BACTERIAL CELL SHAPE

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Abstract | Bacterial species have long been classified on the basis of their characteristic cell shapes. Despite intensive research, the molecular mechanisms underlying the generation and maintenance of bacterial cell shape remain largely unresolved. The field has recently taken an important step forward with the discovery that eukaryotic cytoskeletal proteins have homologues in bacteria that affect cell shape. Here, we discuss how a bacterium gains and maintains its shape, the challenges still confronting us and emerging strategies for answering difficult questions in this rapidly evolving field.

PEPTIDOGLYCAN
A covalently linked macromolecular structure made up of stiff glycan strands crosslinked by somewhat flexible peptide bridges. It gives the cell wall its strength. Also called 'murein', from Latin *murus*, wall.

SACCULUS
A synonym for the 'sac-like' peptidoglycan molecule that surrounds the cytoplasmic membrane of a bacterium.

SPHEROPLAST
A cell in which the cell wall is either absent or disrupted, causing it to adopt a spherical shape.

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Since the advent of microbiology, cell shape has been an important criterion in the description and classification of bacterial species. This is reflected in taxonomy — *Streptococcus* species are named for their spherical or seed-shaped (coccus) cells, bacilli for their rod shape and spirochaetes for their spiral shape. For many years, the basis for generation of these various cell shapes remained obscure. It became increasingly clear that the bacterial cell wall, with its PEPTIDOGLYCAN layer (or SACCULUS), was important in maintaining the shape of the cell and protecting against osmotic pressure. Disruption of the cell wall of rod-shaped *Bacillus* species or *Escherichia coli* with lysozyme or penicillin resulted in the formation of round, osmotically sensitive cells (SPHEROPLASTS)[1,2]. Moreover, peptidoglycan sacculi isolated from *E. coli* retained the rod shape of intact cells[3,4]. But what was responsible for the shape of the cell wall? Schwarz and Leutgeb[5] reported in 1971 that *E. coli* spheroplasts, produced by omission of a peptidoglycan amino-acid precursor for which they were auxotrophic, quickly resynthesized spherical sacculi with unaltered chemical composition after reintroduction of the precursor. Two hours later, the spherical cells had regained their rod shape. Therefore, they postulated that there was a 'distinct morphogenetic apparatus' that directed cell-wall shape[5]. Subsequently, genetics revealed clusters of genes that were important for the rod-shaped morphology of *Bacillus subtilis* and *E. coli*. Consistent with the importance of the cell wall in overall morphology, some of these genes encoded factors that were involved in peptidoglycan synthesis and remodelling, including PENICILLIN-BINDING PROTEINS (PBPs)[6–9], whereas other genes were involved in the synthesis of TEICHOIC ACIDS in Gram-positive cells[10–12]. However, the rod shape also depended on the *mre* genes (*mreB, mreC, mreD*) and *rodA*, the products of which had unknown functions[13–17]. Recently, MreB was identified as a bacterial homologue of the eukaryotic cytoskeletal protein actin[18–20], and was shown to form helical structures along the long axis of the cell, probably just beneath the cytoplasmic membrane[19,21–23]. These data lent support to the notion that the MreB structure might be a morphogenetic apparatus that dictates cell shape. Crescentin, an intermediate filament-like protein with an essential role in the curved-rod shape of *Caulobacter crescentus*, was observed to form a filamentous structure along the inside curvature of cells[24], further bolstering the case that internal structures can be important determinants of bacterial cell shape.

Despite these recent advances, the field of bacterial morphogenesis is still in its infancy. The molecular mechanisms that allow bacterial cytoskeletal elements to affect the cell wall remain to be elucidated, as do the cellular processes that regulate cytoskeletal structure and activity. In this review, we discuss the elements responsible for bacterial cell shape, such as the cell wall, the cytoskeleton and the membrane-bound shape determinants and enzymes that probably link them together. We also relate cell growth to cell shape, discuss outstanding questions and consider the future of the bacterial cell-shape field.

Box 1 | **Determination and maintenance of cell shape**

The concepts of shape determination and shape maintenance are related but distinct. Determination refers to the guidance of something new, whereas maintenance refers to the preservation of something previously determined. In the case of a poured-concrete wall, its shape is determined by wooden formwork when the concrete is poured, but is maintained not by the formwork but by the cured concrete itself. Once hardened, the shape of the wall would be maintained even if the formwork were destroyed. What if, however, a structural element has both determination and maintenance roles? The shape of a wall of sandbags is both determined and continually maintained by the bags — if they were ripped open, the sand would spill out and the wall would lose its shape. In terms of bacterial cell shape, distinguishing these two scenarios is complicated by the constant degradation of the peptidoglycan cell wall to allow insertion of new wall material. Here, a cytoskeletal shape determinant might have no structural role but still be constantly required for shape maintenance. Cytoskeletal elements (the formwork) might direct the shape of the peptidoglycan cell wall (the concrete) by modulating the location and activity of peptidoglycan synthesis. If the cytoskeleton is required to direct the insertion of new cell wall, depletion of cytoskeletal proteins — in an attempt to isolate their function — occurs concurrently with cell growth, so the cell loses its shape. Whether that loss of shape is caused by an absent structural support or insufficient guidance for continual synthesis cannot be distinguished. Resolution of this question might be accomplished through rapid destruction of cytoskeletal structures by using drug treatments, temperature-sensitive mutations favouring cytoskeletal disassembly, or targeted proteolysis. Morphological changes that occur after such disruption would indicate that the cytoskeleton has a structural role, and more gradual, growth-dependent changes would indicate a loss of cytoskeleton-mediated guidance. In *Caulobacter crescentus*, low concentrations of the MreB-depolymerizing drug A22 cause cells to lose their shape as they grow, whereas high concentrations cause immediate cessation of growth but no shape change[103]. These results support the hypothesis that MreB is required for peptidoglycan synthesis but plays no structural role. Further elucidation of the relationship between cytoskeletal elements and cell shape as a whole will give insight into the strategies available to bacterial cells for altering and maintaining particular shapes.

