycan strands are oriented along the circumferential direction. This orientation allows us to examine whether helical insertion produces any global ordering of the glycans. In our model, strand synthesis and crosslinking initiates from a randomly chosen point along an MreB segment, and then follows the existing orientation of the surrounding peptidoglycan network until the strand terminates (Fig. 4A). This mechanism for strand insertion is inspired by previous experiments that link PBP2-mediated glycan-strand crosslinking to nearly circumferential motion of MreB (18).

To determine if growth twisting is an emergent property of the spatial patterning of cell-wall synthesis, we elongated a cell wall to twice its original length and tracked the positions of vertices connecting the glycan and peptide springs (a subset of which are marked with cyan spheres in Fig. 4B). The cell-wall network twisted with the same handedness as the MreB helix during elongation, similar to the experimentally observed behavior. The growth angle was quantitatively similar to the value measured in our experiments on *E. coli* (Fig. 4C; approximately −8° for simulation in Fig. 4B). Moreover, the magnitude of the growth angle decreased roughly linearly with the MreB helix angle (Fig. 4D), suggesting that our experimental measurements of peaked distributions of MreB/C/D helix angles and growth angles are associated by their common link to cell-wall synthesis.

Growth twist in simulations does not require a single, coherent helical structure. To test whether our results depend on the spatial extent of the MreB, we instead generated MreB patterns selected randomly from the distributions measured in Fig. 1 and modeled elongation as in Fig. 4B. In all cases, the resulting growth angle was similar to growth from a single helix extending between the two ends of the cell. This similarity in growth angle was true even for an MreB distribution similar to that in Fig. 1C, viii, which had a small fraction of right-handed MreB filaments.

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**Fig. 4.** Modeling of cell elongation demonstrates that helical peptidoglycan insertion gives rise to growth twist. (A) After glycan-strand insertion initiates near the MreB helix (yellow), synthesis and crosslinking of new strands (blue) proceeds along the direction defined by the existing glycan strands (green), which are initially oriented roughly circumferentially. Peptide crosslinks are in red. (B) Cell wall grown in silico to twice its initial length. Teal spheres represent beads bound to a single vertex of the peptidoglycan network. (C) Circumferential versus longitudinal distance between pairs of beads (blue curves) bound to vertices along the cell walls in B, with the average overall bead pairs shown in red. Twisting remains left-handed in a simulation with a small percentage of right-handed MreB helical segments (maroon), but handedness is abolished with equal levels of left-handed and right-handed segments (gray). (D) The growth angle decreases linearly with the MreB helix angle. Light blue region indicates one standard deviation above and below the average growth angle for pairs of beads on a single cell. Experimental measurements of MreB helix angle and growth angle are shown in black.
(average bead trajectories shown as magenta line, Fig. 4C). To systematically assess the importance of extended MreB filaments, we separated a left-handed MreB helix into increasing numbers of segments and randomly positioned them with respect to each other around the circumference. The steady-state growth angle remained constant as long as the MreB filaments were longer than the glycan strands being inserted, indicating that growth twist could be maintained by MreB filaments smaller than a diffraction-limited spot as long as the underlying pattern of insertion sites had an overall left-handed orientation. In contrast, simulations of growth from an MreB pattern containing an equal proportion of right- and left-handed MreB exhibited very little twist (Fig. 4C, gray line). Taken together, our results indicate that the precise spatial distribution of MreB is less critical for growth twist than the dominant left-handed geometry.

In our discrete model of cell-wall growth, we discovered that growth twist is coupled to a chiral organization of the peptidoglycan. Insertion from a left-handed MreB helix led to a reorientation of newly inserted strands away from the helix in a right-handed fashion, even in cell walls initiated with circumferential (achiral) glycan strands (Fig. 5A). The average strand orientation underwent an early phase of rotation to a glycan angle of approximately 82°, followed by a partial recovery and stabilization at an angle of approximately 86° (Fig. 5A). The peptidoglycan architecture close to and distant from the MreB helix during each of these phases illustrates this emergent chiral self-organization (Fig. 5B–D). The steady-state glycan angle is highly reproducible (Fig. S3) and is not sensitive to the number of fragments composing the MreB distribution. Moreover, the close agreement between the growth angle in our simulations and our experimental measurements supports the notion that the glycan angle is a signature of MreB-mediated helical cell-wall insertion.

