Determination of bacterial rod shape by a novel cytoskeletal membrane protein

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Cell shape is critical for growth, and some genes are involved in bacterial cell morphogenesis. Here, we report a novel gene, *rodZ*, required for the determination of rod shape in *Escherichia coli*. Cells lacking *rodZ* no longer had rod shape but rather were round or oval. These round cells were smaller than known round mutant cells, including *mreB* and *pbpA* mutants; both are known to lose rod shape. Morphogenesis from rod cells to round cells and *vice versa*, caused by depletion and overproduction of RodZ, respectively, revealed that RodZ could regulate the length of the long axis of the cell. RodZ is a membrane protein with bitopic topology such that the N-terminal region including a helix-turn-helix motif is in the cytoplasm, whereas the C-terminal region is exposed in the periplasm. GFP-RodZ forms spirals along the lateral axis of the cell beneath the cell membrane, similar to the MreB bacterial actin. Thus, RodZ may mediate spatial information from cytoskeletal proteins in the cytoplasm to a peptidoglycan synthesis machinery in the periplasm.

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Introduction

Bacteria display a variety of cell shapes, including round, rod, spiral, and amorphous (Young, 2006). Maintenance of cell shape is vital for cell growth and cell division in most bacteria. The distinct shape of most bacteria is retained by a peptidoglycan layer, or sacculus, enveloping bacterial cells because the sacculus is a single giant macromolecule (Vollmer and Bertsche, 2008). The sacculus serves as a rigid body against mechanical stress, including osmotic pressure of the cytoplasm. In fact, inactivation of peptidoglycan synthesis by some antibiotics causes the cells to burst by osmotic pressure, leading to cell death. Gram-positive *Bacillus subtilis*

and Gram-negative *Escherichia coli* bacteria are enveloped in the peptidoglycan layer made of glycan strands cross-linked by short peptides in different manners. Obviously, the peptidoglycan layer of Gram-positive bacteria is thicker than that of Gram-negative bacteria.

Morphogenesis of the peptidoglycan layer is regulated not only by peptidoglycan biosynthesis, but also by cytoskeletal proteins beneath the cytoplasmic membrane (Osborn and Rothfield, 2007). It has been shown that most bacteria have homologues of eukaryotic cytoskeletal proteins, such as tubulin and actin (Carballido-Lopez and Formstone, 2007; Dye and Shapiro, 2007; Erickson, 2007; Graumann, 2007; Osborn and Rothfield, 2007; Pichoff and Lutkenhaus, 2007). The bacterial tubulin, FtsZ, has an important function in cell division (Margolin, 2005) and contributes to cell morphogenesis. FtsZ forms a ring-like structure at mid-cell, which serves as a scaffold for other cell division proteins. The other bacterial cytoskeletal protein is actin homologue MreB. MreB has been originally identified from a mutant showing aberrant cell morphogenesis in *E. coli* (Wachi *et al.*, 1987). *mreB* is conserved in a wide range of bacterial species, and it has been shown that MreB in *B. subtilis* has a cytoskeletal function in bacterial cell morphogenesis (Jones *et al.*, 2001), and this finding has opened the door to research on the regulation of cell morphology through cytoskeletal proteins. In addition to MreB, *B. subtilis* has two other MreB paralogues, Mbl and MreBH (Jones *et al.*, 2001; Carballido-Lopez *et al.*, 2006), and at least two of the three MreB paralogues seem to be required to maintain the rod shape. MreB determines the length of the short axis of the cell, whereas Mbl determines the length of the long axis of the cell (Jones *et al.*, 2001). It is reported that MreB mutants affect the length of the short axis of the *E. coli* cells (Kruse *et al.*, 2003). Moreover, Mbl has been shown to be required for helical insertion of peptidoglycan (Daniel and Errington, 2003). Both MreB and Mbl form a helix or filament just beneath the cytoplasmic membrane along the long axis of the cell in *B. subtilis* (Jones *et al.*, 2001).

Penicillin-binding proteins (PBPs), including PBP2, are enzymes involved in peptidoglycan metabolism. Some of the PBPs and enzymes required for synthesis of the peptidoglycan layer have been shown to interact with the multi-complex, including MreB, MreC, and MreD (Divakaruni *et al.*, 2005, 2007; van den Ent *et al.*, 2006; Mohammadi *et al.*, 2007). The absence of these components of the complex results in abnormal cell shapes, such as round or Y shape, because the cells cannot synthesize the peptidoglycan layer and divide correctly (Young, 2003); these cells eventually die (Bendezu and de Boer, 2008). It is known that MreB and PBP2 serve to maintain the length of the short axis in *E. coli* (Den Blaauwen *et al.*, 2003; Kruse *et al.*, 2003). In fact, both MreB and PBP2 seem to have important functions in synthesis of the peptidoglycan layer in *E. coli* (Uehara and Park, 2008). A variety of proteins, including PBPs, bacterial actin homologues (MreB, Mbl, and MreBH), and their interaction partners (RodA,

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MreC, and MreD), most likely orchestrate to synthesize an intact peptidoglycan layer so that bacterial morphogenesis can continue during cell proliferation.

Results

A novel round-shaped mutant among knockouts

Escherichia coli K-12 genomic sequencing has been completed and 4288 open-reading frames are found in the genome (Hayashi *et al*, 2006; Riley *et al*, 2006). A set of precisely defined, single-gene deletions of all non-essential genes in *E. coli*, or the KEIO strains, has been systematically made, and 3985 mutants have been obtained among the genes targeted (Baba *et al*, 2006). We visually screened the KEIO strains to find mutants of interest with morphological deficiencies. Although several characterized genes showed expected phenotypes, one deletion mutant in a putative gene, JW2500, exhibited a round or oval shape (Figure 1A and B).