PENICILLIN-BINDING PROTEINS
A class of enzymes first discovered by their ability to bind labelled penicillin. They catalyse the reactions that are necessary to synthesize and modify peptidoglycan.

TEICHOIC ACIDS
Phosphate-rich, anionic polysaccharides that are attached to the peptidoglycan of Gram-positive bacteria. In *Bacillus subtilis*, most are polyglycerol phosphate or polyribitol phosphate and, in the case of lipoteichoic acids, have lipid modifications that allow association with the cytoplasmic membrane.

TRANSGLYCOSYLASE
An enzyme that catalyses the attachment of a peptidoglycan disaccharide-pentapeptide precursor molecule to an existing glycan strand by a β-1,4 glycosidic bond.

TRANSPEPTIDASE
An enzyme that catalyses the formation of a peptide bond between adjacent polypeptide side chains, forming a flexible peptide bridge between glycan strands.

PEPTIDE INTERBRIDGE
Additional amino acids that bridge the D-alanine in position 4 from one peptide with the dibasic amino acid in position 3 of the adjacent peptide. In the Gram-positive bacterium *Staphylococcus aureus*, for example, interbridges comprise five glycine residues.

Cell shape — growth and remodelling

Keeping in mind the basic function of the cell wall — a structure that maintains cell shape and rigidity[1–3] — it is clear that its alteration will affect cell morphology. A bacterial cell might therefore control its shape either by directing the location of new wall synthesis during cell growth or by remodelling the peptidoglycan independently of growth (BOX 1). For example, bacteria such as *E. coli* and *B. subtilis* preferentially synthesize new peptidoglycan along their lateral walls as they grow[25–29], to maintain a rod shape. By contrast, the composition of *Helicobacter pylori* peptidoglycan changes when cells shift from a curved rod to a coccoid morphology in extended culture[30]. Therefore, at least some morphogenetic determinants are predicted to be cellular factors that govern the synthesis[26] or remodelling of wall material. The importance of PBPs in cell morphology[6–9,31] is consistent with this idea, as these enzymes catalyse the actual synthetic reactions that are required for peptidoglycan growth and remodelling.

The bacterial cell wall

Most bacteria have a cell wall that maintains cell shape and protects against osmotic lysis. The strength and rigidity conferred by the cell wall results from a layer of peptidoglycan, which is a covalent macromolecular structure of stiff glycan chains that are crosslinked by flexible peptide bridges[32] (FIG. 1). Peptidoglycan comprises disaccharide-pentapeptide precursors that are composed of two aminosugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), connected by a β-1,4 glycosidic bond. The lactyl group on the MurNAc allows attachment of the five amino acids that comprise the pentapeptide. TRANSGLYCOSYLASES link a disaccharide precursor to an existing glycan strand by another β-1,4 glycosidic bond, which produces long, strong strands of alternating GlcNAc and MurNAc residues. The peptides, which extend at right angles from the glycan strands, can then be connected to pentapeptides that extend from adjacent glycan strands by TRANSPEPTIDASES, forming peptide cross-bridges that link the glycan strands together (FIG. 1). The presence of a dibasic amino acid (*meso*-diaminopimelic acid in *E. coli*) is required to crosslink the peptides. This transpeptidation event occurs between the D-alanine at position 4 of one peptide and the dibasic residue at position 3 of the other peptide. In *E. coli* and many other Gram-negative species, this is a direct link, but additional amino acids, the sequences of which can vary considerably, can form PEPTIDE INTERBRIDGES between peptides that are attached to adjacent glycan strands. By crosslinking the glycan strands together with peptide bridges, a strong mesh is created that protects the cell from osmotic lysis. The structure of the peptidoglycan can be further modulated through the action of carboxypeptidases and endopeptidases (FIG. 1).

There are two general classes of bacterial cell walls, first distinguished over a century ago by Hans Christian Gram based on their different retention of crystal-violet dye. Gram-positive cell walls are composed of a thick (20–80 nm), multilayered peptidoglycan sheath that includes embedded teichoic and lipoteichoic acids (FIG. 2a). These anionic polysaccharides are essential for viability in *B. subtilis* and contribute to cell morphology[10,33]. Gram-negative cell walls include an outer membrane that surrounds a thin (1–7nm, depending on measurement technique) peptidoglycan layer, with a periplasmic space between the inner and outer membranes (FIG. 2b). The outer membrane and peptidoglycan

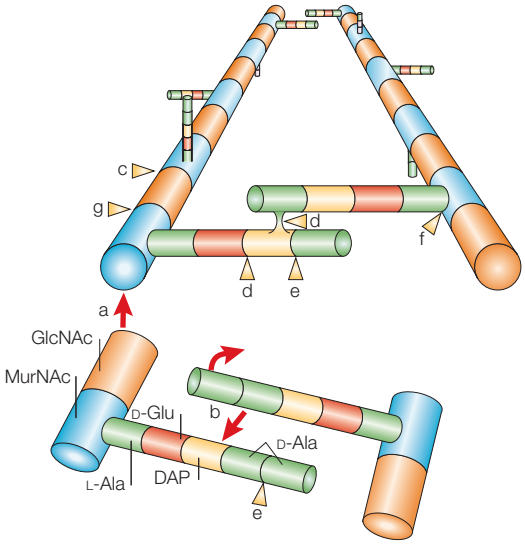

Figure 1 | **Chemistry of peptidoglycan synthesis and processing.** Glycan strands are built from repeating disaccharide subunits composed of *N*-acetylmuramic acid (blue; MurNAc) and *N*-acetylglucosamine (orange; GlcNAc). Pentapeptides are attached to the MurNAc; adjacent glycan strands are linked by peptide cross-bridges. The generation of cross-bridges is dependent on a dibasic amino acid such as diaminopimelic acid (DAP). Red arrows indicate synthetic reactions and yellow arrowheads indicate cleavage activities. a, transglycosylase activity; b, transpeptidase activity, resulting in the loss of the terminal D-alanine on one of the pentapeptides; c, lytic transglycosylase activity; d, endopeptidase activity; e, carboxypeptidase activity; f, amidase activity; g, *N*-acetylglucosaminidase activity.

