CELL SHAPE DETERMINATION IN ESCHERICHIA COLI

by

FELIPE O. BENDEZÚ

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Advisor: Piet de Boer, PhD.

Department of Molecular Biology and Microbiology
CASE WESTERN RESERVE UNIVERSITY

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CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of

____________________Felipe O. Bendezú______________________________

candidate for the _______Doctor of Philosophy_________________________degree *.

(signed)_______Erik Andrulis________________________________________
(chair of the committee)

_______Piet de Boer________________________________________

_______Patrick Viollier________________________________________

_______Pieter de Haseth________________________________________

_______Arne Rietsch________________________________________

(date) __June 10, 2008__________________________

*We also certify that written approval has been obtained for any proprietary material contained therein.
This work is dedicated in loving memory to my mother-in-law, Judy “Juju” Hilyard (1954-2005), who accepted and cared for me as a son and to my loving parents, Eve and Rafael Bendezú, whose generous and unwavering support made it possible for me to complete this work
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Cell Shape Determination in \textit{Escherichia coli}

Abstract

by

FELIPE O. BENDEZÚ

The load-bearing exoskeleton, known as the murein sacculus, is believed to impart the shape of bacterial cells. The chemical composition and enzymes responsible for building this bag-like structure are fairly well understood. What is lacking, however, is a detailed understanding of how the bacterial cell coordinates the spatio-temporal synthesis of the sacculus to achieve a complex shape. It is now clear that many genera of bacteria use the cytoskeletal actin protein MreB to accomplish this. However, the precise mechanism by which MreB actin contributes to complex cell shape determination is not known. A popular model states that MreB spiral-like cyto-filaments serve as a track to direct the placement of new murein material. In the well-studied bacterium \textit{Escherichia coli}, MreB is one of five known proteins needed for rod-shape growth, with the others being MreC, MreD, PBP2 and RodA. How loss of one or more of these proteins affects cell physiology has been a confusing issue. In attempts to bring clarity into this field of research, I created a defined and comprehensive set of shape mutants and analyzed their phenotypes. These studies showed that all five are only conditionally essential. Cells lacking one or more of these proteins were able to survive when grown under conditions of slow
mass increase or when the essential division protein FtsZ is over-expressed. Intriguingly, when grown under conditions of fast mass increase, mutant cells grow into giant spheres that are unable to regulate important cellular processes and eventually die. These spheres accumulate excess membrane in their interior that compete with the cell membrane for essential division proteins and I propose that this contributes significantly to the lethal division defect under non-permissive conditions. I used the fact that extra FtsZ can rescue life to shape-defective mutants as the basis for a genetic screen to identify additional shape mutants. This identified an uncharacterized membrane protein we named RodZ. Cells lacking RodZ display an impressive set of media and temperature-influenced shape defects. Also, using the first functional fluorescent fusion protein to MreB, I was able to show that RodZ is essential for proper MreB assembly.
Chapter 1

Introduction
Introduction

How a cell determines and maintains its shape is a fundamental question in biology. In eukaryotic organisms, cytoskeletal elements including actin, tubulin, and intermediate filaments play an integral role in cell shape maintenance, among other functions. In contrast, the cell shape of prokaryotic organisms has long been believed to be determined by the load-bearing exoskeleton composed of the amino-sugar peptidoglycan, or murein. As will be discussed in more detail below, the murein exoskeleton is indeed a primary determinant of cell shape. However, it is now clear that bacteria encode homologues of all three eukaryotic cytoskeletal elements that contribute intimately to cell morphogenesis.

Cell shape and the murein sacculus

Many genera of bacteria encounter environmental conditions that can vary in temperature, nutrients, pH, and allo-tonic surroundings. To ensure survival in spite of these often harsh conditions, bacteria have evolved, among other systems, an exoskeleton that aids in resisting intracellular turgor pressure which can exceed 75 lb/in² (101). The exoskeleton, known as the murein sacculus, is essentially a tight fabric of covalently linked glyco-peptide strands (reviewed in (87)). The glycan strands are composed of the alternating amino-sugars N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) linked by β 1-4 glycosidic bonds (Fig.1-1). The presence of a lactyl group on NAM molecules allows for the attachment of short peptides, which have several important features. In
Escherichia coli, (E. coli) the peptides are primarily composed of five amino acids including D isomers unique to murein (87). This is thought to restrict undesired proteolysis of murein by non-specific peptidases. Furthermore, the peptides contain a di-basic amino acid, which in E. coli is meso-diaminopimelic acid, an intermediate in the lysine biosynthetic pathway (87). This feature allows for the cross-linking of the amino-glycan strands through peptide bonds. The result is essentially a large covalently linked molecule that forms a bag-like net enveloping the cell (Fig.1-1).

The murein sacculus is believed to be the major determinant of cell shape due to the following observations. First, it is possible to isolate intact sacculi from bacteria that, remarkably, still retain the overall shape of the cell from which it was isolated (87). Second, the degradation of the murein sacculus by treatment with lysozyme, an enzyme that cleaves the glycosidic bond between NAG and NAM, results in a rapid loss of rod-shape. If maintained in isotonic medium, lysozyme treated cells will retain a stable spherical shape. Accordingly, the key to understanding cell shape determination in bacteria will likely require a detailed knowledge of the factors governing synthesis of the murein sacculus.

The life cycle of Escherichia coli: the sacculus perspective

E. coli is an excellent model organism for studying several aspects of cell biology. E. coli are unicellular haploid bacteria whose genetic information is held in a single circular chromosome. The life cycle of E. coli can be thought of a process that has to overcome several challenges. The chromosome must be
replicated and the cell must double in size and divide in a manner to ensure equal inheritance of the genetic information (165). The morphological changes associated with the life cycle of *E. coli* have been well established. A young daughter cell with a rod shape first elongates along the long axis of the cell maintaining a constant diameter throughout the length of the cylinder. When the cell has doubled in length, a cytokinetic septum is formed at mid cell and division is executed giving rise to two cells that are morphologically identical to the parent cell (149) (Fig.1-2).

As discussed above, the murein sacculus is the defining morphological factor of the cell. The shape of a cell at any point in the life cycle can therefore be thought of as being determined by the spatial and temporal synthesis of the sacculus. Under this murein-centric model for shape determination, the maintenance of rod-shape can further be considered to involve two separate phases of growth; cylindrical murein synthesis (CMS) and septal murein synthesis (SMS) (Fig.1-2). The two phases can be visualized by labeling nascent murein during progression of the cell cycle (46, 47).

Accordingly, cells deficient in CMS or SMS could be considered defective in proper shape maintenance. However, several lines of evidence suggest that CMS and SMS are executed by two separate systems that share a common murein precursor. The inability to carry out SMS results in a failure to divide but does not affect the cells ability to elongate by CMS and maintain a constant diameter (63) (Fig.1-2). The result is cylindrical growth without division and the formation of long filamentous cells. Cells unable to execute CMS can no longer
maintain a constant diameter along their cylinder and are therefore widely considered to be truly shape-defective (Fig.1-2).

**Synthesis of the murein sacculus**

Synthesis of the murein sacculus can be thought of as a multi-step pathway that requires the cell to; (i) generate precursor molecules, including NAM, NAG, and peptides, (ii) transport precursors across the inner membrane to the periplasmic space, and (iii) incorporate precursors into the existing sacculus. This multi-step pathway involves the action of multiple gene products and three cellular compartments; the cytoplasm, the inner membrane and periplasm. Each step in the pathway will be reviewed briefly with relevant proteins involved in each step listed in brackets.

**Generation of murein substrate**

The first step in the pathway entails the production of the final substrate used in the polymerization reactions, a lipid-linked disaccharide pentapeptide known as lipid II (Fig.1-3). The production of this substrate involves three groups of reactions; formation of UDP-NAG, the step-wise attachment of the pentapeptide to UDP-NAG, and formation of the lipid-linked substrate. The first committed step in this pathway is the two-step formation of UDP-NAM from UDP-NAG [MurA and MurB] (6, 25, 144). This is followed by the sequential addition of the five amino acids by specific and dedicated ligases. Two of these include amino acids unique to murein; D-glutamate [MurI] (56, 57), and D-alanyl-D-alanine [DdIA, DdIB]
The order of addition is L-alanine [MurC] (91, 112), D-glutamate via a γ-D bond [MurD] (123, 143), meso-diaminopimelic acid [MurE] (127, 128), and finally D-alanyl-D-alanine [MurF] (141). The resulting NAG-pentapeptide is attached to the membrane anchoring lipid undecaprenyl phosphate (also known as bactoprenol), giving rise to NAM(pentapeptide)-pyrophosphoryl-undecaprenol (lipid I) [MraY] (90). Undecaprenol is a C55 prenyl-derived molecule that is made via the non-mevalonate pathway of isoprenoid synthesis (7, 62). The final step in the synthesis of the murein precursor is attachment of NAG to lipid I [MurG] (124, 125), yielding NAG-NAM(pentapeptide)-pyrophosphoryl-undecaprenol (lipid II).

**Transport of the murein substrate**

The second step in the synthesis of murein requires the cell to transport the lipid II precursor across the cell membrane to the periplasmic space. This is clearly a crucial step in murein synthesis as it requires flipping of the hydrophilic glyco-peptides across the hydrophobic membrane layer. It is therefore surprising how little is known about its mechanism or the role of specific factors. Several lines of evidence suggest this process must be aided by specific factors, unlike the transport of phospholipids, which is believed to be facilitated by the presence of transmembrane proteins (102). Translocation of lipid II is also believed to occur independent of ATP or a proton motive force (171).

*E. coli* is thought to contain approximately 1000 lipid II molecules per cell and incorporate this same number of precursors into the existing murein layer per second (174). Therefore, the transport must be fast indeed, capable of
translocating 1000 molecules per second and thus recycling the undecaprenol pyrophosphate carrier molecule (174). A recent study found that a fluorescent lipid II analogue was unable to spontaneously translocate across lipid vesicles (171). However, translocation of this same substrate was found to occur when *E. coli* derived inner membrane vesicles were used, suggesting a membrane protein(s) is required (171). The possibility that the enzyme responsible for the last step in lipid II synthesis, MurG, is required for translocation was excluded. Interestingly, evidence suggests that ongoing transglycosylation is required for translocation (171).

Eukaryotic cells produce a related molecule remarkably similar to undecaprenol, called dolichol (26). It is used for the transport of glycan molecules to the lumen of the endoplasmic reticulum and protein glycosylation. Similar to the transmembrane transport of lipid II in bacteria, the factor(s) responsible for translocation of dolichol substrate is not known (152). It is intriguing to think that a conserved factor(s) is responsible for both processes.

**Incorporation of the murein substrate**

The third step in synthesis of murein is the incorporation of the precursor into the existing sacculus. This requires the formation of two distinct sets of chemical bonds; a peptide and glycosidic bond (88) (Fig. 1-4). *E. coli* encodes monofunctional murein transpeptidases [PBP2 and PBP3] (8, 132), a monofunctional murein transglycosylase [Mgt] (53) and bifunctional murein
synthases [PBP1A, PBP1B and PBP1C] (24, 92, 131, 153) that possess both
activities.