In our model, the direction of a new strand is dictated by the orientation of the surrounding strands. Thus, once MreB-mediated helical insertion establishes a right-handed glycan angle, new strands will adopt the same orientation even without MreB. However, without an underlying MreB structure to reinforce the helical pattern of growth that gives rise to the glycan chirality, there is no driving force to maintain the overall right-handedness of the glycan angles. Indeed, after switching from a helical to a uniform pattern of growth to mimic treatment with A22, the average glycan angle slowly decays to zero (Fig. 5E), with a coincident decrease in the bead twisting (Fig. 5F).

Recent experiments have also shown that the MreB structure in E. coli cells is dynamic, moving in the circumferential direction with a frequency of approximately 4 complete rotations per doubling time (18). The incorporation of this rotation into our simulations has little effect on the steady-state growth angle (−6°) or glycan angle (88°). Moreover, our simulations have shown that MreB rotation helps to maintain the cell width during growth and osmotic shock (18). Thus, our simulations support the hypothesis that a left-handed orientation of MreB segments is sufficient for robust left-handed cell twisting during elongation.

**Osmotic Shock Reveals the Global Chiral Peptidoglycan Ordering.** For a chirally organized peptidoglycan structure, longitudinal stretch-
ing or compression of the network will also cause twist that reflects the helical stiffness anisotropy between the stiffer glycan strands and weaker peptide links (Fig. 6A). Because turgor pressure is balanced by elastic extension of the cell wall, longitudinal stretching or compression can be affected by osmotic down- or up-shock, respectively (31, 32). For a cell wall constructed from glycan strands oriented chirally, the angle of the strands will increase in response to an osmotic up-shock, leading to cell twisting opposite to the strand handedness. Because strand handedness is predicted to be opposite the MreB handedness (Fig. 5), we expect the osmotic twist to have the same left-handedness as the MreB structures (Fig. 1). In contrast, if the glycan strands were primarily oriented in the circumferential direction, vertices near the two poles would move closer to each other along the cell axis without twisting.

To experimentally verify our prediction of chiral peptidoglycan organization, we again used beads as landmarks of surface motion and applied an osmotic up-shock from PBS buffer to PBS + 1 M NaCl to rapidly cancel the turgor pressure across the cell membrane. Similar to our growth-twist experiments, polylysine-coated beads bound to cephalaxin-treated filamentous E. coli cells twisted in a left-handed fashion immediately after up-shock (Fig. 6B). We used this osmotic twist data to extract a peptido-

glycan-stiffness angle, defined as the helix angle of bead motion under osmotic shock ($\pm 11.4 \pm 0.9^\circ$, $N = 6$ cells), which was significantly higher than the growth angle (Fig. 2F). Shocks from PBS to PBS + 2 M NaCl gave the same stiffness angle ($\pm 11.5 \pm 0.6^\circ$, $N = 5$ cells), indicating that the turgor pressure has been fully abolished in these experiments. An ensemble distribution of the stiffness angle is shown in Fig. 6C. Similar to Fig. 6A, we interpret this osmotic twist to reflect an underlying helical anisotropy in the cell-wall stiffness, with a left-handed orientation of the more compliant peptide crosslinks and a right-handed orientation of the stiffer glycan strands.

To demonstrate that osmotic twist depends only on the order of the cell-wall material, we measured the stiffness angle after short-term and long-term A22 treatment. For the short-term experiments, up-shock was performed 3 min after A22 addition, just long enough to allow MreB to fully depolymerize. The stiffness angle was unaffected in these cells (Fig. 6D). In long-term experiments, cells were preincubated with A22 for 1.5 h at 37°C to allow substantial MreB-independent elongation. These cells were still rod-shaped, but the stiffness angle was reduced, within error, to zero (Fig. 6D). These observations again suggest that the helical stiffness anisotropy in E. coli cells is established by MreB-mediated cell-wall growth, rather than by direct elastic coupling of MreB to the cell wall.