are linked to each other with lipoproteins[34,35], and loss or altered expression of outer-membrane proteins and lipoproteins in *E. coli* can affect cell shape[36–38], indicating that the outer membrane is important for shape generation and/or maintenance. Some bacteria completely lack a cell wall, but still retain distinct morphologies (BOX 2).

Macromolecular structure and assembly. Although the components and assembly of peptidoglycan are well characterized, its construction and higher-order structure are not well understood. Several different models have been proposed for the mechanisms of new peptidoglycan insertion and the arrangement of glycan strands and peptide cross-bridges within peptidoglycan. One popular view of peptidoglycan architecture is that the glycan strands are arranged parallel to the cytoplasmic membrane, primarily forming a single layer in Gram-negative cells and multiple crosslinked layers in Gram-positive cells. This model, at least for Gram-negative cells, is in accordance with experimentally determined values for the quantity of peptidoglycan per cell, the thickness of peptidoglycan and the length distribution and degree of crosslinking of glycan chains[39]. Recent evidence also shows that hydrated *E. coli* sacculi are more deformable along their long axes, consistent with the orientation of glycan strands along the short axis of the cell, parallel to the cytoplasmic membrane[40]. The 'scaffold model', a computer-simulation-based model in which the glycan strands are oriented perpendicular to the cytoplasmic membrane, recently challenged this traditional view[41,42]. Although doubt has been cast on the scaffold model for Gram-negative bacteria[39], its more recent application to the Gram-positive *Staphylococcus aureus* cell wall fits well with experimental data[41].

Insertion of new glycan strands into the peptidoglycan is problematic, as the peptidoglycan is under constant stress from intracellular turgor pressure. In Gram-positive bacteria, new subunits are attached to the layer of glycan strands nearest to the cytoplasmic membrane and are then pushed outwards into the stress-bearing layer by continued peptidoglycan synthesis until degradation occurs near the exterior peptidoglycan surface[43]. Maintenance of Gram-negative peptidoglycan, which is mainly composed of a single layer of glycan strands[44], is trickier, as the bond breaking that is required to insert new material into the covalently closed structure endangers its integrity. Therefore, peptidoglycan hydrolase activity must be carefully controlled to break bonds and generate new insertion sites. The mechanism that allows such insertion of new material has not yet been elucidated, but might include the cleavage of old glycan strands by PEPTIDOGLYCAN HYDROLASES, rapidly followed by insertion of new subunits[45], or the attachment of three new glycan strands to the existing structure, which are then automatically pulled into the stress-bearing layer by the cleavage and removal of one old strand[46]. The large number and variety of hydrolases have so far hindered rigorous testing of these hypotheses.

Mechanical properties. As isolated peptidoglycan sacculi retain the shape of intact cells[3], the cell wall was thought to be inherently rigid. However, several lines of evidence indicate that it is both flexible and elastic[40,47–49]. As early as the 1960s, electrostatic effects within the peptidoglycan were postulated to cause expansion and contraction of isolated sacculi[48]. This notion was supported by experiments that combined manipulation of the charge on isolated *E. coli* sacculi with low-angle laser light-scattering measurements of their surface area, in which it was determined that peptidoglycan could expand up to 300% from its relaxed state[49]. The properties of isolated and hydrated *E. coli* sacculi were later assessed mechanically using ATOMIC FORCE MICROSCOPY (AFM) (see Supplementary information S1 (box)), confirming its flexibility and elasticity[40]. These experiments are in agreement with theoretical calculations based on peptidoglycan intramolecular bonds[50] and suggest that the bacterial cell wall is not a 'hard shell' but a structure that retains flexibility in living cells.

New wall synthesis. It is plausible that selective synthesis of new cell wall at particular locations contributes to cell morphology as cells grow and divide. Therefore, knowledge about the location and nature of specific synthesis regions is important for understanding morphogenesis. Currently, there are three main strategies

PEPTIDOGLYCAN HYDROLASES
A class of enzymes that break molecular bonds in peptidoglycan. They are required to allow insertion of new peptidoglycan and to enable cell division, but must be tightly regulated to prevent autolysis.

ATOMIC FORCE MICROSCOPY
A technique in which a sharp tip is scanned across the surface of a sample, probing sample-tip interaction forces. The resulting 'image' is high resolution and, as no light is required, the sample can be hydrated in aqueous solutions.