The transglycosylation reaction is believed to involve transfer of the existing
murein strand, which is still lipid-linked, onto the glucosamine residue of the lipid
II precursor. This would then result in the release of undecaprenyl diphosphate,
which is then dephosphorylated [YbjG, YeiU and LpxT] and “flipped” to the
cytoplasmic side of the cell membrane (reviewed in (170)). From here it is reused
for murein synthesis by accepting another NAG-pentapeptide from MraY and
yielding lipid I.

The overwhelming majority of cross-linking between murein strands are
formed by D,D peptide bonds between D-Ala and DAP (Fig.1-4) (87). The energy
for the transpeptidation reaction is provided by hydrolysis of the D-Ala-D-Ala
peptide bond of the precursor pentapeptide (87). It involves a two-step reaction in
which a PBP-substrate intermediate is formed releasing the terminal D-Ala. (This
is the step at which penicillin, a structural analogue of D-Ala-D-Ala, interferes
with murein synthesis by becoming a suicide substrate and permanently
attaching itself to the PBP (72)). In summary, for D,D transpeptidation the
pentapeptide D-Ala-D-Ala, which is considered the donor in this reaction, loses
the terminal D-Ala and has the $\alpha$-carboxyl group of the penultimate D-Ala
attached to the $\varepsilon$-amino group of the acceptor DAP molecule. This leaves the
amino group of the donor DAP free to accept a carboxyl group from other
strands. In theory, this could lead to many cross-links, however, only di-, tri- and
tetrameric cross-links have been reported for \textit{E. coli} (87).
In addition to D,D transpeptidation, *E. coli* also generates L,D cross-links between two DAP moieties. This represents about 2% of the total cross links in exponentially growing cells and increases significantly during stationary phase (87). L,D transpeptidation is also required for the attachment of Braun’s lipoprotein, or Lpp, to the murein sacculus (23). Lpp is one of the most abundant proteins in *E. coli* and is modified by attachment of two fatty acids at its N terminus and covalently attached to every 10 to 12 DAP molecules through a C terminal lysine residue (157). Despite its abundance and attachment to murein, Lpp is dispensable for proper rod shape in *E. coli* (85).

*E. coli* encodes five genes who’s products show homology to the recently discovered L,D transpeptidase from *E. faecalis* [ErfK, YbiS, YcfS, YnhG and YcbB] (117). A quadruple mutant lacking four of these were still able to perform murein L,D transpeptidation (the fifth was not assayed (116)). This mutant was, however, unable to attach Lpp to the murein sacculus. Interestingly, three were able to restore Lpp attachment to the quadruple mutant, with YbiS capable of fully complementing the mutant. This is the first example of enzymes with L,D transpeptidase activity in *E. coli*. This still leaves the question of what enzyme is responsible for the L,D transpeptidase activity necessary for the minor cross-linking of murein. Perhaps this activity is carried out by the fifth putative L,D transpeptidase, YcbB.

**Modification, maturation and recycling of murein**
The majority of *E. coli* murein (75-80%) is believed to exist as a monolayer, with the remaining sacculus being triple-layered (106). This has led to the question of how the cell is able to build new murein while maintaining the integrity of this stress-bearing sacculus. In order to be able to insert new precursor molecules the cell needs to break existing bonds. It is widely accepted that this process must be highly regulated so as to prevent disrupting the integrity of the sacculus.

*E. coli* encodes many murein hydrolytic enzymes that are capable of hydrolyzing nearly every different type of bond found in murein. These include lytic transglycosylases [SltY, MltA, MltB, MltC and EmtA], N-acetylmuramyl-L-alanine amidases [AmiA, AmiB, AmiC and AmiD], D,D endopeptidases [PBP4, PBP7, PBP8 and MepA] and D,D carboxypeptidases [PBP5, PBP6 and PBP6b] ((168) and reviewed in (87)) (Fig.1-5). Conspicuously absent from this list is a known L,D carboxypeptidase responsible for maturation of the murein sacculus. As described above, the major transpeptidation reaction yields tetra-tetra cross-linkages. As murein matures the tetra-tetra cross-links are converted to tetra-tri cross-linkages, which would be the product of a L,D carboxypeptidase. One is known, LdcA, but it is involved in recycling imported muropeptides and is active in the cytoplasm (164). Therefore, the L,D carboxypeptidase responsible for the maturation of murein remains unknown.

The abundance of enzymes capable of digesting murein is remarkable, and has led to the widely held notion that their action must be tightly regulated as to prevent unwanted degradation of the sacculus. How this is accomplished,
however, remains unknown. Nonetheless, the concerted action of murein hydrolytic enzymes results in the turnover of nearly 50% of murein per generation (75). Of this, nearly 90% is efficiently recycled and inserted back into the sacculus. Several recycling pathways exist in E. coli that can efficiently import the periplasmic material which is then digested sufficiently for reincorporation into the murein synthetic pathway (reviewed in (87)).

**Shape mutants of *Escherichia coli***

A number of mutants are known that can result in a variety of cell shape defects. These include lesions in genes involved in the two well-defined steps in murein synthesis: generation (28, 84, 120) and incorporation of substrate (52). Shape defects are also caused by mutations affecting maturation, modification and hydrolysis of murein (52, 134). Many of these mutants, however, are most likely still able to perform CMS to some degree or another. In contrast, two loci appear to be absolutely required for CMS, the *mrd* and *mre* operons (reviewed in (27, 29, 189). Mutations in either operon lead to a defect in CMS that forces the cell to use SMS to synthesize the murein sacculus and results in spherical shape (45).

**The *mrd* operon**

The *mrd* (murein D) operon contains two genes, *mrdA* (*pbp2*), and *mrdB* (*rodA*) (162, 163) (Fig.1-6). The protein product of the first gene, PBP2, is a bitopic membrane protein with D,D transpeptidase activity and is the only PBP
(penicillin-binding protein) in *E. coli* that is specifically required for CMS (93). Its counterpart in the SMS system is PBP3, which is likewise dispensable for CMS (161). The second protein encoded by the *mrd* operon is RodA, a polytopic membrane protein with 10 transmembrane helices (83). It belongs to a family of proteins that includes the division protein FtsW (118). Several lines of evidence suggest that RodA and FtsW interact with and are required for proper transpeptidase activity of PBP2 and PBP3, respectively (61, 89, 93, 126). The function of RodA and related proteins is unknown. A role, however, in lipid II translocation has been suggested (87).

The *mre* operon

The *mre* operon is comprised of three genes; *mreB*, *mreC* and *mreD* coding for the MreB, MreC, and MreD proteins, respectively (Fig.1-5). MreB is the sole actin protein in *E. coli* (29, 172). Next to PBP2, MreB is the most widely investigated shape protein and will be discussed in more detail below.

MreC is a bitopic cytoplasmic membrane protein with a large C terminal domain located in the periplasm (105, 110). The crystal structure of the periplasmic domain from the Gram positive bacterium *L. monocytogenes* revealed several interesting insights. This portion of MreC has two β-strand domains buttressing a central α-helical domain (173). The helical domain appears to mediate a close dimeric contact between two molecules. One of the β-strand domains revealed a structure with similarity to an RNP protein and the other has a chymotrypsin-like fold. However, the functional implication of a
protease-like fold remains unknown. The role of MreD, a polytopic membrane protein, is presently unclear but it may function to stabilize the shape protein complex.

The bacterial actin MreB

Several lines of evidence suggest MreB is a bona fide bacterial actin. Primary sequence homology, however, is not one of them. MreB and actin share only ~15% amino acid identity, much less than the 20% commonly used to determine homologues (29). Despite this weak similarity, it was nonetheless sufficient to place MreB into a large super-family of functionally diverse proteins that includes actin, sugar kinases, Hsp70 heat shock proteins, and the bacterial division protein FtsA (20). The super-family is defined by the conservation of residues surrounding a nucleotide-binding pocket. MreB was firmly labeled as a bacterial actin protein following two landmark publications. In 2001, van den Ent et al showed that one of the two MreB proteins from the Gram positive organism Thermotoga maritima polymerized into actin-like polymers in vitro and had a three-dimensional structure that was remarkably similar to that of actin (172). Earlier that same year, Jones et al showed that two of the three MreB proteins from the Gram positive organism Bacillus subtilis assembled into helical structures underneath the cytoplasmic membrane that span the length of the cell resembling a true actin cytoskeleton (97).

Sub-cellular localization of MreB
Sub-cellular localization studies involving several different organisms have shown that MreB can form a variety of cytoskeletal-like structures. To date, no two organisms encode a complement of MreB proteins that share the same pattern of sub-localization. For example, the Gram positive organism *Bacillus subtilis* encodes three MreB-like proteins, MreB, MreBH and Mbl that all appear to co-localize to form one mixed structure (31). In the Gram negative organisms studied thus far, MreB can form rings in addition to the helical-like spirals found in *B. subtilis*. In the α-proteobacteria member *Rhodobacter sphaeroides*, MreB appears to exclusively form rings at mid-cell (159). In contrast, in the related α-proteobacteria *Caulobacter crescentus*, MreB localizes in a dynamic fashion that is dependent on the cell cycle; switching from helical structures in growing cells to mid-cell rings in dividing cells (66, 73). In *E. coli*, a γ-proteobacteria member, MreB has also been reported to form both helical as well as ring structures (104, 156, 175). The sole MreB protein in the cyanobacteria *Anabaena* is found in patches at the poles as well as in short fibers throughout the cell (86). It is important to note that studies in *E. coli* and *C. crescentus* are limited to immunofluorescence and non-functional fluorescent fusion proteins.

Although the localization patterns of MreB and its related proteins differ between various organisms, in nearly all cases these proteins appear to be involved in the general cellular process of rod shape determination and maintenance. The one exception to the involvement of MreB proteins in morphogenesis has been described for the filamentous Gram positive *Streptomyces* spp. Interestingly, MreB is dispensable for vegetative growth and
localizes to pre-spore septa (119). Here it is thought to be critical for the sporulation process, as MreB mutants can no longer regulate the shape and integrity of aerial hyphae. It therefore appears that in *Streptomyces* spp. MreB is involved in SMS and not CMS.

**MreB filament assembly and dynamics**

As discussed above, MreB and actin share structural properties and assemble into cytoskeletal-like structures in vivo. Despite these similarities, MreB and actin have some notably different biochemical properties. Several methods including light scatter, FRET, fluorescence and electron microscopy have been used to study polymerization and assembly dynamics. It should be further noted that all studies have been limited to the MreB1 protein from *T. maritima*, for which no phenotypic or cytological data exists. Nevertheless, using this system, *T. maritima* MreB1 has been shown to assemble into polymers in a nucleotide-dependent manner, a property it shares with actin (11, 64, 65, 172). However, unlike actin, MreB1 is an equally effective GTPase, and the rate of polymerization is significantly faster than actin with little or no need for nucleation (11, 65). Furthermore, there is no evidence of end-to-end joining, a feature which contributes significantly to actin assembly (64). Lastly, the critical concentration for polymerization of MreB1 has been found to be strongly influenced by temperature, ranging between 3 and 2100 nM, with optimal conditions yielding a concentration over 100 times lower than actin (11, 64).
Another difference between MreB and actin can be found at the filament level. Unlike actin, which forms intertwined double helical protofilaments, MreB has been shown to polymerize into a wide array of structures ranging from single protofilaments to filamentous bundles, or sheets (11, 64). The latter property appears to occur spontaneously without the need for accessory factors and has been proposed to be a reflection of the state of nucleotide hydrolysis throughout the filament (65).