JW2500 has a *yfgA* gene deletion, and the gene product was estimated by the *E. coli* K-12 genomic sequence and has not been well characterized until now. We cloned the *yfgA* gene under the *trc* promoter into the plasmid vector pWM2784 with the FLAG epitope tag, yielding pDS69. The rod shape was restored by the introduction of pDS69 but not by that of the pWM2784 vector (Figure 1C and D and Supplementary Figure S1), indicating that the *yfgA* gene was responsible for maintenance of the rod shape in *E. coli*. Therefore, we named the *yfgA* gene *rodZ* as a novel determinant of rod shape of *E. coli*.

To exclude the possibility that additional mutations occurred in the mutant to suppress the *rodZ* deletion defect, the deletion mutation in JW2500 was transduced into the parental strain BW25113 carrying a plasmid (pDS111), which containing the *rodZ* gene under the arabinose-inducible promoter. The transductants (DS151) grew as rod shape in the presence of arabinose (see below). On the other hand, they showed round shape in the absence of arabinose, suggesting that any suppresser mutations were not accumulated in the *rodZ* deletion mutant. In fact, round cells of JW2500 and transductants were able to form colonies on agar plates. However, their growth was much slower than that of BW25113, and only minute colonies formed after long incubation (Figure 1E–G and data not shown). Although *rodZ* was not essential for minimal cell growth, the deletion of *rodZ* affected cell growth.

Smaller round cells in the *rodZ* deletion mutant

Quantitative analysis of the size of cells was carried out to clarify morphological phenotypes of the *rodZ* deletion mutant (Figure 2A). Shapes of rod and round cells in images were considered as ellipses, and the lengths of the major and the minor axes of cells were automatically measured by processing digital images. The length of the major axis represents the length of the long axis in a cell, and minor axis represents short axis. In a population of exponentially growing cells, cell size was distributed from the single unit cell size of a new daughter cell to longer dividing cells with constriction at the mid-cell (Supplementary Table S1). In the wild-type cells, the distribution of cell size was relatively broad. Wild-type rod-shaped cells had a ratio of the length of the longest axis to the shortest axis of 4.0. On the other hand, the ratio was almost 1.6 in the *rodZ* deletion mutant (Figure 2A and

Figure 1 Cell shape and cell growth of the *rodZ* deletion mutant. (A) Phase-contrast image of wild-type BW25113 cells. (B) Phase-contrast image of $\Delta rodZ$ mutant cells of JW2500. (C) Phase-contrast image of JW2500 harbouring the vector plasmid pWM2784. Arrowhead shows two spheres stuck together. (D) Phase contrast image of JW2500 harbouring pDS69. Scale bars indicate 5 μ m. (E) Growth of wild-type and $\Delta rodZ$ mutant on a plate incubated at 30°C for 18 h. (F) Magnified colonies of wild-type BW25113. (G) Magnified colonies of $\Delta rodZ$ mutant JW2500.

Supplementary Table S1). Almost all of the mutant cells with relatively higher ratio of the long axis versus the short axis (~ 2) were dividing, or dividing cells that looked like two spheres stuck together (shown by arrow heads in Figures 1C and 2C). The quantitative analysis indicates that the dividing cells in the *rodZ* deletion mutant are ellipsoid or round. Deletion of *rodZ* affected the lengths of both long and short

Figure 2 Characterization of cell proportions in round mutant cells. (A) Cell proportions of the $\Delta rodZ$ mutant of JW2500. The average length and s.d. of the length of the long axis is in blue, and those of the short axis is in magenta. (B) Phase-contrast image of BW25113 cells. (C) Phase-contrast image of JW2500 cells. Arrowhead shows two spheres stuck together. (D) Phase-contrast image of BW25113 cells treated with A22. (E) Phase-contrast image of BW25113 cells treated with mecillinam. (F) Comparison of cell proportions among wild-type, $\Delta rodZ$, and wild-type cells treated with A22 or mecillinam. The average length and s.d. of the major axis is in blue and that of the minor axis is in magenta. Scale bar indicates 5 μ m.

axes of the cell. However, notably the average diameter of the mutant was close to the average length of the shortest axis in the wild-type cells rather than that of the longest axis (Figure 2A and Supplementary Table SI). This result suggests that RodZ mainly affects the length of the long axis of the cell rather than that of the short axis of the cell. It is already known that several genes such as *mreB* and *pbp2* product defects result in round cells instead of rod cells. The morphology in the *rodZ* deletion mutant invited comparison of the morphology of the other round cells.

The *mreB* and *pbp2* genes as well as *rodZ* are responsible for the maintenance of rod cell shape, and *mreB* and *pbp2* are both essential for cell growth unlike *rodZ*. However, functions

of each gene product are easily inhibited by the addition of antibiotics, A22 (Iwai *et al*, 2002; Gitai *et al*, 2005) and mecillinam (Wientjes and Nanninga, 1991), respectively. A22 causes MreB to diffusely localize from the cytoplasmic membrane to the cytoplasm and the helical structure of MreB completely disappears (Karczmarek *et al*, 2007). Mecillinam is a beta-lactic antibiotic specific to PBP2 and blocks cell-wall elongation. As both antibiotics affect synthesis of the peptidoglycan layer (Uehara and Park, 2008), the cells became round or lemon shaped 2 h after the addition of either antibiotic, as reported previously (Iwai *et al*, 2002; Den Blaauwen *et al*, 2003) (Figure 2D and E). After the cells reached their maximum size after 2–3 h post-treatment, the length of the longest and the shortest axes in each round cell was measured as described above (Figure 2F and Supplementary Table SII). For both the wild-type and the *rodZ* mutant, cells with constriction at mid-cell were omitted in this analysis. As cell division was completely blocked after treatment with A22 or mecillinam, there were no elongated cell with constriction at mid-cell.