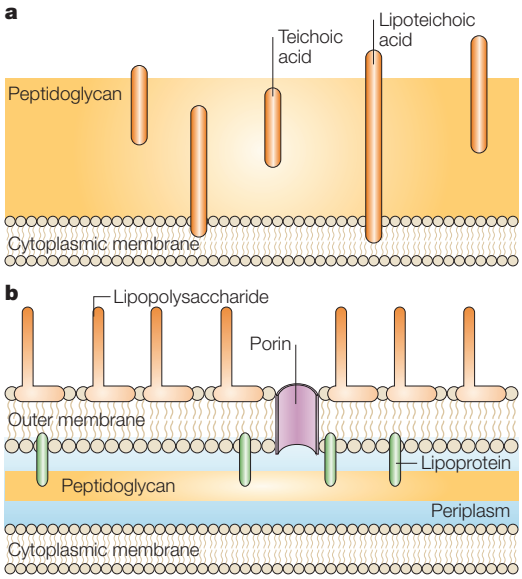

Figure 2 | **Gram-positive and Gram-negative cell walls.** **a** | The Gram-positive cell wall is composed of a thick, multilayered peptidoglycan sheath outside of the cytoplasmic membrane. Teichoic acids are linked to and embedded in the peptidoglycan, and lipoteichoic acids extend into the cytoplasmic membrane. **b** | The Gram-negative cell wall is composed of an outer membrane linked by lipoproteins to thin, mainly single-layered peptidoglycan. The peptidoglycan is located within the periplasmic space that is created between the outer and inner membranes. The outer membrane includes porins, which allow the passage of small hydrophilic molecules across the membrane, and lipopolysaccharide molecules that extend into extracellular space.

for differentiating between pre-existing and newly incorporated peptidoglycan (see Supplementary information S1 (box)). However, it is difficult to resolve areas of new synthesis with precision, and the processes that govern synthesis localization remain largely unclear. In *E. coli* and *B. subtilis*, cell poles are subject to far less synthesis and turnover than sidewalls and division sites[25–29,51], and in spherical *S. aureus* and *Streptococcus* species, new synthesis occurs primarily at division sites[52–55] (FIG. 3). New peptidoglycan insertion in *E. coli* and *B. subtilis* seems to be distributed among discrete patches and circumferential bands along the sidewall, in a pattern indicative of a helix[26,27,29]. There might, therefore, be guidance systems to direct peptidoglycan synthesis at particular cellular locations.

Tracking the insertion and fate of peptidoglycan as cells grow and divide has led to the concept of 'inert peptidoglycan' — peptidoglycan that does not undergo growth or turnover, or does so at a greatly reduced rate[25,27,29]. In species such as *B. subtilis* and *E. coli*, it has been hypothesized that inert peptidoglycan at the cell poles functions as a rigid support for overall cell morphology[56]. In this hypothesis, a mislocalized patch of inert peptidoglycan would function as an ectopic pole, causing cell branching. This prediction is supported by the association of morphological abnormalities with deposition of inert peptidoglycan at sites along the sidewall[57].

MIN
The Min system comprises three proteins in *Escherichia coli*: MinC, MinD and MinE. Mutations in the *min* genes produce characteristic mini cells. The cooperative action of MinC, MinD and MinE proteins ensures the placement of the division site at the midcell.

Z RING
The ring-shaped structure that is formed during cell division from FtsZ polymers. The Z ring recruits proteins that are required for septal wall synthesis and cell division.

The bacterial cytoskeleton

Eukaryotic cells contain three major cytoskeletal systems: microfilaments, microtubules and intermediate filaments, which are assembled from actin, tubulin and intermediate filament proteins, respectively. These systems function to help maintain cell shape and integrity. They also participate in many cellular functions, including motility (which results in cell shape changes), chromosome segregation, signal transduction and cytokinesis. For many years, the prevailing view was that bacteria contained no cytoskeletal elements and were instead shaped by an 'exoskeleton' — the cell wall. However, homologues of all three eukaryotic cytoskeletal elements have now been found in bacteria (FIG. 4). Mounting evidence indicates that these proteins have important roles in cellular functions such as DNA segregation, cell polarity and sporulation. Other uniquely bacterial proteins, the MIN proteins, assist in division-site placement and also seem to form cytoskeletal structures. The structure and function of bacterial cytoskeletal elements have recently been reviewed[58,59], so we focus here only on their functions that are most closely related to cell shape during growth and division.

The tubulin homologue FtsZ. FtsZ, the first of the bacterial cytoskeletal homologues to be discovered, is required for cell division in nearly all bacteria, where it forms a ring structure (the Z RING) at the cell-division site[59]. This hinted at a possible cytoskeletal function[60] and, along with the ability of FtsZ to hydrolyze GTP with a tubulin signature motif[61–63] and to form filaments *in vitro*[64,65], made a case for FtsZ as a prokaryotic tubulin homologue. X-ray crystallographic structures revealed remarkable similarities between FtsZ and tubulin, confirming this hypothesis[66,67]. FtsZ has a crucial role in cell division, as it is required for recruitment of all the other division proteins[68]. During cell division, the Z ring assembles and constricts at the division site, directing the peptidoglycan synthesis that is required for formation of new cell poles[68]. Therefore, the role of FtsZ at the cell-division site implicates FtsZ as a shape determinant, as cell size is determined by cell division. Moreover, mutations in FtsZ can cause aberrant cell morphology in some genetic backgrounds[69–71], further linking it to shape determination. Interestingly, recent evidence indicates that FtsZ is not only highly dynamic within the Z ring itself [72], but also forms dynamically oscillating helix-like structures independently of Z ring formation[73], the significance of which has not yet been determined.

The actin-like MreB family. MreB was originally discovered as a protein with a function in rod shape, as deletion of the *E. coli mreB* gene resulted in round or irregular cell morphology[14,15]. The *mreB* gene is also present in *B. subtilis* (*B. subtilis mreB*)[16], which contains two additional *mreB* homologues: *mbl* (*mreB*-like)[74] and *mreBH*. Notably, most spherical bacterial species lack *mreB*, whereas it is well-represented among bacteria with more complex shapes[19]. By comparing sequences

Box 2 | What about cell-wall-less bacteria?