In vivo dynamics of MreB

As discussed above, MreB from various organisms have been found to exist in a number of different structures in vivo, including spirals and rings. The dynamics of these structures has been examined in detail for the MreB proteins of B. subtilis and C. crescentus. By tracking the fluorescence recovery after photobleaching (FRAP) of a functional GFP-Mbl fusion in B. subtilis, cables were found to be continuously remodeled during the life cycle (30). Turnover did not appear to carry polarity and displayed a half-time of recovery of 8 minutes. A different study looking at the dynamics of GFP-tagged MreB and Mbl found filaments to move rapidly throughout the cell (49). MreB filaments were measured at a speed of 4.2 nm/s. Interestingly, the direction of Mbl appeared to be opposite that of MreB and both were suggestive of a treadmilling mechanism (polymerization at one end and depolymerization at the other) (49).

Treadmilling of MreB in vivo was recently confirmed using a non-functional MreB-YFP fusion protein in C. crescentus. In this study, quantitative imaging of
single MreB-YFP molecules was done in live cells and led to several conclusions about the dynamic properties of MreB (100). MreB was found to exist in both filamentous forms (fMreB) and monomeric, or globular forms (gMreB). The rate of diffusion of gMreB was slower than that of cytoplasmic proteins and similar to membrane proteins (100). This suggests that MreB associates with a membrane protein when not incorporated into a filament. Also, the rate, distance, and direction of MreB polymer movement was indicative of a treadmilling mechanism, as was previously suggested (100). Furthermore, the rate of monomer addition was estimated to be 1.2 s$^{-1}$ and filaments had an average length of 400 nm. In summary, in vivo dynamics of MreB are consistent with it being a member of the actin family. However, factors involved in regulating the dynamics of MreB are not known.

**Model of MreB-directed murein growth zones**

At this point it is well established that the murein sacculus dictates cell shape. The chemical composition and enzymes responsible for the production of the precursor molecule, lipid II, are known. Numerous enzymes are known that synthesize and modify murein as well. Furthermore, five proteins (MreBCD and MrdAB) have been implicated in CMS but are dispensable for SMS. The next logical question is precisely how the cell is able to accomplish CMS. The following model has been proposed and has garnered considerable support. It states that the MreB cytoskeleton serves as a scaffold to direct murein synthesis machineries, which would ensure growth along the lateral cell wall and control
the cell diameter (40) (Fig.1-2). Put another way, the helical nature of the MreB related proteins topologically constrains the murein synthetic and/or hydrolytic machinery located in the periplasm and ensures murein growth along the long axis.

The idea that MreB provides a scaffold for murein synthesis is supported by a series of experiments that localized zones of nascent murein insertion. This has been done by utilizing fluorescent derivatives of the antibiotics vancomycin (Van-Fl) and ramoplanin (Ramo-Fl), which bind the terminal D-Ala-D-Ala dipeptide at the end of lipid II and diphospho-NAM (which immediately follows the C55 portion of bactoprenol), respectively (Fig.1-3). In this way, sites of murein insertion can be visualized directly by fluorescent microscopy following a brief staining period. Staining of vegetative *B. subtilis* cells with Van-Fl (40, 167) or Ramo-Fl (167) revealed a spiral-like pattern of lipid II localization reminiscent of MreB/Mbl/MreBH patterns (31), in addition to periods of intense septal staining during division. In summary, the pattern of nascent murein visualized with fluorescent antibiotics targeting lipid II reveal a pattern of murein incorporation similar to that of MreB.

Further evidence supporting the scaffold model is found in the protein-protein network of interactions that have been found between some of the shape proteins and murein synthetic and hydrolytic enzymes. Using a bacterial two-hybrid assay, interactions between *E. coli* MreB and MreC, and MreC and MreD have been reported (104). Affinity purification as well as bacterial two-hybrid assays have also proven useful in identifying interactions between *B. subtilis* and
*C. crescentus* MreC and several murein synthases including PBP2 (55, 173). Interactions between *E. coli* MreB and the glycosyltransferase MurG have also been found by a cross-linking/immunoprecipitation method (129). This method also identified an association between MurG and MraY. If the protein-protein interactions from the various organisms mentioned can be unified, then a network of interactions can be established that link the enzymes responsible for synthesis of lipid I and II (MraY and MurG) to murein synthetic enzymes (PBP2 etc.) through MreB and MreC.

The MreB-scaffold model, supported by the pattern of nascent murein and numerous protein-protein interactions, lends further support to the idea that a multi-enzyme complex, or holo-enzyme, is responsible for the regulated synthesis of the sacculus (179). The holo-enzyme model, put forth by Vollmer and Holtje, proposes that a complex of murein synthases and hydrolases enlarge the sacculus in an inside to outside mechanism. It states that a triplet of monomers gets attached to the existing sacculus by transpeptidation and the existing strand is removed, yielding a net growth of two monomers. This would be accomplished by the concerted action of a complex of enzymes, or a holo-enzyme. This model is satisfying for several reasons. First, it agrees with the observed rate of murein turnover, estimated to be 50% per generation (75). Secondly, it provides a reasonable explanation for how the cell can enlarge a stress-bearing sacculus without compromising its integrity. And lastly, an inside to outside mode of growth is consistent with the observation that the polymerizing enzymes, the transpeptidases and transglycosylases, are inner membrane
associated while the lytic transglycosylases are outer membrane lipoproteins (179).

**Bacterial actin and DNA segregation**

MreB and related bacterial actin proteins have been implicated in DNA segregation (48, 74, 103, 105, 130, 160). The first bacterial actin protein found to play a direct role in DNA segregation was the ParM protein (for review see (69)). Together with the DNA-binding protein ParR and the centromere-like parS element, ParM is part of the partitioning system of the low-copy number R1 plasmid of *E. coli*. These three factors are sufficient to partition the plasmid, with ParM filament formation providing the mechanical force for segregation (130).

Several lines of evidence suggest MreB-like proteins also play a role in DNA segregation. Different experimental approaches have been used to show that MreB is necessary for segregation of both the origin of replication and bulk DNA (nucleoid and terminus) in *B. subtilis*, *C. crescentus*, and *E. coli* (48, 74, 103, 105, 160). However, unlike the ParM paradigm of DNA segregation, a centromere-like element and bridging factor between DNA and MreB has not been well established. In *C. crescentus*, MreB was found to associate with the origin region of the chromosome (74). In this case, however, it is unclear if this association is direct or indirect. In *E. coli*, RNA polymerase (RNAP) may be the bridging factor that connects MreB with DNA (103). In this example, two mechanisms were proposed by which MreB and RNAP could segregate DNA. MreB could either immobilize RNAP thus allowing the force generated by
transcription to segregate DNA, or, that MreB filaments actively segregate RNAP and its associated DNA (103).

The role of DNA segregation by MreB in *B. subtilis* and *E. coli* is controversial, however, as their involvement in this process has recently been questioned (67, 99). Additional studies will likely need to be performed to address these conflicting findings and firmly establish this role for MreB-like proteins.

**The bacterial tubulin FtsZ and cell division in *E. coli***

In the way MreB is the proposed spatial director of CMS, the functional analogue of the SMS system is FtsZ (185). Many of the similarities that exist between MreB and actin also apply to FtsZ and eukaryotic tubulin. For example, although primary sequence similarity between the two is low, FtsZ shares biochemical properties with tubulin that extend to its behavior in the cell (22). The structure of FtsZ closely mirrors that of tubulin and purified FtsZ self-assembles into polymers following the binding and hydrolysis of GTP (42, 115). FtsZ is the first known protein to assemble at the future site of division and therefore the determining factor dictating the site of SMS.

The spatial regulation of FtsZ assembly is a well-studied process that involves the action of two partially redundant systems, the Min system and nucleoid occlusion, both of which work to restrict FtsZ assembly to midcell. The Min system is a negative regulatory system that consists of three proteins, MinC, MinD and MinE, that oscillate from pole to pole (43, 147). This allows for the FtsZ-inhibitor, MinC, to have a greater inhibitory effect at the poles, and a lesser
effect at midcell (149). Nucleoid occlusion works to inhibit FtsZ formation over nucleoids through the negative action of the DNA-binding protein SlmA (16). Together, these two systems are remarkably efficient at restricting FtsZ ring formation, and thus SMS, to the middle of the cell.

**Regulation of phospholipid synthesis**

Synthesis of the cell envelope; the inner membrane, murein and outer membrane, require fatty acid and isoprenoid derived lipids. The isoprenoid lipids, described above, are needed for transport of murein precursor as well as sugars destined for attachment to the outer membrane (169). Fatty acid derived lipids are used in membrane phospholipids, making lipid A, which is the anchor lipid for lipopolysaccharide (LPS), and for the acylation of pro-lipoproteins (39). The enzymes involved in fatty acid synthesis are located in the cytoplasm (38). The cell shields growing fatty acid molecules from water by attaching them to highly hydrophilic molecules including acyl carrier protein (ACP) and coenzyme A (CoA) (39, 148). Mature fatty acids are delivered to the enzymes that synthesize phospholipids and all but one is an integral membrane protein, with the remaining enzyme membrane associated only when active (38).

It is assumed that synthesizing too little of either of the cell wall components would have a detrimental effect on the cell, and too much could at least prove to be a hindrance. It is therefore surprising that the mechanisms that regulate synthesis of the three components of the *E. coli* envelope are poorly understood.

In *E. coli*, the rate of phospholipid synthesis is closely coupled to the growth
rate (38). That is to say, at any particular growth rate the cell “knows” how much phospholipid to make. However, how this is achieved is not known. The overproduction of the enzymes responsible for phospholipid synthesis does not lead to increased amounts (38). This suggests the rate of synthesis is exerted on the enzymes themselves. This may be accomplished through feedback inhibition, where the product of one enzyme would have an inhibitory effect on itself. This form of regulation could, in theory, prevent the overproduction of intermediates in the pathway. There is indeed evidence that the accumulation of acylated-ACP molecules inhibit fatty acid synthases (38). However, feedback inhibition of phospholipid synthases has not been demonstrated, likely due to the fact that these enzymes are membrane proteins, which complicates experimental methods. Exponentially growing E. coli cells have a phospholipid composition of 75% phosphatidylethanolamine (PE), 20 % phosphatidylglycerol (PG) and 5% cardiolipin (CL) (39). Cells deficient in the production of one of these classes of phospholipids are viable, albeit with special growth requirements (38). Cells are able to survive without one of these classes by adjusting the synthetic rate of the others. Though the mechanism responsible for this adjustment is unknown, it is postulated to be the same for all three classes of phospholipids (38). In summary, the mechanism responsible for regulating the rate of phospholipid synthesis in E. coli is not well understood.