Both A22- and mecillinam-treated cells showed similar morphology in terms of shape and size. However, they are significantly larger than the *rodZ* deletion mutant (compare Figure 2B–F and Supplementary Table SII). Interestingly, the average lengths of the long axis in A22- and mecillinam-treated cells were close to the length of the longest axis of BW25113 rather than the length of the shortest axis, whereas the average diameter of the *rodZ* mutant was close to the average length of the short axis in the wild-type cells rather than that of the long axis (Figure 2F and Supplementary Table SII). These results suggest that the RodZ protein could function in a different pathway from both MreB and PBP2 to maintain the rod shape of *E. coli*. Thus, we had considered that MreB and PBP2 regulate the length of the short axis of a cell, whereas RodZ regulates the length of the long axis instead.

Regulation of the length of the long axis of a rod-shaped cell by RodZ

To test this possibility, we attempted to observe the process of reshaping cell morphology from a rod cell to a round cell. The plasmid pDS111 was able to complement the deletion mutation of *rodZ* in the presence of arabinose. After 2 h of arabinose removal, the cells still retained their rod shape in addition to cells in the presence of arabinose (Figure 3A and B and Supplementary Table SIII). In the cells harbouring pDS111, the length of the short axis was similar to that of cells grown in the presence of arabinose (Figure 3B; 2 h). After more than 5 h of depletion, the length of the long axis of cells slightly decreased, whereas the length of the short axis of cells remained constant (Figure 3B; 5.5 h). Round cells began to emerge in a population with heterogeneous shapes after 9 h incubation (Figure 3A; 9 h); the length of the long axis decreased by about 40%, whereas the length of the short axis slightly increased (Figure 3B; 9 h) (see Discussion). This result suggests that the *rodZ* gene is involved in regulation of the length of the long axis. Further incubation of the cells did not significantly affect heterogeneous cell shape, probably because of remaining gene products or leaky expression.

We next tested whether overexpression of the *rodZ* gene would elongate cell length. When the plasmid that overproduced the *rodZ* gene product under the *trc* promoter

Figure 3 Cell morphogenesis under controlling expression of RodZ. (A) Depletion of RodZ in DS151, which harbours pDS111. Cells were grown in the presence of 0.002% arabinose overnight at 30°C. Cells were diluted into fresh L supplemented with (+) or without (-) 0.002% arabinose (time 0). (B) Cell proportions were measured at the indicated time points. The major axis is in blue and the minor axis is in magenta. (C) Overproduction of RodZ in wild-type BW25113 carrying either pDSW208F or pDS63 (FLAG-RodZ). RodZ was induced by 1 mM IPTG. (D) Cell proportions were measured at the indicated time points. The major axis is in blue and the minor axis is in magenta. (E) Restoration of rod-shaped DS151 cells by RodZ. DS151 cells were grown in the presence of 0.1% glucose overnight. Cells were diluted into fresh L supplemented with 0.2% arabinose (time 0), incubated and observed at the indicated time points. Scale bars indicate 5 μm.

was transformed into the *rodZ* deletion mutant, the length of the long axis was extended to some degree (Figure 3C and D, and Supplementary Table SIV). However, the length of the short axis remained nearly constant. Obviously, the overproduction of RodZ by the addition of the inducer IPTG resulted in an increase in the length of the long axis instead of the short axis. Cell division was not inhibited by overproduction of RodZ because doubling time was not significantly different between these strains (Supplementary Table SIV). These results suggest that the amount of RodZ might correlate with the length of the long axis to some extent.

To further confirm that *rodZ* regulates the length of the long axis of a cell, we observed how round-shaped cells restored their rod shape after the *rodZ* gene was expressed in deletion mutant cells (Figure 3E). The *rodZ* deletion mutant carrying pDS111 (DS151) was grown in the presence of glucose overnight. Round cells were diluted in fresh L medium, including arabinose (time 0). Cell tips started to elongate after incubation for 2.5 h, and the round cells became filamentous. Rod-like cells were emerged at 4 h, and then almost all the cells exhibited a rod shape after incubation for 5.5 h. This observation also suggests that *rodZ* can regulate the elongation of a cell along the long axis of the *E. coli*.

The RodZ protein spanning the cell membrane

Bioinformatic analysis indicated that the *rodZ* gene encodes a polypeptide of 337 amino acids of 35.9 kDa of an estimated molecular weight, and is predicted to have a single trans-membrane domain (residues 111–133) (Newitt *et al*, 1999). To

Figure 4 Subcellular localization of RodZ. (A) Immunoblotting analysis of RodZ in cell fractionations. T, total lysates; S, soluble fractions; IM, inner-membrane fractions; OM, outer-membrane fractions. (B) PhoA fusion assay. TH1276 (*ΔphoA*) cells producing RodZ, PhoA, PhoA_{ΔSS} or RodZ-PhoA_{ΔSS} were streaked onto L supplemented with 50 μg ml⁻¹ BCIP. The plate was incubated at 30°C for 18 h.

confirm the subcellular localization of RodZ, cells producing FLAG-tagged RodZ were fractionated into soluble, inner (cytoplasmic) membrane and outer membrane fractions. As

seen in Figure 4A, FLAG-tagged RodZ was fractionated into the cytoplasmic membrane fraction. The RodZ protein migrated as a significantly larger species in SDS–PAGE gels. Possibly, the FLAG epitope affected the mobility of the RodZ protein. However, the RodZ protein migrated as a larger species even if a His tag or GFP was fused (data not shown). Similar aberrant migrations in SDS–PAGE are occasionally seen in case of the *E. coli* membrane proteins such as ZipA (Hale and de Boer, 1997).