The MOLLICUTES are some of the simplest self-replicating cells in nature. Despite being phylogenetically related to Gram-positive bacteria, these organisms lack cell walls, and instead have only a cholesterol-containing cell membrane. It is perhaps surprising that these organisms have clearly defined shapes, ranging from the simple *Acholeplasma* cocci to the tapered flask-like shape of some *Mycoplasma* species and the distinct spiral shape of *Spiroplasma* species. Interestingly, Mollicutes seem to contain internal cytoskeletal structures that govern their shapes and enable motility[104]. Small helical structures have been isolated from the cytoplasm of *Acholeplasma laidlawii*[105], and ultrastructural analysis of *Mycoplasma pneumoniae* revealed a highly complex, asymmetric cytoskeletal network that is composed of many unknown proteins[106]. In *Spiroplasma* species, meanwhile, the cytoskeleton is primarily composed of fibril protein[107], which forms a flat, helical ribbon that is probably an important determinant of the spiral shape of the cells[104]. Additionally, the *Spiroplasma citri* genome includes five putative *mreB* homologues[108]. Using CRYO-ELECTRON TOMOGRAPHY the helical cytoskeleton of *Spiroplasma melliferum* cells was recently observed within cells, and has been postulated to include MreB[108]. The cytoskeletal ribbon is probably responsible for the motility of *Spiroplasma* species by contractile action, driven by conformational changes in fibril subunits[109]. No homologues of the fibril protein have been found in other bacteria or eukaryotes[104,107]. However, FtsZ has been found in Mollicutes, and probably has a role in their cell division[110,111]. Notably, *Mycoplasma genitalium* contains an FtsZ protein that is distinct from that found in walled bacteria, and lacks homologues of the other *Escherichia coli fts* cell-division genes[110]. This indicates that the full complement of cell-division proteins is only necessary for division in cells with a peptidoglycan cell wall[111]. The relationship between Mollicute cytoskeletal structures and those of walled bacteria, if any, remains to be determined. Experiments using techniques to visualize these proteins in live Mollicutes will be invaluable to the field, but new genetic tools are needed to make this possible[112]. Nonetheless, the existence of Mollicute cytoskeletons shows that bacteria, in the absence of a shape-maintaining cell wall, can still retain a distinct shape based on internal structures.

and predicted structural motifs, the ATPase domain of MreB was predicted to have a similar structure to that of sugar kinases, Hsp70 heat-shock proteins and actin[20]. The X-ray crystal structure of MreB has striking structural similarity to actin, and purified MreB can assemble into actin-like filaments[18]. Just before its structure was solved, fluorescence microscopy of the *B. subtilis* MreB and Mbl proteins revealed helical cable-like structures beneath the cytoplasmic membrane[19] (see Supplementary information S1 (box)). Mbl formed a double-helix-like structure that runs the length of the cell, whereas MreB formed shorter helices with fewer turns within the cell[19]. Similarly, MreB in *E. coli* forms helical intracellular structures[23] (FIG. 4b). MreB is essential for viability in *B. subtilis*, and cells with a disrupted *mbl* gene are morphologically distorted, with irregular bends, twists and bulges[19,74]. Depletion of the *B. subtilis* MreBH protein, which also forms helical filamentous structures in cells[75], results in cell curvature[76], also linking this actin homologue to cell morphology. Like FtsZ, the helical structures formed by MreB and its homologues are dynamic and can change their pitch and rotate within growing cells[75,77], observations that might have important implications for their roles in cell shape. The observation that nascent peptidoglycan — visualized in live *B. subtilis* cells using fluorescent VANCOMYCIN (see Supplementary information S1 (box))— localizes to Mbl-dependent helices provides a crucial link between a cytoskeletal element and cell-wall synthesis[26]. Additionally, MreB in *C. crescentus* forms intracellular helices that might coordinate peptidoglycan synthesis[22]. Together, these data indicate that bacterial cytoskeletal elements like FtsZ and MreB (or Mbl in *B. subtilis*) govern cell shape by localizing cell-wall synthesis to specific subcellular locations during growth and division.

Intermediate filament-like crescentin. The most recently discovered bacterial cytoskeletal element is crescentin, which has the conserved coiled-coil domain architecture of eukaryotic intermediate filament proteins, as well as the ability to self-assemble *in vitro* into filaments that are structurally similar to intermediate filaments[24]. Disruptions in the crescentin-encoding gene (*creS*) of *C. crescentus* produce mutants with a straight-rod morphology instead of the characteristic crescent shape of wild-type cells[24]. Crescentin localizes as an apparent intracellular filamentous structure at the inner curvature of cells (FIG. 4c), where it is thought to exert its influence on cell shape[24]. In old stationary-phase cultures, *C. crescentus* cells lengthen into helical filaments[78], and crescentin forms a structure following the shortest helical path through the cell[24] (see Supplementary information S1 (box)). This observation indicates that the helical geometry of crescentin structure promotes helical cell growth[24], as crescent-shaped cells in young cultures can be thought of as sections of a helix that are shorter than one helical turn. The molecular mechanism by which crescentin influences cell shape is currently unknown, but the existence of crescentin in *C. crescentus* raises the possibility that other curved or helical bacteria employ similar shape-determining strategies. The amino-acid sequence of crescentin contains long stretches of fairly common coiled-coil-forming repeats[24]. Together with the absence of enzymatic signatures in crescentin and intermediate filament proteins, this makes it difficult to find true homologues in other species. However, there are many uncharacterized proteins with long coiled-coil-forming regions in other curved and helical bacteria, suggesting possible crescentin-like function[24].

PBPs and membrane-bound shape determinants

In order for cytoskeletal structures such as FtsZ, MreB, Mbl and crescentin to influence the assembly of the cell-wall peptidoglycan and therefore overall cell shape, a molecular link must bridge the cytoskeleton and the peptidoglycan. Such a link could be provided by membrane-bound and membrane-associated proteins that can transmit shape information across the cytoplasmic membrane. This group of shape determinants might include PBPs and other proteins that are required for shape maintenance, such as RodA, MreC and MreD.