Interestingly, phospholipid synthesis appears to be coupled to murein synthesis. Inhibition of phospholipid synthesis by treatment with the fatty acid synthase inhibitor cerulenin, or by glycerol starvation, results in the concomitant
inhibition of murein synthesis (61). Moreover, the step of murein synthesis inhibited was concluded to be transport of the murein substrate. It is therefore attractive to consider the existence of a mechanism that ensures coupling of phospholipid and murein synthesis.
Figure 1-1. The murein sacculus. The murein sacculus is a network of covalently linked sugar-peptides composed of the subunits N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). The subunits are linked together by $\beta$ 1,4 glycosidic and peptide bonds.
Figure 1-2. Cylindrical and septal murein synthesis (CMS and SMS). A Shown is the MreB-scaffold model for rod shape determination in *E. coli*. This model states that the MreB spiral-like structures direct cylindrical murein synthesis (CMS) leading to growth along the long axis of the cell. This is then followed by FtsZ-directed septal murein synthesis (SMS). B Cells deficient in CMS fail to elongate along the long axis and grow as spheroids and cells deficient in SMS fail to divide and form long filaments.
Figure 1-3. Generating the murein precursor. Shown is the stepwise generation of the murein precursor molecule lipid II. The enzymes are listed in red. NAM, N-acetylmuramic acid; NAG, N-acetylglucosamine; U, UDP; PEP, phosphoenolpyruvate; IM, inner membrane. Van and Ram indicate the targets of the antibiotics vancomycin and ramoplanin, respectively (40, 167).
Figure 1-4. Incorporating murein precursor. The red arrows show how the murein precursor lipid II is incorporated into the existing sacculus. See text for details.
Figure 1-5. Murein hydrolases. Show are the cleavage sites for each class of murein hydrolase. LTG, lytic transglycosylase; NAGase, N-acetylglucosaminidase; Ami amidase; LD-EP, L,D endopeptidase; DL-EP, D,L endopeptidase; LD-CP, L,D carboxypeptidase; DD-EP, D,D endopeptidase; DD-CP, D,D carboxypeptidase. See text for specific enzymes.
Figure 1-6. The \textit{mrd} and \textit{mre} operons and encoded proteins. Shown is the organization of the \textit{mrd} and \textit{mre} operons and the known or predicted topology and function of the encoded proteins.
Chapter 2

Conditional lethality, division defects, membrane involution and endocytosis in

\textit{mre} and \textit{mrd} shape mutants of \textit{Escherichia coli}.
Summary

Maintenance of rod-shape in *E.coli* requires the shape proteins MreB, MreC, MreD, MrdA (PBP2), and MrdB (RodA). How loss of the Mre proteins affects *E.coli* viability has been unclear. We generated Mre- and Mrd-depletion strains under conditions that minimize selective pressure for undefined suppressors, and found their phenotypes to be very similar. Cells lacking one or more of the five proteins were fully viable and propagated as small spheres under conditions of slow mass increase, but formed large non-dividing spheroids with non-canonical FtsZ assembly patterns at higher mass doubling rates. Extra FtsZ was sufficient to suppress lethality in each case, allowing cells to propagate as small spheres under any condition. The failure of each unsuppressed mutant to divide under non-permissive conditions correlated with the presence of elaborate intracytoplasmic membrane-bound compartments, including vesicles/vacuoles and more complex systems. Many, if not all, of these compartments formed by FtsZ-independent involution of the cytoplasmic membrane (CM), rather than de novo. Remarkably, while some of the compartments were still continuous with the CM and the periplasm, many were topologically separate, indicating they had been released into the cytoplasm by an endocytic-like membrane fission event. Notably, cells failed to adjust the rate of phospholipid synthesis to their new surface requirements upon depletion of MreBCD, providing a rationale for the ‘excess’ membrane in the resulting spheroids. Both FtsZ and MinD readily assembled on intracytoplasmic membrane surfaces, and we propose that this
contributes significantly to the lethal division block seen in all shape mutants under non-permissive conditions.
Introduction

As in many other bacteria, maintenance of cell shape in *E.coli* requires an intact peptidoglycan (murein) layer of the envelope and, at least, five well-conserved proteins (PBP2, RodA, MreB, C, and D) that are each required to prevent a cell shape conversion from rod to prolate spheroid or sphere (for reviews, see (17, 27, 29, 87, 114, 189)).

PBP2 and RodA are encoded by the *mrdA (pbpA)* and *mrdB (rodA)* genes residing in the *mrd* (murein D) operon (162, 163). Penicillin binding protein 2 (PBP2) is a bitopic integral cytoplasmic membrane (CM) species with a large periplasmic domain that possesses murein DD-transpeptidase activity, binds β-lactams, and has a particularly high affinity for the amidino-penicillin mecillinam (FL1060) (93, 161). PBP2 is unique among the PBP's in *E.coli* in that it is specifically required for cylindrical murein synthesis during cell elongation, but dispensible for septal murein synthesis during cell constriction. Its counterpart, PBP3 (FtsI), is similarly unique in that it is specifically required for septal murein synthesis but dispensible for cylindrical murein synthesis (45, 87, 161). RodA belongs to a family of polytopic membrane proteins, which also includes the division protein FtsW (83, 118), and evidence suggests that RodA and FtsW are required for proper functioning of PBP2 and PBP3, respectively (61, 87, 89, 93, 126).

MreB, C, and D are encoded by the *mre* operon. MreB is the sole known bacterial actin (172) in *E.coli*. As in *Bacillus subtilis* (48, 97, 160) and *Caulobacter crescentus* (66, 74), MreB localizes just underneath the cytoplasmic membrane.
in a spiral/banded-like pattern along the length of the cell (105, 156), and is implicated in both shape maintenance and chromosome segregation (103, 181). MreC is a bitopic CM protein and MreD a polytopic one (104, 109, 180). A crystal structure of the large periplasmic domain of *Listeria monocytogenes* MreC revealed a dimer with some structural similarities to chymotrypsins, though it is unlikely to be a protease (173). Affinity purification and bacterial two-hybrid analyses indicate that MreC interacts with MreD as well as with several of the high molecular weight murein synthases (PBP’s), including the PBP2 homologues in *C. crescentus* and *B. subtilis* (55, 104, 173). An interaction between MreC and PBP2 is further supported by co-localization experiments in *C. crescentus* and *Rhodobacter sphaeroides* cells (59, 158). Like MreB, PBP2, MreC and MreD appear to accumulate in a spotty or helical fashion along the cell envelope in *E. coli*, *B. subtilis*, and/or *C. crescentus* (51, 55, 59, 66, 108), and these localization patterns are reminiscent of those of new murein insertion in the cylindrical portion of *B. subtilis* cells (40, 167). These and other observations support models in which the helical organization of bacterial actins in the cytoplasm topologically constrain murein synthase and/or hydrolase activities in the periplasm, resulting in growth of the sacculus as a cylinder in between periods of cell constriction (31, 40, 66). How the location of MreB polymers in the cytoplasm is coupled to that of (mostly) periplasmic MreC/PBP complexes is unclear. Coupling could be quite direct in *E. coli*, as suggested by an MreC-MreB interaction in a bacterial two-hybrid assay (104), but this is probably not the case in *C. crescentus* and *R. sphaeroides* (59, 158).
How loss of rod shape affects the ability of *E. coli* to propagate has been a confusing issue. Loss of the Mre proteins has variously been reported to yield stably propagating spheres (105, 137, 155, 180), or to be lethal (104, 183), unless cells are supplied with extra copies of *ftsQAZ* (104). Inactivation of PBP2 and/or RodA, by treatment of wt cells with mecillinam or by mutation of *mrdA* or *mrdB*, typically results in the formation of giant non-dividing spheroids/spheres, which eventually lyse and die (12, 139, 163, 177). However, PBP2− cells were found to stably propagate as smaller dividing spheres under several conditions including: i) a simultaneous increase in the essential division proteins FtsQ, A, and Z (133, 177); ii) a slow growth rate (10, 98); and/or iii) an increase in the level of the alarmone ppGpp above a certain threshold (21, 98, 176). RodA− spheres were similarly reported to survive on poor medium or upon overexpression of *ftsQAZ* (12, 45, 177).

Taking care not to select for secondary suppressing alterations, we created sets of *mre* and *mrd* mutants in two genetic backgrounds, and compared their properties. Our results indicate that unsuppressed cells lacking either of the Mre proteins behave very similarly to those lacking PBP2 and/or RodA. Thus, like *mrd* cells (45, 98, 177), *mre* cells were conditionally viable in that they propagated stably as small dividing spheres at low growth rates on poor media, but formed giant non-dividing spheroids at higher growth rates. Lethality of *mre* cells at higher growth rates could be partially suppressed by supply of an overactive form of (p)ppGpp synthase (RelA'). In addition, we found that increased expression of
just FtsZ was sufficient to suppress the lethality of both *mre* and *mrd* mutants on rich medium.

One striking feature, common to all unsuppressed shape mutants under non-permissive conditions, was the extensive invasion and elaboration of the CM into the spheroid’s cytoplasm. Some of these elaborations were continuous with the exterior CM, while others were topologically separate. Several lines of evidence indicate that the latter derived from the exterior CM by endocytic-like membrane fission events that release periplasm-filled vesicles in the cytoplasm. Interestingly, MreBCD-depleted spheres synthesize phospholipid at about the same rate per unit cell mass as wt rods, providing a rationale for the ‘excess’ membrane in their interiors. This failure to properly adjust membrane lipid synthesis to actual surface requirements under non-permissive conditions is likely to contribute to the accompanying lethal division defect in the shape mutants. Both FtsZ and MinD assembled aberrantly on internal membrane systems, suggesting that the latter directly interfere with proper assembly of a division apparatus on external segments of the CM by diverting significant fractions of division proteins into non-productive assemblies.
Materials and methods

_E. coli_ plasmids and phages. The most relevant plasmids, phages, and strains used in this study are listed in Table 2-1, and depicted in Fig. 2-1.

Plasmids  pBR322 (19), pZAQ (186), pJF118EH (68), pMEL1 (181), pMLB1113 (43), pCX19 (184), pCP20 (32), pDB346 (146), pDR107 (147), pDR120 (80), pKD13 (41), pCH151 (14), pCH157 (107), and pTB59 (15) were described previously.

Unless indicated otherwise, MG1655 chromosomal DNA was used as template in amplification reactions. Sites of interest (e.g. relevant restriction sites) are underlined in primer sequences.

To construct pCH221 [P_\text{lac}::gfpmut2-t-mrdB], _mrdB_ (rodA) was amplified using primers 5’-TATAGAATTCATATGACGGATAATCCGAATAAAAAACATTCTGG-3’ and 5’-CATTGTCGACTTACACGCTTTTCGACAACATTTC-3’. The product was treated with _EcoRI_ and _SalI_, and the 1127 bp fragment was used to replace the 13 bp _EcoRI-SalI_ fragment of pDR107c, yielding pCH218 [P_\text{T7}::gfpmut2-t-mrdB]. The 1970 bp _BglII-HindIII_ fragment of pCH218 was next used to replace the 20 bp _BamHI-HindIII_ fragment of pMLB1113, resulting in pCH221.