We have investigated the topology of RodZ in the cytoplasmic membrane by the PhoA fusion assay (Figure 4B). In the assay, PhoA is active only when it locates in the periplasm, and the activity can be detected by the colour using BCIP, a chromogenic substrate of alkaline phosphatase (Manoil and Beckwith, 1985). Cells producing PhoA formed blue colonies on L plate supplemented with BCIP; in contrast, cells producing a mutant PhoA, which lacks the signal sequence (Δ ss) thereby unable to be transported to the periplasm, formed white colonies on the same plate (Figure 4B). Cells producing RodZ or PhoA Δ ss did not show blue colonies, but when the PhoA Δ ss was fused at the C terminus of RodZ (RodZ–PhoA Δ ss) cells formed blue colonies on the same plate (Figure 4B). These results indicate that the C terminus of RodZ was exposed to the periplasmic domain. The result is much consistent with that of fluorescent detection of the fluorescent protein-tagged RodZ fusion proteins (Supplementary Figure S2). The GFP-tagged RodZ at the N terminus (GFP–RodZ) was detected at cell periphery, but GFP-tagged RodZ at the C-terminus (RodZ–GFP) was not detected. However, the mCherry-tagged RodZ at the C terminus (RodZ–mCherry) was detected at cell periphery. Whereas mCherry can be folded properly at the periplasm (Pradel *et al*, 2007), GFP is improperly folded outside of the cytoplasm (Feilmeier *et al*, 2000). It should be noted that GFP–RodZ, RodZ–GFP, and RodZ–mCherry could restore the cell shape defect of JW2500 cells (Supplementary Figure S2).

Formation of the MreB cytoskeletal filament in the *rodZ* round mutant

It is possible that RodZ functions as a transcriptional regulator of genes critical to rod-shape determination such as *mreB*, as the N-terminal domain of the RodZ protein is in the cytoplasm and has a helix-turn-helix motif classified as XRE family transcriptional regulator, which is conserved among eukaryote, archaea, and bacteriophages and is similar to that of the well-characterized Cro protein of λ phage (Luscombe *et al*, 2000). To test this possibility, we examined the MreB protein levels in wild-type and the *rodZ* deletion strains. Western blot of whole-cell lysate revealed that the protein levels of MreB were not affected by the absence of RodZ in two different background strains (Figure 5A). These results suggest that RodZ is unlikely a transcription factor of the *mreB* gene.

Furthermore, we tested whether the helical filaments of MreB were dependent on RodZ or not. When the GFP-tagged MreB at the N terminus (GFP–MreB) was produced in the *rodZ* deletion mutant JW2500, several fibres of GFP–MreB were visible and several spots were scattered on the cell periphery (Figure 5B and Supplementary Figure S3). These structures were continuous with changing their intensities in image sections along the *z* axis of a microscope stage (Supplementary Figure S3). These results suggest that these

filaments are actually helical structures under the membrane, probably identical to the ones in wild-type cell, with the exception that they now have to conform to the spherical shape instead of the rod shape. Moreover, these GFP–MreB spots and filaments were made to disappear by the addition of A22 into the medium (Figure 5C). The results suggest that the GFP–MreB complex was assembled in the RodZ mutant.

Helical structure of RodZ along the long axis of the cell

To examine the subcellular localization of RodZ, we have constructed fusion genes of *rodZ* with GFP or mCherry. JW2500 harbouring the plasmids expressing the GFP–RodZ in the *rodZ* mutant cells restored the cell-shape defect (Figure 5D and Supplementary Figures S1 and S2), indicating that the fusion protein retained enough physiological activity to retain the rod shapes. Optical dissection of single cell was carried out, and the resulting series of GFP–RodZ images was processed by a deconvolution algorithm to remove out-of-focus fluorescence. Deconvolved GFP–RodZ showed significant fluorescent signals at the cell periphery (Figure 5D and Supplementary Figure S2), which corresponded to the above result that RodZ was enriched in the cytoplasmic membrane fraction (Figure 4A). As the observation that several spots of GFP-surrounded rod cells and faint fibres extended across the cell was similar to images of the MreB cytoskeletal protein, we examined three-dimensional localization patterns of RodZ. In three-dimensional images reconstituted in Figure 5E, RodZ seemingly formed helical filaments along the long axis of the cell. As the RodZ structure was quite similar to the MreB filaments, we further examined whether the RodZ helical filaments were dependent on the MreB filaments.