To determine the magnitude of this osmotic twist in our discrete model, we first elongated the cell wall until the glycan angle and other cell-wall metrics reached their steady-state values (11). We then varied the turgor pressure from 0.9 to 1.1 atm, and observed an osmotic twist with a mean stiffness helix angle of $-7.2 \pm 3.5^\circ$ (standard error measured across bead pairs in one

![Fig. 6. Twist during osmotic shock reveals the chiral organization of peptido-
glycan. (A) Illustration of the effect of osmotic shock on a chiral peptido-
glycan network with right-handed glycan strands. Arrows indicate the glycan orientation, and teal and purple spheres correspond to virtual beads labeling the same vertices in all schematics. A decrease in turgor pressure increases the helix angle of the glycan strands, leading to a left-handed twist. (B) DIC and fluorescence images of an FM4-64-labeled E. coli cell before and after an osmotic up-shock. The beads twist in a left-handed direction (arrows), similar to the growth twist in Fig. 2. Scale bar represents 2 µm. (C) Histogram of stiffness angles for wild-type cells without A22 treatment. (D) The stiffness angle (mean and standard error) of E. coli Y534 cells with and without A22 treatment. Wild-type: without A22 ($N = 6$). A22 (short): cells were incubated with A22 for 3 min before shock ($N = 7$). A22 (long): cells were incubated with A22 for 1.5 h at 37°C before shock ($N = 7$). Double asterisks (**) indicate significant difference from wild-type in t test, with $p < 0.01$.

![Fig. 7. Simulations reproduce left-handed osmotic twist. (A) Simulations of a peptido-
glycan network with a steady-state average glycan angle after vary-
ing the turgor pressure from 0.9 to 1.1 atm. Circumferential and longitudinal displacement for the cell-wall vertices of the cell wall relative to $P = 0.9$ atm. The majority of the vertices display a left-handed osmotic twist. (B) Stiffness angle measurements from simulations in which the cell wall in A was further elongated with a random insertion pattern to mimic growth in the presence of A22, after which the turgor pressure was varied from 0.9 to 1.1 atm. (C) Circumferential and longitudinal displacement for the vertices of the cell wall in B relative to $P = 0.9$ atm. The average osmotic twist is nearly eliminated by nonhelical growth.](image)
cell) (Fig. 7A). To illustrate that this osmotic twist is also dependent on growth from a helical pattern, we elongated the cell wall examined in Fig. 7A to double its initial length via randomly distributed synthesis, mimicking experimental cell growth in the presence of A22 prior to osmotic shock (Fig. 6D). Osmotic shock simulations at various time points during growth determined that the stiffness angle monotonically decreased (Fig. 7B), coincident with a reduction in the degree of ordering of the glycan strands to $-0.3 \pm 12^\circ$ after one doubling with random insertion (Fig. 7C).

**Growth Twist, Stiffness Angle, and MreB Fluorescence Patterns are all Right-Handed in *B. subtilis*.** Our results indicate that growth twist and chiral wall organization should be general features of rod-shaped bacteria with a helical bias for peptidoglycan insertion, due to the ensuing global ordering of the peptidoglycan. To test this hypothesis, we repeated our bead-labeling experiments using the Gram-positive bacterium *B. subtilis*. *B. subtilis* possesses mreB and a homologue mbl, both of which are required for rod-shaped growth (10). Although recent studies have suggested that fluorescent MreB homologues in *B. subtilis* often appear as diffraction-limited foci in which orientation is difficult to assess with conventional microscopy, these and previous studies also show that *B. subtilis* MreB can form arcs or helical structures, all of which are right-handed (10). Using beads coated with polylysine, we found that wild-type *B. subtilis* cell growth is accompanied by a right-handed twist (Fig. 8A), with a growth angle of $4.3 \pm 0.3^\circ$ ($N = 11$ cells, Fig. 8B). Like *E. coli*, *B. subtilis* growth twist has the same handedness as the cytoskeleton, suggesting that twisting in this organism also arises from a global helical ordering of the peptidoglycan.