For pCH222 [P_\text{lac}::mrdB-gfpmut2], _mrdB_ was amplified using primers 5’-TATAGAATTCATATGACGGATAATCCGAATAAAAACATTCTGG-3’ and 5’-GCACCTCGAGCAGCCTTCGACAACATTTC-3’. The product was treated with _NdeI_ and _XhoI_, and the 1119 bp fragment was used to replace the 77 bp _NdeI-XhoI_ fragment of pET21a, yielding pCH219 [P_\text{T7}::mrdB-h]. The 1152 bp
XbaI-XhoI fragment of pCH219 was used to replace the 1025 bp XbaI-XhoI fragment of pCH151 [P_{lac}::zipA-gfpmut2], resulting in pCH222.

Construction of plasmid pCH235 [P_{lac}::mreD-le] involved several steps. The annealed product of oligo's 5'-TCGAGTAAGTCGACACGGTACCA-3' (sense) and 5'-AGCTTGGTACCGTGTCGACTTAC-3' (anti-sense), was used to replace the 122 bp XhoI-HindIII fragment of pCH157 [P_{lac}::gfpmut2-t-minD, minE-h]. This resulted in pCH181 [P_{lac}::gfpmut2-t-minD, minE-le], in which the His\textsubscript{6} tag sequence in pCH157 was substituted with an XhoI site, encoding the dipeptide LE (le), followed by the TAA stop codon. The mreD gene was amplified using primers 5'-TATAGAAATTCATATGGCGAGCTATCGTAGCCAGGGACGCTG-3' and 5'-CGTTCTCGAGTTGCACTGCAAACTGCTGACGGAC-3', and digested with EcoRI and XhoI. The 494 bp fragment was used to replace the 34 bp EcoRI-XhoI fragment of pDR107c, resulting in pCH217 [P_{T7}::gfpmut2-t-mreD-h].

Circularization of the 5837 bp NdeI fragment of pCH217 yielded pCH223 [P_{T7}::mreD-h]. pCH235 was finally obtained by replacing the 1859 bp XbaI-XhoI fragment of pCH181 with the 512 bp XbaI-XhoI mreD fragment of pCH223.

Plasmid pCH244 [P_{lac}::mreB, mreC, mreD, yhdE] was obtained after several steps. The 3359-bp Apol fragment of pMEL1 was inserted in the EcoRI site of pDR107a, yielding pDB364 [P_{T7}::gfpmut2-t-mreB(5-347), mreC, mreD, yhdE]. The 4223 bp BglII-HindIII fragment of pDB364 was next used to replace the 20 bp BamHI-HindIII fragment of pMLB1113, resulting in pDB366 [P_{lac}::gfpmut2-t-mreB(5-347), mreC, mreD, yhdE]. The mreB gene was amplified using primers 5'-CGACTCTAGACAGCTTTCAGGATTATCCCTTAGTATG-3' and 5'-
GCAAAAGCTTACTCTTCGCTGAACAGGTCGCC-3’. The product was treated with XbaI and HindIII, and the 1072 bp fragment ligated to the 7639 bp XbaI and HindIII fragment of pCH151 [Plac::zipA-gfpmut2], generating pCH214 [Plac::mreB]. Finally, replacement of the 534 bp KpnI-HindIII fragment of pCH214 with the 2879 bp KpnI-HindIII fragment of pDB366 resulted in pCH244.

To obtain pCH268 [Plac::gfpmut2-t-zapA], zapA was amplified using primers 5’-GAAGGATCCATGTCTGCACAACCGTC-3’ and 5’-CGAGTCGACTCATTCAAAGTTTTGGTTAG-3’. The product was treated with BamHI and SalI, and the 336 bp fragment was used to replace the 1164 bp BamHI-SalI fragment of pDR120 [Plac::gfpmut2-t-ftsZ].

For plasmid pFB112 [tet sdiA], the 1312 bp EcoRI-PstI fragment of pCX19 was ligated to the 3615- EcoRI-PstI bp fragment of pBR322.

For pFB118 [Plac::mreB], the 2696 bp ClaI fragment of pCH244 was deleted.

To obtain pFB120 [Plac::mreC-le], mreC was amplified using primers 5’-CTAGTCTAGAATACGAGACATCGATAACT-3’ and 5’-CGTTCTCGAGTTGCCCTCCCCGCGCAGCGCAGGC-3’. The product was treated with XbaI and XhoI, and the 1128 bp fragment was used to replace the 512 bp XbaI-XhoI fragment of pCH235.

For pFB121 [Plac::mreC, mreD-le], an mreCD fragment was amplified using primers 5’-CTAGTCTAGAATACGAGACATCGATAACT-3’ and 5’-CGTTCTCGAGTTGCAGCTGCAAACTGCTGACGGAC-3’. The product was treated with XbaI and XhoI, and the 1616 bp fragment was used to replace the 512 bp XbaI-XhoI fragment of pCH235.
Plasmid pFB124 [cl857(ts) P\text{\textsubscript{\textlambda}}R\text{::} mre\text{C}, mre\text{D-le}] was obtained by replacing the 1196 bp XbaI-SalI fragment of pDB346 [cl857(ts) P\text{\textsubscript{\textlambda}}R::ftsZ] with the 1625 bp XbaI-SalI fragment of pFB121.

In turn, pFB128 [cl857(ts) P\text{\textsubscript{\textlambda}}R:: mre\text{D-le}] was created by replacing the 1625 bp XbaI-SalI fragment of pFB124 with the 521 bp XbaI-SalI fragment of pCH235.

Plasmid pFB142 [P\text{\textsubscript{lac}}::mre\text{B}, mre\text{C-le}] was created in two steps. The 1271 bp XbaI-XhoI fragment of pCH217 was used to replace the 1859 bp XbaI-XhoI fragment of pCH181, yielding pCH233 [P\text{\textsubscript{lac}}::gfpmut2-t-mre\text{D-le}]. An mreBC fragment was amplified using primers 5'-

\text{CGACTCTAGACAGCTTTCCAGATTATCCTTGTAG-3'} and 5'-

\text{CGTTCTCGAGTTGCCCTCCCGGCGACGCAGGC-3'}. The product was treated with XbaI and XhoI, and the 2240 bp fragment was used to replace the 1271 bp XbaI-XhoI fragment of pCH233.

For pFB149 [P\text{\textsubscript{lac}}::mre\text{B}, mre\text{C}, mre\text{D-le}], the 1033 bp BamH\text{I}-SalI fragment of pCH244 was replaced with the 359 bp BamH\text{I}-SalI fragment of pFB124.

To create pFB174 [P\text{\textsubscript{BAD}}::mre\text{B}, mre\text{C}, mre\text{D-le}], the 1451 bp XbaI-HindIII fragment of pLL116 (a pBAD33 derivative that will be described elsewhere) was replaced with the 2743 bp XbaI-HindIII fragment of pFB149.

For pFB185 [P\text{\textsubscript{lac}}::mrd\text{B}], the 508 bp NsiI-HindIII fragment of pCH221 was used to replace the 1252 bp NsiI-HindIII fragment of pCH222.

To construct pFB190 [P\text{\textsubscript{lac}}::mrd\text{A}], pTB59 [P\text{\textsubscript{lac}}::mrdAB] was used as template to amplify mrd\text{A} (pbp\text{A}) with primers 5'-

\text{CTCTGAATTCCGCTGAGTGATAAAGGGAGGTTGAGTAG-3'} and 5'-
GCCAAGCTTTGGTCGACTTAATGGTCCTCCGCTGC-3'. The product was treated with EcoRI and HindIII and the 1954 bp fragment was used to replace the 3084 bp EcoRI-HindIII fragment of pTB59.

For pFB194 [cl857(ts) P_{\lambda R}::mrdB], the 1155 bp XbaI-SalI fragment of pFB185 was used to replace the 1625 bp XbaI-SalI fragment of pFB124.

Plasmid pTB182 [ftsQAZ] was obtained in several steps. The HindIII site within ftsA on pZAQ was removed by the Quickchange procedure (Stratagene) using the mutagenic primers 5'-

CAGTTGCAGGAAAAGCTCCGCAACAAGGG-3' and its reverse complement, resulting in a silent change (underlined) of FtsA codon 319 (Leu). The resulting plasmid (pTB178) was next mutagenized using primers 5'-

TTATGAGGCGACGATCTAGACGGCCTCAGGCGACAG-3' and its reverse complement, creating an XbaI site in between ftsA and ftsZ. The 4377 bp PstI-HindIII fragment of the resulting plasmid (pTB179) was then used to replace the 12 bp PstI-HindIII fragment of pGB2, yielding pTB182. The direction of ftsQAZ transcription from this plasmid is opposite that of the aadA gene.

For pTB188 [P_{\lambda R}::ftsZ], pDB346 [cl857(ts) P_{\lambda R}::ftsZ] was used as a template in a PCR with 5'-

CGTAGCGATCCGCATGCGGGATAATATCTAACCCTGCGTG-3' and 5'-

GCTCAAGCTTTGTCGACTTAATCACGCTTGCTTACGGAAATG-3'. The product was treated with BamHI and HindIII, and the 1359 bp fragment was used to replace the 20 bp BamHI-HindIII fragment of pGB2, yielding pTB188. Note that
this plasmid lacks lambda repressor, and that \(ftsZ\) is constitutively transcribed in the direction opposite that of \(aadA\).

For pYT11 [\(P_{\text{tac}}::relA\)], a portion of \(relA\) was amplified with primers 5’-CTTTTCTAGATTTCGGCAGGTCTGGTCCCTAAAGG-3’ and 5’-GGTCCTCGAGCTGGTGAACCGCACAATGC-GCC-3’. The product was treated with \(XbaI\) and \(XhoI\), and the 1401 bp fragment was used to replace an \(XbaI-XhoI\) fragment of pCH276, a plasmid whose construction will be detailed elsewhere. The 1500 bp \(EcoRI-HindIII\) fragment of the resulting plasmid (pYT5) was next used to replace the 30 bp \(EcoRI-HindIII\) fragment of pJF118EH, yielding pYT11. The plasmid encodes the first 455 residues of RelA, followed by an glutamic acid residue and a stop codon.

Phages \(\lambda\)CH221, \(\lambda\)CH235, \(\lambda\)CH268, \(\lambda\)FB120, \(\lambda\)FB185, \(\lambda\)FB190 and \(\lambda\)TB59 were obtained by crossing \(\lambda\)NT5 with pCH221, pCH235, pCH268, pFB120, pFB185, pFB190 and pTB59, respectively, as described (43).