Although we showed that MreB assembled independently of RodZ in a cell (Figure 5B), structures of these filaments were very similar to each other. We then compared MreB and RodZ filaments in a single cell. GFP–MreB and RodZ–mCherry were produced in JW2500 (Δ rodZ) cells. Distributions of both filaments were well overlapped in the cells, suggesting that MreB and RodZ colocalized in the cell (Figure 5F left). A22 was added into the medium of cells producing GFP–MreB and RodZ–mCherry. GFP–MreB was diffuse in the cytoplasm after 1 h of the addition of A22, whereas RodZ–mCherry still formed filaments in the cell (Figure 5F, right). This result strengthened the implication that maintaining of both filaments can be independent of each other. After 2 h of the addition of A22, cells became round shaped because the MreB filaments were disassembled. However, the RodZ filaments still formed in round cells treated with A22 (Figure 5G and Supplementary Figure S4A). The fluorescent spots were distributed at cell periphery and irregular fibers were visible within the round cells. In addition, a similar result was obtained in round cells that were treated with mecillinam (Figure 5H and Supplementary Figure S4B). These results indicate that MreB and PBP2 are not required to maintain RodZ filaments that were already formed before MreB and PBP2 activity was inhibited.

We also examined the localization of RodZ in cells depleted of FtsZ, which is a bacterial tubulin and responsible for formation of a constriction ring. As FtsZ needs to form a ring-like structure known as the Z-ring at division site, depletion of FtsZ caused inhibition of cell division, so the

Figure 5 Cytoskeletal protein structures in round cells. (A) Immunoblotting analysis of MreB using anti-MreB antibody. BW25113 (WT), JW2500 (BW25113: $\Delta rodZ$), PA340 (WT), PA340-678 (PA340: $\Delta mreBCD$), and DS165 (PA340: $\Delta rodZ$). Arrowhead indicates MreB protein. The upper band of the MreB protein shows a non-specific protein that cross-reacts with anti-MreB antibody. (B) Fluorescent image of GFP-MreB in JW2500. (C) Fluorescent image of GFP-MreB in JW2500 after the addition of A22. (D) Sectional fluorescent images of GFP-RodZ in JW2500. (E) Three-dimensional reconstruction of GFP-RodZ on the basis of sectional fluorescent images. (F) Fluorescent images of RodZ-mCherry and GFP-MreB in a single cell treated with none (left) or A22 (right). (G) Fluorescent image of GFP-RodZ in BW25113 treated with A22. (H) Fluorescent image of GFP-RodZ in BW25113 treated with mecillinam. (I) Fluorescent image of GFP-RodZ in WM2767 in the absence of sodium salicylate, which is needed to express *ftsZ*. (Inset) Enlarged fluorescent image of GFP-RodZ in WM2767. Scale bars indicate 2 μ m.

cells became filamentous. Nevertheless, a number of fluorescent spots surrounded the long filamentous cells, and RodZ helices were easily detected (Figure 5I). These results indicate that the RodZ helix was formed independently of two cytoskeletal proteins, the actin homologue MreB and the tubulin homologue FtsZ, and another rod-shape determinant, PBP2. We have already shown that the MreB filament was formed in the $\Delta rodZ$ strain and this mutant was able to divide at mid-cell, therefore formations of the MreB helix and the Z-ring are most likely independent of RodZ.

Requirement of the cytoplasmic domain for the RodZ helical structure

To reveal the molecular mechanism by which the RodZ protein regulates cell morphology, we have carried out molecular anatomy of the RodZ protein on the basis of its predicted amino-acid sequence. Bioinformatic analysis indicated that the 337 amino acids of the RodZ polypeptide should consist of three parts: a cytoplasmic domain, including the HTH motif (residues 1–110), a transmembrane domain (residues 111–133), and a large periplasmic domain (residue 134–337) (Newitt *et al*, 1999). A series of deletion mutant of GFP-RodZ have been constructed on purpose to

determine the region required for the helical formation of RodZ (Figure 6A). Among them, four truncated GFP-RodZ proteins with deleted C-terminal residues were able to form similar filaments to the full-length GFP-RodZ: GFP-RodZ_{1–252}, GFP-RodZ_{1–204}, GFP-RodZ_{1–155}, and GFP-RodZ_{1–142} (Figure 6A). An image of the helical filaments of GFP-RodZ_{1–142}, which are representative, is shown in Figure 6B. We also made truncated GFP-RodZ proteins, GFP-RodZ_{1–111} and GFP-RodZ_{1–48}, which completely lacked both the periplasmic domain and the transmembrane domain. If the cytoplasmic domain, including the HTH motif, is sufficient for interaction with normal RodZ proteins or other proteins required for assembly, the truncated proteins would form the helical filaments in the wild-type (*rodZ*⁺) cells. Both the GFP-RodZ_{1–111} and GFP-RodZ_{1–48} were diffused within the whole cytoplasm in the wild-type cells as observed in the *rodZ* deleted mutant (Figure 6A and B and data not shown). These results suggest that the HTH motif is not sufficient for assembly of the truncated proteins and the full proteins. It is known that the GFP tag can interfere with protein interactions in some cases. We then tested how deletion of the HTH motif affected the helical formation of the mutants of GFP-RodZ (Figure 6A).

Figure 6 Functionality of RodZ and its deletion mutants. (A) Schematic illustrations of RodZ and its deletion mutants and summary of their localization and complementations. Black and green rectangles indicate the transmembrane (TM) and the helix-turn-helix domains of RodZ, respectively. Pink and purple rectangles indicate the transmembrane (MalF_{TM1}) and the periplasmic domains of MalF. Localization of GFP-RodZ and cell shape were observed in JW2500 harbouring pDS62 (GFP-RodZ) or in its derivatives that encoded truncated GFP-RodZ. In the 'Localization' column: H, helix; Dm, diffuse in membrane; Dc, diffuse in cytoplasm. In the 'Shape' column: NR, normal rod; FR, fatter rod; SFR, shorter and fatter rod; S, sphere. Growth defect was measured by colony size on L plate incubated at 30°C for 20 h. In the 'Growth' column: +, large colonies; -, small colonies; +/-, medium colonies. (B) Localization of GFP-RodZ deletion mutants in the indicated strain. (C) Morphology of cells expressing various RodZ mutants in JW2500. Scale bar indicates 5 μ m.