Penicillin-binding proteins. PBPs are categorized according to their molecular weight, sequence and enzymatic and cellular functions (TABLE 1). Biochemical evidence from *E. coli* and *C. crescentus* so far supports the proposal that PBPs form complexes with peptidoglycan hydrolases, in which each protein contributes its specific enzymatic activity to insert and modify new

VANCOMYCIN
An antibiotic that binds to the C-terminal D-alanine–D-alanine polypeptide of peptidoglycan precursors, preventing the transpeptidation reaction that is required for peptide crosslinking of glycan strands.

MOLLICUTES
A class of wall-less bacteria that includes acholeplasmas, mycoplasmas and spiroplasmas. They have the simplest genomes of any self-replicating, free-living organisms but can retain defined shapes by virtue of internal cytoskeletons.

CRYO-ELECTRON TOMOGRAPHY
A technique in which a specimen, embedded in vitreous ice, is imaged from multiple angles using electron microscopy. The resulting images are then combined to reconstruct the 3D structure of the specimen.

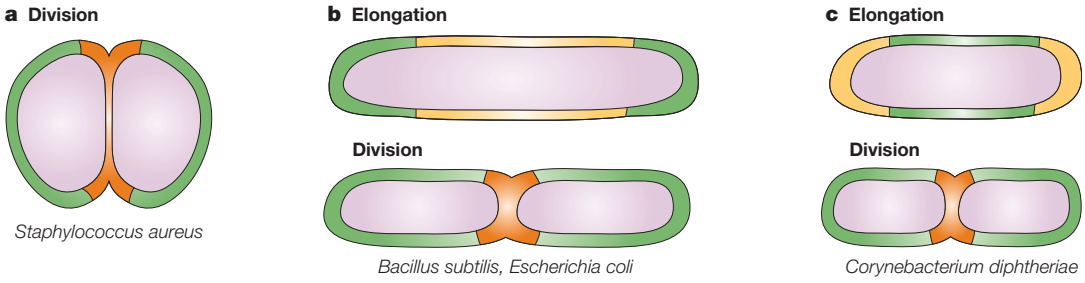

Figure 3 | **Where does cell-wall growth occur?** In virtually all eubacteria, division is accomplished through synthesis of new peptidoglycan (red), and division planes can therefore be considered as regions of cell-wall growth. **a** | In spherical cells such as *Staphylococcus aureus*, this is the primary means of cell growth, and the peptidoglycan composing the septum becomes a hemisphere in each daughter cell. **b** | In rod-shaped cells like *Bacillus subtilis* and *Escherichia coli*, new peptidoglycan is inserted not only at division sites during cell division but also along the sidewalls during cell elongation (yellow). The poles, meanwhile, remain relatively inert. **c** | In *Corynebacterium diphtheriae*, cell elongation is mainly accomplished by polar growth, not sidewall growth.

peptidoglycan[22,79–81]. Moreover, in *Haemophilus influenzae*, two different multienzyme complexes have been found: one that is associated with the cell-elongation-specific transpeptidase PBP2 and one that is associated with the cell-division-specific transpeptidase PBP3 (REF. 82). As rod-shaped cells seem to have two important peptidoglycan-synthesis activities — elongation and division — the difference between the two could be the identity of the particular transpeptidase present in the synthesis complex.

Synthesis of new peptidoglycan at a specific location might occur through recruitment of one or more PBPs to a localized cell-shape determinant. This seems to occur during septal synthesis, when FtsZ recruits PBP3 (FtsI) to the division plane[68,83]. Similarly, in *C. crescentus*, PBP2 localizes in a band-like pattern that is not observed in spherical MreB-depleted cells, indicating that MreB might recruit PBP2-containing peptidoglycan-synthesis complexes to function in cell elongation[22] (FIG. 5). MreB in *C. crescentus* also localizes in a FtsZ-dependent manner to the division plane, hinting at a possible function for MreB in the switch from cell-wall elongation to cell-wall synthesis at the cell-division site[22]. Therefore, cytoskeletal structures might have a role in determining the location and timing of peptidoglycan-synthesis activities in cell elongation and division.

Membrane-bound shape determinants. Just as two class B high-molecular-weight PBPs (PBP2 and PBP3; see TABLE 1) function in cell elongation and division in *E. coli*, respectively, each of these distinct synthesis functions also requires a second membrane-bound protein. Elongation requires both PBP2 and RodA[7,17,84], and division requires both PBP3 and FtsW[85]. RodA and FtsW are structurally similar to each other and to the *B. subtilis* SpoVE protein, which functions in spore formation[86]. These three proteins are the prototypes of the SEDS (shape, elongation, division and sporulation) protein family, with members probably present in all walled eubacteria[31]. In *E. coli*, *rodA* and *ftsW* are OPERONIC with *pbpA* (PBP2) and *ftsI* (PBP3), respectively[87,88], highlighting the need for both a PBP and a SEDS family member for effective peptidoglycan synthesis. In *B. subtilis*, *rodA* is MONOCISTRONIC, but it is essential for viability and necessary for maintenance of rod-shaped cells[31], indicating that it has a similar function to *rodA* in *E. coli*. Evidence from *E. coli* suggests that PBP2 requires RodA to perform its enzymatic role[89] and that FtsW is required for the localization of PBP3 to the cell-division site[90]. However, the mechanism of action of RodA and FtsW remains unknown.