**E.coli strains.** \(mre\) knockout strains were constructed by \(\lambda\)red recombinating, using pKD13 as a template for amplification of an \(aph\) cassette consisting of \(aph\) flanked by FLP recombinase substrate sites (\(frt\)) and appropriate \(mre\) sequences (41, 190). Knockout alleles on linear fragments were recombined with the chromosome of strain DY329 carrying plasmid pCX16 [\(sdiA\)]. When plated under standard conditions (LB-kan at 30°C), the number of recombinants recovered in the presence of pCX16 were, at least, two to three logs higher than in its absence.
We used the following primer sets (chromosomal sequences are underlined):

For \textit{mreB}<>\textit{aph}, 5'-
\begin{align*}
\text{GACCTGGGTACTGCGAATACCCTCATTTATGTAAAGGACAAGGCATCGT} \\
\text{TGTAGGCTGGAGCTGCTTC-3'} \quad \text{(primer mreB(KO)5')} \text{ and 5'-} \\
\text{AGCCATCGGTTCCTCAATCAGGAAGACTTCACGGGCAACGCCCTGCGA} \\
\text{TTCCGGGGATCCCGTCGACC-3'} \quad \text{(mreB(KO)3')}; \text{ for mreC<>aph, 5'-} \\
\text{ATCGGATGCGAGGGAAGTGTCTGTTACCTGCGTGATCTGATACGA} \\
\text{TAAGTGTAGGCTGGAGCTGCTTC-3'} \quad \text{(mreC(KO)5')} \text{ and 5'-} \\
\text{AGCGATCCCCCGTTGCGGGGATCCCGTCGACC-3'} \quad \text{(mreC(KO)3')}; \text{ for mreD<>aph, 5'-} \\
\text{GTGGCGAGCTATCGTAGCCAGGGACGCTGGGTAATCTGGCTCTCTTTC} \\
\text{TAAGTGTAGGCTGGAGCTGCTTC-3'} \quad \text{(mreD(KO)5')} \text{ and 5'-} \\
\text{TCAGCAAGAAAATCCACGGCCAGAGCACCCCATTGACTACACTCCAGA} \\
\text{ATTCCGGGGATCCCGTCGACC-3'} \quad \text{(mreD(KO)3')}; \text{ for mreBCD<>aph, primers} \\
\text{mreB(KO)5'} \text{ and mreD(KO)3'}; \text{ for mreBC<>aph, primers mreB(KO)5'} \text{ and} \\
mreC(KO)3'; \text{ and for mreCD<>aph, primers mreC(KO)5'} \text{ and mreD(KO)3'}. \\
\end{align*}

Recombination yielded a set of six \textit{mre<>aph} derivatives of DY329/pCX16, which all showed a spherical phenotype. The six strains were transformed with pCH244 [\text{P}_{\text{lac}}::\textit{mreB}, \textit{mreC}, \textit{mreD}, \textit{yhdE}], and transformants of each reverted to rod-shape in an IPTG-dependent manner. Phage P1 was grown on a transformant (containing both pCX16 and pCH244) of each strain in the presence of 250 \(\mu\text{M}\) IPTG, resulting in a high-titer transducing lysate for each \textit{mre<>aph} allele. These lysates were then used to transduce \textit{Mre}^+ strains, PB103 or TB28,
using various strategies to avoid selective pressure for the accumulation of undesired suppressor mutations. Generally, this was accomplished by the introduction of appropriate correcting or suppressing \textit{mre}, \textit{sdiA}, or \textit{ftsZ} plasmids or phages into the \textit{Mre}^+ recipient before introduction of a chromosomal \textit{mre}<->\textit{aph} allele by transduction. For example, to obtain the \textit{MreBCD}-depletion strain FB30/pFB174 [\textit{mreBCD}<->\textit{aph}/\textit{cat araC P}_{BAD}::\textit{mreBCD}], TB28 was transformed with pFB174 prior to transduction of \textit{mreBCD}<->\textit{aph} and transductants were recovered at 30\textdegree C on LB-kan supplemented with chloramphenicol and 0.5\% arabinose.

Similarly, derivatives of PB103 carrying chromosomal \textit{mre}<->\textit{frt} alleles (Table 2-1, Table 2-2) were obtained by introduction of pFB112 [\textit{tet sdiA}] prior to transduction with the corresponding \textit{mre}<->\textit{aph} lysates. The resulting \textit{mre}<->\textit{aph}/\textit{pFB112} strains were then transformed with pCP20 [\textit{bla cat repA(ts) cl857(ts) P}_{\lambda R}::\textit{flp}] (32, 41), and plated at 30\textdegree C on LB containing ampicillin and tetracycline. Transformants were streaked on LB lacking ampicillin and incubated at 42\textdegree C to simultaneously induce production of Flp recombinase and block replication of pCP20. Kanamycin and ampicillin sensitive clones were purified, resulting in the desired \textit{mre}<->\textit{frt}/\textit{pFB112} strains. These strains were transformed with appropriate \textit{mre} plasmids and used for complementation analyses (Table 2-2). Growth of some of these transformants at 37\textdegree C and in the presence of IPTG led to simultaneous correction of the rod phenotype and competitive loss of pFB112 (Table 2-2), giving rise to depletion strains that lacked extra copies of
sdiA, such as the MreB-depletion strain FB17/pFB118/pFB124

\[ mreBCD<>frt|P_{\text{lac}}::mreB| P_{\lambda}R::mreCD].\]

For construction of mrd mutants we used the following primer sets
(chromosomal sequences are underlined):

For \( mrdAB<>aph, 5' \)-

\[
\text{CATCCTTATCACCCTGAGTGAATAAGGGAGCTTTTGAGTAGAAGACGCAGCGG}
\]

\[
\text{GTGTAGGCTGGAGCTGCTTC -3' (pbp2(KO)5') and 5'-}
\]

\[
\text{CGCCACCCATGACGCAGCTCGCTGCCTTC -3' (rodA(KO)3'); and for mrdB<>aph, 5'}-
\]

\[
\text{CGATCTGCCTGCAGCGAGTGTTGGTCTGCGCTCTGGCGGCTATCCATT}
\]

\[
\text{CCGGGGATCCGACGCGTCGACC-3' (rodA(KO)5') and (rodA(KO)3').}
\]

Recombination with the chromosome of DY329/pCX16 resulted in

FB29/pCX16 [\( mrdAB<>aph/sdiA \)] and FB20/pCX16 [\( mrdB<>aph/sdiA \)], which propagated as spheres. These strains were transformed with pTB59

\[[P_{\text{lac}}::mrdAB], which caused transformants to revert back to a rod shape in the presence of IPTG. P1 lysates were prepared on FB29/pCX16/pTB59 and FB20/pCX16/pTB59 transformants, and these were used to transduce

\( mrdAB<>aph \) and \( mrdB<>aph \) into PB103 or TB28 derivatives carrying appropriate complementing plasmids and/or phages.

For the P1 transduction experiments in Tables 2-4 and 2-7, we used the

\( mre<>aph \) and \( mrd<>aph \) lysates described above, except for the \( mrdB<>aph \) and \( lacIZYA<>aph \) transducing lysates (Table 2-4), which were prepared on strains FB22(\( \lambda.CH221 \)) and TB12, respectively.
**Growth conditions.** Cells were routinely grown in LB (0.5% NaCl), or M9 minimal medium supplemented with 0.2% casamino acids, 50 µg/ml L-tryptophan, and 0.2% maltose. Low-phosphate (LP) medium contained 100 µM Tris-Cl (pH 7.5), 10 mM KCl, 17 mM NaCl, 29 mM NH₄Cl, 0.3 mM KH₂PO₄, 2 mM MgSO₄, 1 mM CaCl₂, 50 µM thiamine, 20 µg/ml L-tryptophan, 0.2% casamino acids, and 0.2% of maltose or glucose. When appropriate, medium was supplemented with 50 µg/ml ampicillin (Amp), 50 µg/ml spectinomycin (Spec), 25 µg/ml kanamycin (Kan), 25 µg/ml chloramphenicol (Cam), and/or 12.5 µg/ml tetracycline (Tet), except for cells carrying pTB63 in which case 5.0 µg/ml Tet was used. Other additions are specified in the text. Unless indicated otherwise, strain DY329 and derivatives were incubated at 30°C and other strains at 37°C.

**Immunofluorescence and confocal microscopy.** On-slide immunofluorescence staining methods that work well with rods and filaments (1,80) did not result in consistent labeling of FtsZ structures in large spheroids. We therefore developed a protocol for immunostaining of cellular structures in which incubations with lysozyme and antibodies are done in solution. Briefly, cells were fixed by adding 1 ml of culture directly to a mixture of formaldehyde and gluteraldehyde in NaPO₄ buffer (pH 7.5), giving final concentrations of 2.4%, 0.04%, and 30mM, respectively. The suspension was incubated for 10 minutes at room temperature followed by 50 minutes on ice. Cells were washed twice in 1 ml PBS (10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH
7.4), once in 1 ml GTE (20mM Tris.Cl, 50mM glucose, 10mM EDTA, pH 7.5), and resuspended in 0.4 ml of GTE. Aliquots (0.1 ml) of cells were treated with egg white lysozyme from a freshly prepared stock of 0.4 mg/ml in GTE to a final concentration of 16 µg/ml and incubated for 2-4 minutes at room temperature. Cells were washed once in 1 ml PBS and resuspended in 1 ml PBS containing 2% BSA. During the subsequent blocking and antibody incubation steps, samples were gently mixed in an end-over-end tube mixer. After 1 hr at RT, affinity-purified rabbit polyclonal anti-FtsZ antibodies (80) were added, and incubation was continued overnight at 4°C. Cells were washed once with 1 ml PBS, resuspended in 1 ml PBS containing 2% BSA and a 1:2500 dilution of Alexa-488 conjugated anti-rabbit IgG (Molecular Probes), and incubated for 2 hr at RT. Cells were washed twice with 1 ml PBS, resuspended in 50 µl PBS, and aliquots were spotted onto poly-L-lysine coated coverslips. Confocal microscopy was performed with a Zeiss LSM 510 inverted laser-scanning microscope using a 100X (NA 1.45) oil immersion objective. Images were collected using 488 nm excitation light from an argon/krypton laser, a 560-nm dichroic mirror, and a 500-550-nm band pass barrier filter. For all images, a z series was collected at 0.2 µm increments. Image processing, including projections and 3D rotations, were performed using the LSM 510 software (Version 2.5).

**Wide-field microscopy.** The cells in figures 2-9 and 2-13G were imaged on a Leica DM IRE2 microscope outfitted with a CoolSnap HQ camera (Photometrics) and a piezo driven 100X (NA 1.4) oil objective. Optical sections
were collected at a 0.2 µm step size, and with Cy3- and/or GFP-specific filter sets. Images were deconvolved through 40 iterations of a blind deconvolution algorithm provided in the Leica AS MDW package. When indicated, either deconvolved individual slices or maximum projections of the deconvolved image stack are shown. All other wide-field images, including time-lapse series, were obtained with a Zeiss Axioplan-2 microscope setup as previously described (95). Live cells were imaged using clean but otherwise untreated microscope slides.