We also performed osmotic up-shock experiments on surface-labeled *B. subtilis* cells and found that, like *E. coli*, the cell surface twists as the turgor pressure is relaxed. The stiffness angle in these cells was right-handed, again opposite that of *E. coli* ($3.7 \pm 1.1^\circ$, $N = 5$ cells). This right-handedness indicates that the stiffness of the Gram-positive cell wall in *B. subtilis* has a chiral anisotropy with stiffer left-handed components, in contrast to the *E. coli* cell wall with stiffer right-handed glycan strands. The Gram-positive cell wall is significantly thicker and more complex than the Gram-negative wall and much less is known about its 3D architecture and mechanical properties. As such, we cannot draw more specific conclusions that relate the stiffness angle to microscopic details of the peptidoglycan architecture. Nevertheless, the common features of cell twisting in *E. coli* and *B. subtilis* reinforce the notion that MreB-mediated cell-wall growth leads to emergent order within the cell wall. Future experiments will be required to develop a more concrete link between helical insertion, cell twisting, and wall stiffness in these more complex mechanical systems.

**Discussion**

Our experimental, 3D fluorescence measurements demonstrate that MreB forms patterns of helical segments with a well-defined, left-handed pitch that direct the synthesis of new cell-wall material (Fig. 1). Our experiments link the presence of these helical patterns of cell-wall synthesis with a well-defined pitch to a constant twist during the growth of rod-shaped bacteria (Fig. 2, Fig. 8), whereas our simulations support the hypothesis that this twist is coupled to chiral self-organization of the peptidoglycan network with predominantly right-handed glycan strands (Fig. 5). The twist can be similarly realized following an osmotic shock that alters the cell length, and can be eliminated via growth with A22, a small molecule that disassembles MreB (Fig. 6). Thus, MreB establishes elongation with a constant radius that is accompanied by a shearing twist of the cell wall and the maintenance of chiral peptidoglycan ordering. Other rod-shaped bacteria also exhibit twist during growth, although *B. subtilis* cells always twist in a right-handed manner with the opposite handedness to *E. coli*. Our model suggests that this reversal probably arises from the molecular chirality of the MreB cytoskeleton, with MreB and Mbl in *B. subtilis* forming right-handed helices (10).

Imaging studies to date have been inconclusive regarding the length of MreB structures in live cells (Fig. 1) (18-20, 33, 34). Nevertheless, our experimental measurements of growth twist indicate that the pattern of cell-wall synthesis mediated by other MreB fusions must have an underlying left-handed bias. Moreover, our simulations indicate that growth twist is insensitive to the type of fusion and variations in the length or dynamics of the MreB segments, as long as the handedness is preserved.

The observation that cell twisting, a previously unrecognized but likely common bacterial behavior, is coupled mechanistically to the underlying organization of the peptidoglycan network opens several unique lines of general inquiry into bacterial growth. First, the quantitative consistency of the growth angle suggests that cells may use growth twist to mediate intercellular interactions in a colony or biofilm (35), or to moderate interactions with the local microenvironment, such as the invasion of pathogens into host cells. Second, chiral peptidoglycan organization may be mechanically favorable, allowing the cells to endure larger perturbing forces even during extreme osmotic shocks. As a test of this mechanical functionality, our studies motivate the structural investigation of highly osmotically resistant cells such as *B. subtilis* spores. Last, the molecular chirality of the cytoskeleton may have served as the evolutionary origin of cellular and organismal chirality. Similar chiral motion has been observed in *Arabidopsis* plant cellulose deposition (36), *Drosophila melanogaster* oogenesis (37), and *Xenopus laevis* embryonic development (38).

In conclusion, our work presents a mechanistic description of cell-wall structural dynamics for rod-shaped bacteria such as *E. coli*. Moreover, our approach integrates complementary biophysical approaches that are generally applicable to a wide range of questions about the relationship between cell-wall growth and cytoskeletal control. Future experiments will evaluate the functional role of MreB in other organisms and morphologies, as well as the involvement of other cytoskeletal elements in these phenomena. In *E. coli* and *C. crescentus*, the MreB helix compresses to a small region near midcell before cell division (6, 26), suggesting that twist rate and cell-wall organization are transiently affected during cytokinesis. These MreB dynamics must be incorporated into future models of cell division. In *B. subtilis*, cells possess multiple MreB homologues (10, 39); our biophysical approach will likely be necessary to determine which of these structures directly control the initiation of cell-wall synthesis. The experimental and computational technologies developed...
in this work provide a biophysical link between genetic or cell-biological discoveries and morphological behaviors.