The *mre* locus in *E. coli* includes not only *mreB* but also *mreC* and *mreD*, which are important for maintenance of cell shape[14,15]. The same gene cluster is also

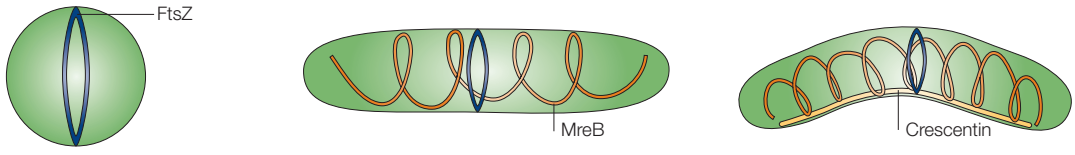

Figure 4 | **Cytoskeletal elements and cell shape. a** | Cells such as *Staphylococcus aureus* contain the tubulin-like division protein FtsZ, which is present in virtually all eubacteria. Whereas FtsZ forms a ring-shaped structure (blue) during cell division that is required for the division process, it seems to impart no shape to non-dividing cells. Therefore, most cells containing FtsZ as the sole cytoskeletal element are spherical. **b** | When actin-like MreB homologues are present, cells can take on a rod-shaped morphology like that seen in *Escherichia coli*. MreB and its homologues often appear as intracellular helical structures (red) when viewed with fluorescence microscopy. **c** | *Caulobacter crescentus* cells contain crescentin (yellow) in addition to FtsZ and MreB, and show a crescent-shaped cell morphology. In *C. crescentus* cells, MreB localizes to apparent helices during cell elongation and to the division plane with FtsZ during cell division.

OPERONIC
Describes multiple genes in an operon, a single transcriptional unit driven by a single promoter. Operons often contain genes encoding protein products that act in the same pathway.

MONOCISTRONIC
Transcribed as a single gene.

Table 1 | **The penicillin-binding proteins (PBPs) of *Escherichia coli***

PBP	Molecular function	Physiological function
HMW class A		
1a	Transglycosylase/transpeptidase[113]	General peptidoglycan synthesis
1b	Transglycosylase/transpeptidase[114]	General peptidoglycan synthesis
1c	Transglycosylase[80]	Unknown
HMW class B		
2	Transpeptidase[89]	Cell elongation[115]
3	Transpeptidase[116]	Cell division[117]
LMW		
4	Endopeptidase/carboxypeptidase[118]	Unknown
5	Carboxypeptidase[96,119]	Cell shape (shows phenotype in combination with other LMW PBP deletions)[96]
6	Carboxypeptidase[119]	Unknown
6b	Carboxypeptidase[120]	Unknown
7/8	Endopeptidase[121]	Unknown

HMW, high-molecular weight; LMW, low-molecular weight.

present in *B. subtilis* and has a similar function[16,91]. MreB, MreC and MreD are essential for viability in *E. coli*, where they form a membrane-bound complex[92] (FIG. 5). Moreover, MreB localization in *E. coli* is disrupted in RodA-depleted cells, and depletion of MreC or MreD leads to progressive delocalization of MreB, adding further support to a model in which cytoskeletal elements coordinate with a complex of proteins to maintain their localization and direct peptidoglycan synthesis[92,93].

Outstanding questions

The recent discovery of the bacterial cytoskeleton, combined with continued characterization of the cell wall and its associated enzymes, places us in an excellent position to begin to develop a complete picture of how bacteria generate and maintain their shape. Still, many questions regarding the molecular interactions between cytoskeletal and peptidoglycan synthesis elements, the biochemical functions of each element and differences in morphogenetic apparatus among different bacterial species remain unanswered. Fortunately, we have many of the tools that are required to begin elucidating these processes, and knowledge already gained will assist the interpretation of new data.

What is the composition and location of peptidoglycan synthesis complexes? PBPs, membrane-bound shape determinants and cytoskeletal elements might all interact to form localized protein complexes that coordinate peptidoglycan synthesis to generate a specific cell shape. More rigorous experimentation is required to confirm this model. In *C. crescentus*, it is probable that PBP2 interacts with MreB[22]. In *B. subtilis*, helical Mbl localization correlates with new peptidoglycan insertion[26]. However, the helical pattern of nascent peptidoglycan in *B. subtilis* does not seem to correlate with PBP localization[94], making it unclear how Mbl might induce localized peptidoglycan synthesis. Instead of recruiting PBPs to a particular location, Mbl might activate adjacent PBPs to synthesize peptidoglycan.

Another key to solving this puzzle is further characterization of RodA and FtsW. Do these proteins interact with MreB, Mbl and FtsZ, bridging the cytoskeleton with PBPs? Do they translocate lipid-linked peptidoglycan precursors? An interaction between FtsW and FtsZ has been shown in *Mycobacterium tuberculosis*, but that interaction occurs through C-terminal tails with extensions that are absent from the *E. coli* counterpart proteins[95]. Therefore, additional factors might mediate similar interactions in *E. coli* and other bacteria. An *E. coli* scaffolding protein, MipA, interacts with PBP1b (TABLE 1) and the LYTIC TRANSGLYCOSYLASE MltA[79], indicating that one or more structural proteins might serve as scaffolds on which a peptidoglycan synthesis complex is built. Further biochemical characterization of these complexes is needed to identify which factors are required for complex formation and whether multiple proteins can fulfil the same role. For example, the mild morphological phenotypes of mutants with deletions in low-molecular-weight PBPs (TABLE 1) other than PBP5 (REF. 96) might be a result of PBPs substituting for each other. This could be tested by determining the contents of PBP complexes[82] in strains that lack one or more low-molecular-weight PBPs.

Are there two main synthetic complexes, one for cell elongation and one for division? Does the structure of peptidoglycan depend on the complex that synthesized it? Are other complexes required for growth-independent peptidoglycan remodelling or maintenance? Additionally, *B. subtilis* has a different PBP complement from *E. coli*, and whereas *E. coli* requires either PBP1a or PBP1b (TABLE 1) for survival, a *B. subtilis* mutant that lacks all four known class A PBPs is viable[97]. Are these differences reflected in the composition of synthesis complexes among different species?