The following RodZ proteins with deleted N-terminal residues were constructed: GFP-RodZ_{49–337}, GFP-RodZ_{76–337}, and GFP-RodZ_{104–337}. All three truncated proteins lacking the HTH motif lost the ability to form filaments, but were still on the periphery of cells (Figure 6A and B and data not shown). These results strongly indicate that the N-terminal 48 amino acids, including the HTH motif, are needed for formation of the RodZ helix. As shown above, the N-terminal 48 amino acids or the cytoplasmic domain (residues 1–111) were not sufficient to form the helix. As RodZ is a cytoplasmic membrane protein, it is likely that the transmembrane domain is crucial for proper assembly of RodZ helical filaments. We then fused GFP-RodZ_{1–48} and GFP-RodZ_{1–111} with the transmembrane domain 1 of the MalF protein (MalF_{TM1}). MalF is a component of the maltose transporter but is not a determinant of cell shape. MalF_{TM1} has been used previously to analyse function of the transmembrane domain of FtsQ (Guzman *et al*, 1997) or the amphipathic helix of FtsA (Shiomi and Margolin, 2008). GFP-RodZ_{1–48}-MalF_{TM1} localized at the cell periphery, but did not form a helix (data not shown). However, GFP-RodZ_{1–111}-MalF_{TM1} formed the helix in both wild-type and JW2500 cells (Figure 6A and B). These results indicate that the cytoplasmic domain of RodZ is sufficient for the RodZ-helix formation when the domain is anchored to the inner membrane.

Domains sufficient for maintenance of the rod shape

We revealed that the cytoplasmic domain of the RodZ is required for the formation of the helical filaments. However, the helical filaments of GFP-RodZ_{1–111}-MalF_{TM1} were not able to complement spherical shape and slower growth rate of the *rodZ*-deleted cells (Figure 6A and C). As helical formation might not be sufficient for maintenance of the rod shape, we next focused on domains that are responsible for cell morphology. Cells producing GFP-RodZ_{1–252}, GFP-RodZ_{1–204}, and GFP-RodZ_{1–155} exhibited normal rod shapes and adequate cell growth, as did GFP-RodZ (full length) (Figure 6A–C). These truncated RodZ proteins also formed the helical structure, indicating that they retained almost full activity of RodZ. However, cells producing GFP-RodZ_{1–155} or GFP-RodZ_{1–142}, which formed a helix in JW2500, showed slightly ‘fatter’ or ‘shorter and fatter’ rods than the wild-type cells and had slower growth, respectively (Figure 6A and C). These results suggest that a very small portion (residues 134–155) of the periplasmic domain of RodZ contributes to maintenance of the normal rod shape. Similarly, a series of N-terminal deletions, including GFP-RodZ_{49–337}, GFP-RodZ_{76–337}, and GFP-RodZ_{104–337} also showed slightly ‘fatter’ and ‘shorter and fatter’ rod cells, respectively (Figure 6A and C). These results suggest that helix formation contributes to maintain the normal rod shape. GFP-RodZ_{1–111}-MalF_{TM1} and GFP-RodZ_{1–48}-MalF_{TM1} did not complement all of the phenotypes of JW2500 (Figure 6A–C). Interestingly, in spite of almost the same topology of RodZ_{1–142} with RodZ_{1–111}-MalF_{TM1}, the latter did not allow JW2500 cells to be rod shaped at all, although it formed filamentous structure in the cells. These results suggest that domains required for the maintenance of rod shape and formation of the RodZ helical structure can be separated into at least two domains: the vicinity of the transmembrane domain (RodZ_{136–155}) and the HTH domain. The transme-

brane domain might be involved in both functions of the two domains.

Discussion

Our study provides a new insight into the mechanism of maintenance of cell shape in the rod-shaped bacterium, *E. coli*, and points to a possible function of the *rodZ* gene in bacterial cell morphogenesis.