Materials and Methods

Strain Construction. All the strains and plasmids used in this paper are listed in Table S1. To construct pSW1, we cloned mCherry with a Shine–Dalgarno sequence (AGGAGGT) and a linker sequence (CACAT) into the expression vector pBAD18 between the EcoRI and XbaI restriction sites.

Growth Twist Measurements. Overnight cultures of E. coli strains YS34, MC1000, or MC4100 ara-repSW1 (P_ara-rep::mCherry) grown in LB broth were diluted 50-fold into fresh LB and grown for 1.5 h at 37 °C. For MC1000 ara-repSW1, 50 μg/mL ampicillin (Sigma-Aldrich), and 0.2% arabinose (Acros Organics) were added to all media. We then added 25 μg/mL cephalixin (Sigma-Aldrich) to the YS34 or MC1000 culture, or 10 μg/mL cephalixin to the MC4100 ara-repSW1 culture to induce filamentous growth. We also added 1 μg/mL FM4-64 (Invitrogen) to label the membrane of YS34 and MC1000 cells in all subsequent media. Cultures were further incubated for 30 min at 37 °C. Polyethyleneimine-coated (PEI, Sigma-Aldrich) coverslips were made by flowing 1% PEI diluted in water into a flow chamber and washing with water. Rates of growth and division were similar on agarose pads and PEI-coated coverslips in the presence of LB media, as long as all free PEI was removed by washing before adding the cells.

We then flowed the cell culture into the chamber and washed after 3 min using LB + 25 or 10 μg/mL cephalixin to remove unattached cells. Polysynemisc-coated beads were made by incubating 0.5-μm diameter Dragon Green-labeled polystyrene beads (FS03F, Bangs Laboratories) for 1 h in 0.1% poly-l-lysine (PB8290, Sigma-Aldrich) diluted in water. Beads were then washed in water and resuspended in water. The bead solution was diluted twofold into LB + 25 or 10 μg/mL cephalixin before being introduced into the flow chamber. After the beads were attached to the cells using the optical trap, the chamber was washed in LB + 25 or 10 μg/mL cephalixin to remove unattached beads. Differential-interference contrast (DIC) and fluorescence imaging was delayed for 10 min to allow the cells to recover from the osmotic shock before acquiring DIC and fluorescence images. The cells were then shocked into PBS + 1 M NaCl (Sigma-Aldrich). The postshock DIC and fluorescence images were compared to the original images (see Image Analyses). For short-term A22 treatment, 10 μg/mL A22 was added 3 min before imaging; for long-term treatment, 10 μg/mL A22 was added when cephalixin was first added to the culture to induce filamentous growth and the cells were grown for 1.5 h at 37 °C.

Image Analyses. To extract cell shape and bead position, we used the MATLAB (R2009a, The Mathwork) Canny edge detector to define the edges of the cell and the beads in the fluorescence images. The two side edges of the cell parallel to its longitudinal axis were averaged toward the longitudinal axis to determine the cell's centerline. The fluorescence profile of each bead was averaged to obtain its position. The angular coordinate of a bead in the cylindrical coordinate system defined by the cell's centerline is then

\[ \beta = \arcsin \frac{x}{r + r_0} \]

where \( r \) is the radius of the cell, \( r_0 \) is the radius of the bead, and \( x \) is the shortest distance between the bead and the centerline (\( x \) is defined to be positive when the bead is on the right of the centerline, and negative on the left). The circumferential distance between two beads on the same cell is then

\[ r_c (\beta_1 - \beta_2) \]
α = 0.1, which penalizes large kinking angles between glycans while allowing small deviations of θ from π. The values of \( k_p \) and \( α \) were determined using experimental estimates of the Young's modulus [25 MPa (41)] and glycan persistence length [10 nm (42)].