How is peptidoglycan oriented? No chemical differences have been detected between the poles and sidewall of *E. coli*, despite years of research. Is the orientation of glycan strands in inert regions of peptidoglycan different from that of other peptidoglycan regions? How does the structure of septal peptidoglycan differ from that in the sidewall? Are glycan strands highly ordered, or arrayed more randomly? Does strand orientation change when different PBPs are inactivated? Interestingly, structural elements of the Gram-positive *Lactobacillus helveticus* cell wall have been observed using AFM, revealing striations along the short axis of the cell[98]. Although these striations are larger than individual glycan strands[98], AFM will probably be a useful tool for probing peptidoglycan structure in the future.

How does the bacterial cytoskeleton function? What regulates the assembly, localization and function of bacterial cytoskeletal proteins? How is crescentin asymmetrically localized, and how does it induce cell curvature? Purified cytoskeletal elements such as MreB and crescentin assemble *in vitro* into non-helical

LYTIC TRANSGLYCOSYLASE
An enzyme that cleaves the bonds between adjacent aminosugar moieties in glycan strands of peptidoglycan, enabling new precursor molecules to be added.

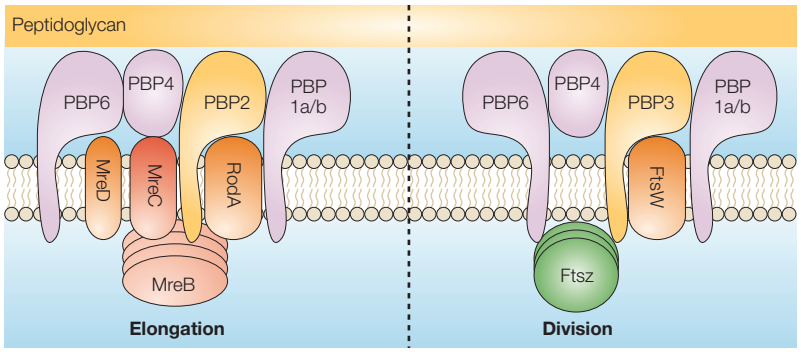

Figure 5 | **Shape information: cytoplasm to cell wall.** This highly speculative model, derived from multiple lines of evidence in different bacterial species, illustrates how shape information might be transferred from cytoplasmic cytoskeletal structures, through membrane-bound shape determinants, to peptidoglycan synthesis complexes during cell elongation and cell division. During cell elongation (left panel), MreB might interact with MreC and MreD to form a shape-determining structure that interacts with an elongation-specific PBP2-containing peptidoglycan synthesis complex. During cell division (right panel), FtsZ and its associated proteins might interact with division-specific PBP3-containing peptidoglycan synthesis complexes. For simplicity, other cell-division proteins have been omitted from the diagram. Additionally, it is probable that synthesis complexes also include other peptidoglycan-modifying enzymes and scaffolding proteins that are not shown here. PBP, penicillin-binding protein.

filaments[18,24], so how are helical filamentous structures assembled in living cells? Are additional proteins required? Biochemical experiments to determine the factors that interact with these bacterial cytoskeletal elements, combined with careful microscopic analysis in living cells, might help resolve some of these questions. What sorts of higher-order structures are formed, and how similar are they to eukaryotic cytoskeletal structures? How do bacterial cytoskeletal elements accomplish their observed dynamism, and how does this movement relate to cell shape? Here, *in vitro* studies on bacterial cytoskeletal filament dynamics have begun to shed light on their assembly and turnover properties[99,100]. Finally, it will be interesting to see if the cytoskeleton comprises the primary cell-shape determinant in bacteria, or if there are additional morphogenetic factors that dictate the structure of the cytoskeleton itself. The observed dependence of MreB helices in *E. coli* on MreC, MreD and RodA[92,93] might indicate that perhaps these proteins act in a co-dependent manner, instead of MreB having the primary shape-determining role.

Are there other shape-determining strategies? Even if holoenzyme complexes directed by cytoskeletal elements mediate peptidoglycan synthesis, some bacteria seem to use other shape-determining strategies. For example, both Gram-positive and Gram-negative bacteria such as corynebacteria and rhizobacteria lack MreB but have a rod-like shape[26]. Accordingly, *Corynebacterium* species insert peptidoglycan from the cell poles (FIG. 3c) instead of in the helical pattern that is observed in *B. subtilis*[26,101]. The determinants that direct this synthesis are unknown. The spirochaete *Borrelia burgdorferi*, meanwhile, has periplasmic flagella that not only enable motility but are also required for its flat-wave or helical shape[102]. Interactions between the flagella and the peptidoglycan probably mediate this shape determination, and elucidation of the nature of this interaction might allow deductions about how other spiral-shaped bacteria maintain their shape.

The future of bacterial cell biology

With the recent discovery of the bacterial cytoskeleton and new insights into the enzymes that govern peptidoglycan synthesis, bacterial cell biology is poised to answer some of the basic questions that have tantalized microbiologists for decades. Many clues have already been found, and new data about molecular interactions will fill in the missing pieces, enabling the development of more accurate models for shape generation. Once tenable models have been established in popular model bacteria, researchers will surely begin to tackle the mechanisms of shape generation in bacteria with different modes of growth and shapes. It has become clear that the generation of even simple rod shapes is far more complicated than was originally anticipated. Ultimately, elucidation of the mechanisms behind bacterial shape will help us move beyond the "how" of the diverse shapes of bacteria to answer a deep and persistent question — why these shapes?

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