Two axes determine the rod shape

A spherical cell shape would be a prototype for bacterial cell morphology. To maintain a rod shape, many genes are involved in the synthesis of the peptidoglycan layer, which is the primary determinant of the cell shape. In fact, the combined action of two cytoskeletal proteins maintain the rod shape of *B. subtilis*: MreB is responsible for cell width regulation and Mbl is responsible for linear axis regulation (Jones *et al*, 2001). This means that controlling the lengths of the long and the short axes of a rod cell is an elementary system for maintaining rod shape (Figure 7A). In contrast, a Gram-negative bacterium, *E. coli*, has only one cytoskeletal protein that is homologous to *B. subtilis*, MreB (Jones *et al*, 2001; Soufo and Graumann, 2003; Carballido-Lopez *et al*, 2006). One question that arises here is whether the lengths of both the long and short axes in the *E. coli* cells are controlled as well. Similar to *B. subtilis* MreB, it has been proposed that *E. coli* MreB also has an important function in the regulation of the short axis of the cell (Kruse *et al*, 2003). If so, it would be expected that another cytoskeletal protein is involved in the regulation of cell length. Indeed, our data support the elementary system that controls both lengths of the long and short axes in *E. coli* (Figure 7A). The *rodZ* deletion made the cells become shorter and fatter (Figure 2A and F), and the cells formed round or oval shape (Figure 2C). In addition, depletion of RodZ mainly affected the longer length of the cell, although the cell length along the short axis of the cell slightly increased (Figure 3A and B). On the other hand, inactivation of the MreB assembly by the addition of A22 caused the cells to become relatively large and round presumably because they still maintained the long axis control (Figure 2D and F). Although RodZ does not have similarities to the bacterial actin MreB, RodZ showed subcellular localization that was similar to the MreB cytoskeletal protein as helical filaments. Therefore, it is likely that RodZ is a new cytoskeletal element involved in cell size control through regulation of the length of the long axis (Figure 7A). The length of the short axis in cells is also slightly affected by RodZ, but this might be an indirect effect of loss of function of RodZ. When the amount of peptidoglycan and the surface area in $\Delta rodZ$ cells is left the same as in wild-type cells after losing the cell shape of $\Delta rodZ$ cells by shortening the length of the long axis, $\Delta rodZ$ cells need to slightly increase the length of the short axis of cells to keep the surface area same as wild-type cells. This is because cell volume of the $\Delta rodZ$ round cells was about 1.5 times larger than that of wild-type cells, which was calculated using average length of the long and short axes of the cells in Figure 2A.

Mutations in the *pbpA* gene cause cells to assume a spherical shape. The *pbpA* gene encodes PBP2, which is required for lateral peptidoglycan synthesis (Den Blaauwen

Figure 7 Schematic diagram of defects in cell morphogenesis and function of RodZ. **(A)** RodZ (blue) and MreB (black) form regular helical filaments along the long axis of the cell to maintain the lengths of the long and short axes, respectively. A defect in maintaining the long axis results in smaller round cells (top). On the other hand, a defect in maintaining the short axis or strength of the horizontal cell wall results in a larger round cell (bottom). For simplicity, the irregular MreB and RodZ filaments shown in Figure 5G and H were omitted in *rodZ*⁻ or *mreB*⁻ or *pbpA*⁻ cells. Thus, *rodZ* mutant becomes a smaller round cell, and both *mreB* and *pbpA* mutants become larger round cells. **(B)** RodZ is a bitopic membrane protein, and the C-terminal domain (orange) in the periplasm can interact with a factor(s) that is involved in peptidoglycan synthesis. The N-terminal domain (blue), including the HTH motif, can interact with another RodZ molecule, MreB, and/or other molecules to form a helical structure.

et al, 2003). The round *pbpA* mutants are able to grow at a normal rate despite the increased cell volume, which is about 4–6 times larger than those of the rod cells. However, it is known that the average length of the rod mutants or the cell radius is almost identical to the length of the longer axis of the rod cells (Donachie and Begg, 1989). This suggests that inactivation of PBP2 causes cells to swell while maintaining their lateral length because of a weakening of the cylindrical surface. Additionally, PBP2 is required for the

maintenance of the diameter of the new cell pole created after cell division (Den Blaauwen *et al*, 2003). These reports are consistent with our data obtained by inactivation of PBP2 (Figure 2E and F), and provide further evidence of the effect of PBP2 disruption on maintenance of the length of the short axis.

Considerable amounts of the RodZ proteins affect the cell length of the long axis (Figure 3C and D), and the length of the RodZ helix corresponds to the regular cell length of *E. coli*. During recovery from the round cell to the rod cell by the restoration of RodZ, a peculiar shape of cells emerged (Figure 3E). As cells with similar shapes are observed in mutants of PBPs (Young, 2003), the peptidoglycan layer that envelops the round cells might be reorganized to reform the rod cell shape. A direct or indirect relationship between RodZ helical filaments and the peptidoglycan synthesis machinery is an unresolved and interesting problem in the study of cell-shape maintenance.

Diversity of RodZ in γ -proteobacteria

MreB homologues are distributed in a wide range of bacterial species relative to the cell shape (Jones *et al*, 2001). At least one homologue is found in almost all genomes of rod-shaped or more complex-shaped organisms. Species in γ -proteobacteria, such as *E. coli* and *Vibrio cholerae*, have one homologue of MreB. The *rodZ* gene is also well conserved mainly in these organisms (Supplementary Figure S5). Although we could not find the *rodZ* gene in a genome database of *Caulobacter crescentus*, which is a well-characterized rod-shaped α -proteobacterium, it does have a *rodZ* homologue that is important for rod-shape determination (Christine Jacobs-Wagner, personal communication). It seems that the *rodZ* gene was acquired to maintain the rod shape in the last common ancestor of γ -proteobacteria.

Spirals of cytoskeletal proteins to maintain the rod shape

RodZ was embedded in the cytoplasmic membrane and distributed in a helical array surrounding the rod cell. Similar to RodZ, the Sec protein translocation machinery and a chemoreceptor are also arranged into a helical array in cytoplasmic membrane (Shiomu *et al*, 2006). The helical distribution of the chemoreceptor is coincident with the distribution of the Sec protein translocation machinery, which appears distinct from the MreB helix. On the other hand, the RodZ helix was seemingly colocalized with the MreB helix. It is unclear whether direct or indirect interactions between RodZ and MreB are responsible for different functions in maintaining the rod shape or helps to maintain the helical structure enclosing the rod cell. de Boer and colleagues independently isolated a round-shaped mutant of *rodZ* and showed that the RodZ protein was required for proper formation of MreB spirals (Piet de Boer, personal communication). Regardless of irregular arrangement of the spirals, self-assembly of MreB helical filaments was maintained in RodZ deletion mutants (Figure 5B). It is also reported that the purified MreB protein of *Thermotoga maritima* can be assembled into filamentous bundles in the presence of ATP without any other proteins *in vitro* (van den Ent *et al*, 2001; Esue *et al*, 2005). Similarly, RodZ filaments were maintained

in round cells treated with A22 or mecillinam (Figure 5F–H). Alternatively, it is possible that each cytoskeletal protein is independently assembled and then colocalized with each other to regulate the peptidoglycan synthesis for maintaining rod shape. It is likely that MreB is not required to maintain the RodZ filaments in a cell.