We model growth by selecting a peptide crosslink for initiation of glycan synthesis, breaking peptide crosslinks along the path of insertion of a new strand, and crosslinking the new strand to the closest uncrosslinked old material. The wall relaxes to its lowest energy state between successive complete insertion events but not during insertion. The energy is minimized using a nonlinear conjugate gradient algorithm, and all simulations were performed using custom C++ code.

A typical E. coli cell requires roughly \( N_g \sim 1000 \) glycan subunits to surmount the hoop force (13). The cost of the additional expense of simulating such a network is currently prohibitive, but we can nevertheless make quantitative contact with experiments by scaling the biophysical parameters describing the peptidoglycan springs. Given that the orientation and crosslinking of new strands depend on the physical architecture of the surrounding network, we surmised that the growth behavior of a smaller cell wall would scale appropriately if the local architecture could be maintained by adjusting the peptide springs constant \( k_p \) (the glycans are stiff, hence their stretching contributes less to the network topology). The width of the cell is \( L = N_g \Delta d_p/\pi \), where \( \Delta d_p \) is the length of each glycan, so that the cross-sectional area is \( A = N_g \Delta d_p^2 \). For a turgor pressure \( P \), the outward force exerted on the end-caps is \( P A \), resulting in a restoring force \( k_p N_g \Delta d_p / 2 \) borne by the peptide crosslinks. Therefore, the extension in each peptide \( \Delta x_p = P A d_p^2 / 2 k_p N_g \), scales linearly with \( N_g \). To maintain this extension across size scales requires balancing the energies of spring stretching and turgor pressure, which we achieve by rescaling \( k_p \) linearly with \( N_g \). Most simulations were performed on cell walls starting with an average circumference corresponding to the length of a hoop with \( N_g = 100 \) glycan subunits. Given that for an \( E. \) coli wall with \( N_g = 1,000 \), the peptide spring constant is estimated to be \( k_p \sim 10 \) pN/nm, we set \( k_p = 1 \) pN/nm for our model cell walls with \( N_g = 100 \). We have verified that after scaling \( k_p \) with \( N_g \), growth simulations with larger hoops (e.g., \( N_g = 200 \)) give quantitatively similar growth angles.

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Fig. S1. Two-dimensional fluorescence images of YFP-MreB (A), YFP-MreC (B), and YFP-MreD (C). Each image is a standard deviation projection along the axial direction from a fluorescence z-series.

Fig. S2. Cylindrical unwrapping produces a uniform distribution for a cell stained with the membrane dye FM4-64. (A) Sample z-series of a cell stained with FM4-64. (B) Corresponding unwrapped fluorescence pattern.

Fig. S3. Steady-state glycan angle is highly reproducible. Mean glycan angle as a function of cell length averaged over four independent simulations. Shaded areas are one standard deviation above and below the mean.
**Table S1. Bacterial strains and plasmids used in this work**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> MC4100 araR</td>
<td>F-araD139 Δ(argF-lac)U169 rpsL150 relA1 flb5301 deoC1</td>
<td>(1)</td>
</tr>
<tr>
<td><em>E. coli</em> YS34</td>
<td>ΔcheY, fliC::Tn10, Δ pilA, Δ motAmotB</td>
<td>(2)</td>
</tr>
<tr>
<td><em>E. coli</em> MTB9</td>
<td>Flagellar hook labeled with a peptide recognized by biotin ligase BirA</td>
<td>Gift from Mostyn Brown, Oxford University, Oxford, England</td>
</tr>
<tr>
<td><em>E. coli</em> MC1000 araD139</td>
<td>Δ (araABC-leu)7679 galU galK Δ(lac)X74 rpsL thi</td>
<td>(3)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> 168</td>
<td>Wild-type</td>
<td>(4)</td>
</tr>
<tr>
<td>WA221 W3110 zhc-12::Tn10 mreB221 (A22 resistant)</td>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>Plasmid/phage</td>
<td>Constitutively expressing EGFP, KanR</td>
<td>(6)</td>
</tr>
<tr>
<td>pWR20</td>
<td>Expression vector, AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>pBAD18</td>
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<td>(7)</td>
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<tr>
<td>pSW1</td>
<td>P$_{BAD}$-mCherry AmpR</td>
<td>(8)</td>
</tr>
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