Fluorescent dyes (Molecular Probes) were visualized using Cy3-specific (FM4-64 and CellTrace BODIPY TR methyl ester) or GFP-specific (FM1-43 and Lucifer Yellow CH) filter sets. FM1-43 and FM4-64 were added to live cells immediately before imaging at a final concentration of 0.25 and 0.50 µg/ml, respectively, except for the pulse labeling experiment in figure 2-11(D-G). For the latter, cells were concentrated 20-fold, incubated with FM 4-64 (20 µg/ml) for 5 minutes at 37°C, washed once in pre-warmed medium, resuspended in the same to the original cell density, and grown at 37°C for an additional 30 minutes before imaging. CellTrace BODIPY TR methyl ester (BTME) was used at 0.25 µM and was added to live cells 15 min before imaging. For figure 2-12 (A-D), cells were first chemically fixed as described above, washed once in PBS and resuspended in PBS containing BTME. After 15 min at RT, cells were washed again in PBS, and imaged. For Lucifer Yellow (LY) internalization assays, the dye was included in the growth medium at the time of inoculation at a final concentration of 50 µg/ml. Upon reaching the desired optical density, 0.5 ml of culture was subjected to gentle centrifugation (6,000 x g for 20 sec in a microfuge) at room
temperature. The supernatant was carefully removed and cells were resuspended in 1 ml pre-warmed medium by gently pipetting up and down twice. Cells were pelleted as before, resuspended in 50 µl pre-warmed medium, and imaged immediately.

**Phospholipid synthesis assay.** Strain FB21/pFB149 was grown overnight at 37°C in LP-glucose medium supplemented with Amp (LP-Amp) and 200 µM IPTG. Cells were washed once in LP-Amp and then used to inoculate two cultures containing 50 ml LP-Amp and either no or 1 mM IPTG to OD$_{600}$=0.001. Cultures were shaken at 37°C and both the increase in optical densities and changes in cell morphologies were monitored. At the indicated time points (T1 and T2) three aliquots were removed from each culture. A 1.0 ml aliquot was used to chemically fix cells for later determination of average cell dimensions, while a 5.0 ml aliquot was used to prepare a whole cell extract for determination of total cell protein per ml culture. To measure incorporation of $^{32}$P$_i$ in total phospholipid, 0.8 ml of culture was mixed with pre-warmed $^{32}$P$_i$ (8000 Ci/mmol) to 50 µCi/ml, and incubation was continued for 15 min at 37°C. Phospholipids were extracted essentially as described (18). Briefly, 3 ml of a 1:2 chloroform-methanol mixture was added, and the mixture was vortexed vigorously for 30 sec followed by incubation on ice for 15 min. The mixture was vortexed briefly after sequential addition of 1 ml each of chloroform and water, and then subjected to centrifugation at 5,800 x g for 15 min at 4°C. A 1.4 ml aliquot of the lower phase was washed by sequential addition of 1.40 ml methanol and 1.26 ml
water, followed by centrifugation as before. A 0.2 ml aliquot of the lower phase was then mixed with 5.0 ml Econosafe cocktail (RPI) and radioactivity was measured with a Beckman scintillation counter. Values were converted to DPM/µg whole-cell protein.

**Cell dimensions and geometrical considerations** Cell dimensions were measured from DIC images using Object-Image 2.15 (178). The position of the long axis in spheroids was judged by eye and the short axis was measured perpendicular to the long one. The volume \( V \), surface \( S \) and circumference \( C \) of a sphere were calculated using \( V_s = \frac{4}{3}\pi r^3 \), \( S_s = 4\pi r^2 \), and \( C_s = 2\pi r \), and those of a rod (capsule) with \( V_c = \frac{4}{3}\pi r^3 + \pi r^2 h \), \( S_c = 4\pi r^2 + 2\pi rh \), and \( C_c = 2\pi r \), with \( r \)=radius and \( h \)=cylinder length. The volume of a prolate spheroid \((c>a)\) was obtained using \( V_{sph} = \frac{4}{3}\pi a^2 c \) with \( c \)=polar radius and \( a \)=equatorial radius.

**Other methods** Whole cell extracts were prepared as described (80). Protein concentrations were measured using the Non-interfering protein assay (NI™, G-Biosciences), with BSA as standard. Quantitative Western analyses were done essentially as before (96).

**Results**

Construction of unsuppressed *mre* and *mrd* null and depletion strains of *E.coli*. 
To study the physiological relevance of the Mre proteins in *E. coli*, we used λ-red-mediated recombineering to create sets of strains carrying chromosomal *mre* mutations. Careful construction and maintenance of these strains was prompted by our initial observations, consistent with those by others (104, 155), that propagation of *mre* mutants under common growth conditions appears to select for progeny that produce an elevated level of the division protein FtsZ. Thus, our first attempts to create *mre<>aph* lesions (Fig. 2-1A) in the recombineering strain DY329 [λ.cI857 Δ(cro-bioA)] yielded no or very few recombinants (data not shown), suggesting the lesions caused death. This result was not in agreement with the viability of strains carrying the classical *mreB129* or *mre-678 [Δ(mreB-rng)]* alleles (181).

A clue to what hindered the construction of *mre* knockouts came from observing strain PA340-678/pCH244 [Δ*mreBCD*/*P* lac::*mreBCD*], which carries the classical chromosomal *mre*-678 allele (180) and a complementing plasmid containing *mreBCD* downstream the *lac* promoter (Fig. 2-1A). Cells grew as spheres in the absence of IPTG and as rods in its presence. In the latter case, however, a small but significant fraction of rods contained polar septa leading to the production of minicells. Minicells were not observed in strain PA340/pCH244, indicating that their formation by the shape-corrected mutant was neither a property shared by its Mre⁴ parent nor induced by expression of the *mre* genes from the plasmid (data not shown). Because co-overexpression of the *ftsQ*, *A* and *Z* genes both suppresses the lethality of *mrd* lesions (133, 177) and induces minicell formation (186), we hypothesized that similar to *mrd*: i) The *mre* genes in
E. coli might be essential, explaining our difficulties in obtaining null alleles. ii) Existing mre mutants may have accumulated second site mutations that lead to an elevated level of FtsQ, FtsA, and/or FtsZ, and iii) Elevated levels of the division proteins restores viability and allows mre mutants to propagate as spheres.

Accordingly, quantitative Western analyses showed that the classical mreB129 and mre-678 derivatives of strain PA340 (181) contained about two times more FtsZ than the parent (Table 2-3). Moreover, introduction of plasmid pCX16 (sdiA) in the recombineering strain DY329 now allowed the recovery of viable mre knockout derivatives at frequencies that were at least two logs higher than without the plasmid (data not shown). SdiA positively regulates a promoter (ftsQ2p) upstream of ftsQAZ, and cells carrying pCX16 contained 3 to 4 fold the normal level of FtsZ (184)(Table 2-3). The use of DY329/pCX16 for recombineering allowed for viable mrd knockout derivatives (Fig. 2-1B) to be readily obtained as well.

Suppression of mre and mrd lethality by pCX16 (sdiA) was also evident when knockout alleles were transferred by P1-mediated transduction from the original DY329/pCX16 recombinants to strains of different backgrounds (Table 2-4, and results not shown). For example, the presence of pCX16 in the acceptor strain PB103 had little effect on the transduction frequency of a lacIZYA<>aph allele, but it increased the number of viable mre<>aph or mrdB<>aph transductants by about two logs. Thus, the mre<>aph and mrdB<>aph alleles were all detrimental to survival, but viability was enhanced by increased SdiA activity in all cases.
The presence of the sdiA plasmid was not absolutely required for allelic transfer as transduction of mre<>aph alleles to PB103/pGB2 did yield rare spherical transductants (Table 2-4). Further analyses of one of these (FB2sup [mreB<>aph]) showed that its FtsZ level was over two-fold higher than normal (Table 2-3), indicating it had undergone a second alteration leading to increased production of the division protein. To avoid a selective advantage for such undefined (and undesired) suppressors of Mre− or Mrd− lethality, care was taken to provide all knockout strains with complementing (Mre+, or Mrd+) and/or lethality-suppressing (SdiA+, or Fts+) plasmids or phages during subsequent strain manipulations.

Polarity of mreB and mreC lesions.

Initial complementation experiments indicated that the mre<>aph alleles were polar on expression of downstream genes, precluding a firm conclusion as to the role of each gene (not shown). We proceeded with complementation experiments using strains in which the aph gene had been evicted by FLP recombinase, leaving only the 82 bp frt scar sequence on the chromosome (41). To maintain viability, these strains also carried the sdiA plasmid pFB112 [tetA, sdiA], a ColE1 derivative conferring tetracyline resistance. As expected, each of the mre<>frt/pFB112 strains grew as spheres. Complementation was studied with a set of six ColE1 derivatives, which confer resistance to ampicillin and carry one or more of the mre genes downstream of the lac promotor (Table 2-1, Fig. 2-1A). The mre<>frt/pFB112 strains were transformed with each one of the P_{lac::mre}
plasmids. Cells were plated on LB agar containing ampicillin and IPTG, and transformants were examined for both cell morphology and loss of the incompatible pFB112 plasmid.

As summarized in Table 2-2, plasmid pCH244 [P\textsubscript{lac}::\textit{mreBCD}] was capable of restoring a rod-shape to each of the \textit{mre<><frt} strains. In addition, pCH235 [P\textsubscript{lac}::\textit{mreD}] restored rod-shape in the \textit{mreD<><frt} strain. However, the \textit{mreB<><frt} lesion failed to be restored by pFB118 [P\textsubscript{lac}::\textit{mreB}] unless cells also harbored pFB124 [P\textsubscript{\lambda R}::\textit{mreCD}], a compatible plasmid carrying \textit{mreC} and \textit{mreD} downstream a temperature inducible \textit{\lambda P\textsubscript{R}} promotor. Similarly, the \textit{mreC<><frt} allele could only be corrected by P\textsubscript{lac}::\textit{mreC} plasmids that carry \textit{mreD} in-cis (pCH244 or pFB121), or when \textit{mreD} was co-expressed in-trans from pFB128 [P\textsubscript{\lambda R}::\textit{mreD}]. Others previously noted that frameshift or \textit{frt} deletion-replacement lesions in \textit{mreB} are polar on the expression of \textit{mreC} and \textit{mreD} (137, 183). Our complementation results are consistent with this and further show that the chromosomal \textit{mreC<><frt} lesion (Fig. 2-1A) is similarly polar on the expression of \textit{mreD}.

Whereas each transformant in which rod shape was restored had lost pFB112, all transformants that remained spherical had retained this \textit{sdiA} plasmid, even though the antibiotic in the medium (Amp) favored maintenance of the incompatible P\textsubscript{lac}::\textit{mre} competitors (Table 2-2). Apparently, pFB112 provided all spherical \textit{Mre}\textsuperscript{-} cells with a selective advantage, supporting the conclusion that extra copies of \textit{sdiA} allowed them to propagate.
Each *mre* gene is required for both maintenance of rod shape and normal viability.

To study unsuppressed Mre⁻ phenotypes, we used strains that lack an *sdiA* plasmid, and in which transcription of one or all of the *mre* genes can be shut off by omitting IPTG or arabinose from the growth medium. Specific depletion of MreB or MreC was accomplished by supplying cells with an appropriate source of MreC and/or MreD to compensate for the polarities associated with the chromosomal *mreB* and *mreC* lesions described above.