FtsZ has GTPase activity, and other bacterial cytoskeletal proteins including, MreB, ParA, and SopA, are ATPases. The binding of these cytoskeletal proteins for ATP or GTP is crucial for the self-assembly of filaments in the cell. However, RodZ does not have an ATPase domain or ATP-binding site. It has been known that bacterial intermediate filament homologue, Crescentin, which is required for the helical shape of *C. crescentus*, has the ability to form filaments in the absence of ATP or GTP (Ausmees *et al*, 2003). Therefore, it is plausible that RodZ forms filaments in the absence of ATP or GTP. Alternatively, it is possible that other cytoplasmic protein(s) helps to localize RodZ in a helical array.

Surprisingly, the helical structure of RodZ was not essential for determining rod shape in *E. coli* because the RodZ_{49–337} and RodZ_{104–337} truncation mutants showed ‘fatter’ or ‘shorter and fatter’ rod cells, respectively, even though these proteins did not form spirals (Figure 6A). The helical structure could be required to accurately control the lengths of the long and short axes of the cell to form a unit-sized rod cell. The length of the helix might correspond to the lateral cell length so that the amount of the RodZ protein affects the cell length. The dimensions of bacterial cells are easily variable according to nutrient conditions, or the growth circumstances. It is possible that the change of cell size is related to the production level of RodZ proteins.

A bifunctional protein with bitopic topology

RodZ can be divided into at least two functional domains joining by the transmembrane domain (Figure 7B). We found that the cytoplasmic domain of RodZ is responsible for the formation of the helix when anchored to the membrane, whereas the transmembrane domain and its proximity to the periplasm are responsible for the formation of the rod shape of the cell. A helix-turn-helix (HTH) motif in the cytoplasmic domain of the N terminus was involved in the formation of spirals to maintain the rigid rod shape. In general, the HTH motif is known as a typical DNA-binding domain found in a variety of transcription factors. However, the protein level of MreB was not markedly affected in the *rodZ* deletion mutant. Instead, the HTH motif of RodZ may contribute to protein–protein interactions to form the RodZ helix, which is localized beneath the cytoplasmic membrane. Our data do not support a model that a direct interaction of MreB with RodZ must occur for the formation of the RodZ spirals. Nevertheless, it is an attractive possibility that MreB spirals are associated with the RodZ spirals.

The C-terminal domain of RodZ is exposed in the periplasm and has no characteristic domain structure. The C terminus of RodZ may be critical for determination of the rod shape in *E. coli*. It is assumed that this periplasmic domain interacts with enzymes required for synthesis of the peptidoglycan layer, including PBPs in the periplasm. Therefore, the helical structure of RodZ may affect the

formation of the cylindrical peptidoglycan cell envelope across the cell membrane.

The present findings suggest that RodZ directs the lateral length of the cylindrical peptidoglycan cell envelope. Perhaps the spatial information on the lateral length determined by cytoskeletal proteins in the cytoplasm mediate the directed localization of the peptidoglycan synthesis machinery in the periplasm through the RodZ membrane protein. Further analysis of RodZ should reveal which proteins are involved in determining the spatial information of cell morphology and how this information is transferred into peptidoglycan synthesis.

Materials and methods

Bacterial strains and plasmids

All of bacteria used in this study are listed in Supplementary Table SV. All plasmids used in this study are listed in Supplementary Table SV.

Microscopy and optical sectioning

Cells were grown at 30 or 37°C in L broth overnight. The culture was diluted in L broth and exponentially grown at 30 or 37°C. The cells were observed under an epifluorescence microscope (Nikon, E800). Optical sectioning experiments were performed using an Olympus IX70 microscope with a PlanApo X100 1.40 oil-immersion objective lens and the DeltaVision system (Applied Precision). Sectioning images were captured along the z axis at 0.2 µm intervals and treated with a deconvolution algorithm.

Measuring cell proportion

Cells were exponentially grown in L broth, and living cells were photographed by an ORCA-II CCD camera (Hamamatsu) under a phase contrast microscope. Digital images were processed by the software Metamorph and changed into binary images to detect cell outlines. Each cell in the binary images was automatically measured for the length of the longest line (the long axis of the cell) and maximum breadth perpendicular to the longest line (the short axis of the cell).

Immunoblotting

Immunoblotting was performed with blots containing equal amounts of cells per lane, probing with anti-FLAG antibody (M2 antibody, Sigma) or anti-MreB antibody, which was provided by W Margolin.

Cell fractionation

BW25113 carrying pWM2784 (FLAG-GFP) or pDS69 (FLAG-RodZ) were grown in the presence of 1 mM IPTG, harvested and resuspended in Buffer A (50 mM Tris–HCl, pH 7.4). Cell fractionation was carried out according to a previously reported procedure (Kruse *et al*, 2005). Fractions were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting using anti-FLAG (M2) antibody.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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