As shown in figure 2-2, cells of strain FB17/pCH244 [*mreBCD<>/frt/Pₐₐc::mreBCD*] (Row 8) grew about as well as the control strain PB103/pCH244 [wt/ Pₐₐc::mreBCD] (Row 7) on LB at 37°C in the presence of IPTG (Columns A-C). Growth of FB17/pCH244 was negligible in the absence of inducer (Columns D-F), however, confirming that depletion of all three Mre proteins severely limits the ability of cells to propagate. Identical results were obtained upon the specific depletion of MreB, MreC, or MreD separately, using strains FB17/pFB118/pFB124 [*mreB<>/frt / Pₐₐc::mreB / Pₐ₀ₐl::mreCD*] (Row 2), FB10(λ.FB120)/pFB128 [*mreC<>/aph(Pₐₐc::mreC)/Pₐₐl::mreD*] (Row 4), or FB11(λ.CH235) [*mreD<>/aph(Pₐₐc::mreD)] (Row 6), respectively. As detailed further below, depletion of any of the Mre proteins caused cells to grow into large spheres that failed to divide properly (Table 2-5).

These results confirmed that all three *mre* genes are required to maintain the rod shape of *E.coli* cells (180). Others recently concluded that all three *mre*
genes are also essential for viability (104, 183). However, Kruse et al did not consider frt-associated polarity in the mre operon, leaving the possibility that only the loss of MreD affected viability (104). The transduction results by Wachi et al are more convincing, although it is unclear whether each of the mre plasmids used in their study would be capable of correcting the corresponding mre lesion(s) in the chromosome (183). Our complementation and depletion results are comprehensive and show quite conclusively that each of the three Mre proteins is indeed required for normal viability. Note, however, that the proteins are only conditionally essential, as shown by subsequent experiments discussed below.

**Overexpression of FtsZ is sufficient to restore viability to Mre− cells.**

In addition to stimulating transcription of the ftsQAZ division genes (184), SdiA affects the expression of many other genes as well (187).

To test whether an increased level of just FtsZ is sufficient to suppress Mre− lethality, we used strain FB30/pFB174 [mreBCD<->aph/P_{BAD}::mreBCD] carrying either pDR3[P_{lac}::ftsZ], or the vector control pMLB1113. Aliquots of serially diluted cultures were spotted on LB agar supplemented with either 0.5% arabinose, 0.1% glucose, or 0.1% glucose plus 100 µM IPTG. Both strains grew well in the presence of arabinose (MreBCD+) but failed to form colonies in the presence of glucose (MreBCD−) when IPTG was absent. The presence of IPTG, however, specifically allowed pDR3-carrying cells to grow in the presence of glucose.
(MreBCD<sup>–</sup>, FtsZ<sup>++</sup>), showing that overproduction of FtsZ is indeed sufficient to overcome the growth defect of Mre<sup>–</sup> cells (Fig. 2-3A).

The antibacterial compound S-(3,4-dichlorobenzyl)isothiourea (A22) causes a rod to sphere shape conversion in *E. coli* and other Gram- species, and likely interferes directly with the activities of the MreB protein (74, 94, 103). Therefore, the results above predicted that *ftsZ* overexpression might also alleviate A22 toxicity. This was indeed the case as determined by spot-titer analyses of *wt* strain TB28 carrying either pDR3 [wt/Plac::ftsZ] or the vector control pMLB1113. Cells were spotted on LB agar containing either no A22, 10 µg/ml A22, or 10 µg/ml A22 plus 50 µM IPTG. A22 prevented colony formation in the absence of IPTG, but overexpression of *ftsZ* in the pDR3-carrying cells overcame the toxicity of A22 to a significant degree (Fig. 2-3B). Cells in the latter case propagated as spheres (not shown), indicating that *ftsZ* overexpression did not interfere with the effect of A22 on cell shape, but rather allowed resultant spheres to survive and grow.

**The Mre proteins are dispensible for viability at slow growth rates.**

In the experiments described thus far, cells were cultured with rich (LB) medium at 30°C or 37°C. Growth phenotypes of Mre<sup>–</sup> cells were characterized more rigorously by depleting MreBCD from derivatives of two parent strains, PB103 and TB28, on both rich (LB) and poor (M9) medium, and at three different temperatures (37°C, 30°C, and 20°C). The parent strains have distinct backgrounds and TB28 grows significantly faster than PB103, especially on
minimal medium (Table 2-6). Depletion strains FB17/pFB149 and FB21/pFB149 ($\Delta mreBCD/P_{lac}::mreBCD$) and their respective parent controls, PB103/pFB149 and TB28/pFB149 (wt/$P_{lac}::mreBCD$), were subjected to spot-titer analyses on medium containing either 0.1% glucose or 250 µM IPTG. The results (shown in Fig. 2-4) are summarized in Fig. 2-3C and Table 2-6. As anticipated, FB21/pFB149 failed to grow in the presence of glucose (MreBCD−) under most conditions (Row 2 in Fig. 2-3C, and even Rows in Fig. 2-4B). Strikingly, however, it grew almost as well as the parent control on minimal medium at room temperature (~20°C) (Row 4 in Fig. 2-3C, and Row 12 in Fig. 2-4B). Strain FB17/pFB149 (see Fig. 2-4A) similarly failed to grow in the presence of glucose on LB at 37°C and 30°C (Rows 2 and 4), but grew about as well as its parent control on M9 at both 30°C and 20°C (Rows 10 and 12), and even formed some tiny colonies on LB at 20°C and on M9 at 37°C (Rows 6 and 8). When growing in the presence of glucose under permissive conditions, cells of either depletion strain propagated as spheres (not shown), indicating that expression of $mreBCD$ from the plasmid was sufficiently repressed. When compared to the doubling times of the parent strains in liquid medium under comparable conditions (Table 2-6), these results indicated that while Mre functions are essential for viability at moderate to fast growth rates ($T_d<150$ min), they become dispensible during slow growth ($T_d>300$ min).

These observations were further supported by P1 transduction experiments in which wt cells were mixed with a $mreBCD<>aph$ lysate and incubated at
different temperatures on selective M9 plates. The results paralleled those of the
depletion experiments in that normal transduction frequencies of TB28 and
PB103 could be attained, but only at sufficiently low temperatures (Table 2-7).
The resulting mreBCD<>aph derivatives of TB28 and PB103 were spherical and
grew about as well as their wt parents on minimal medium at low temperatures.
Moreover, even after several rounds of purification under permissive conditions,
they failed to grow on richer medium and/or at higher temperature (not shown).
These results indicate that conditions of slow growth relieve the selective
pressure on Mre^- cells to acquire alterations that lead to increased FtsZ
production. One possibility is that slow growth itself leads to an increased FtsZ
level. We detected no significant changes in FtsZ levels between fast and slow
growing cells of PB103 or TB28 (Table 2-6), however, arguing against this
possibility.

The ability of unsuppressed Mre^- cells to propagate under slow growth
conditions suggested that slow growth might also reduce the toxicity of A22.
Figure 2-3D shows that while A22 prevented growth of TB28 on LB agar at
37°C, the drug was indeed markedly less effective at inhibiting colony formation
at 20°C.

**Suppression of Mrd^- lethality by ftsZ or slow growth.**

Co-overexpression of ftsQ and ftsA with ftsZ was previously found to be
required to restore viability to mecillinam-treated cells on rich medium (133). This
predicted that, in contrast to Mre-depleted spheres (see above), overexpression
of ftsZ by itself might not be sufficient to rescue Mrd-depleted ones. We addressed this issue using strain FB40(λ.TB59) [mrdAB<\rightarrow frt λ.Plac::mrdAB] carrying either pTB182 [PQaz::ftsQaz], pTB188 [PλR::ftsZ], or a vector control (pGB2). Aliquots of serially diluted cultures, including the Mrd+ parent controls, were spotted on LB agar containing either 100 µM IPTG or 0.1% glucose. All strains grew well in the presence of IPTG (MrdAB+). MrdAB-depleted cells carrying the vector control failed to grow, but those that carried either plasmid pTB182 or pTB188 (MrdAB−, FtsQAZ++ or MrdAB−, FtsZ++, respectively) plated efficiently (Fig. 2-5). Moreover, pTB188 [PλR::ftsZ] rescued MrdAB-depleted cells about as well as cells that were depleted for MreBCD in a parallel control experiment (Fig. 2-5). We conclude that elevated expression of ftsZ is sufficient to alleviate the growth defects of both Mre− and Mrd− cells.

Wildtype cells resist killing by mecillinam on poor medium (10, 98), and a mrdB (rodA) null mutant was reported to survive on poor medium as well (45). Hence, it was likely that slow growth conditions would also allow unsuppressed MrdAB− spheres to propagate. To verify this, spot titer analyses were performed with the MrdAB-depletion strains FB39(λ.TB59) and FB40(λ.TB59) [ΔmrdAB(Plac::mrdAB)], and their parent controls, PB103(λ.TB59) and TB28(λ.TB59) [wt(Plac::mrdAB)], respectively. As summarized in Table 2-6, the results (provided in Fig. 2-6) were similar to those obtained with the MreBCD-depletion strains. Although the depletion strains failed to grow in the presence of glucose (MrdAB−) on LB agar at 37°C and 30°C, they grew about as well as the parent controls under conditions favoring slower mass increase. Transduction
experiments again supported these observations in that stable \textit{mrdAB<->aph} derivatives of both PB103 and TB28 could be obtained at normal frequencies as long as they were selected for and maintained under conditions avoiding too rapid growth (Table 2-7, and data not shown).

**Suppression of MreBCD\(^{-}\) lethality by RelA\(^{-}\).**

Cells become resistant to killing by mecillinam when concentrations of the stringent alarmone ppGpp rise above a threshold that is still well below that needed to stop growth altogether (98, 176). Given the similar growth requirements of Mrd\(^{-}\) and Mre\(^{-}\) spheres noted above, we suspected that the lethality associated with depletion of the MreBCD proteins on rich medium might be suppressed by increased ppGpp as well. To test this, we stimulated alarmone synthesis in \textit{wt} and Mre-depleted cells by IPTG-induced expression of an overactive form of (p)ppGpp synthase (RelA\(^{-}\), lacking residues 456-744) (98, 154) from plasmid pYT11[P\(_{lac}\)::relA\(^{-}\)]. As expected, inducer caused a reduction in the growth rate of pYT11-carrying cells, and growth ceased completely at 250 \(\mu\)M IPTG. In LB medium without arabinose and containing only 50 \(\mu\)M IPTG, mass doubling times increased from 59 to 103 min in WT rods (TB28) and from 76 to 108 min in MreBCD-depleting spheres (FB30/pFB174) (Table 2-8). Spot-titer analyses showed that expression of RelA\(^{-}\) under these conditions was sufficient to suppress the lethality of MreBCD-depleted spheres (Fig. 2-3E). Although the modestly reduced growth rate might have contributed to the ability of Mre\(^{-}\) spheres to survive in this experiment, this is unlikely to be the sole explanation,
as spheres in which relA is not artificially induced fail to survive unless the
doubling time surpasses 150 min, at least (Table 2-6).

Production of RelA was accompanied by a substantial reduction in the
average size of both WT rods and MreBCD spheres (Table 2-8), indicating an
elevated division frequency. Quantitative Western analyses indicated a modest
increase (~30-60%) in relative FtsZ levels in the exponentionally growing, RelA'-
producing, rods and spheres (Table 2-8). Whether the combination of a reduced
mass doubling rate with this small increase in FtsZ is sufficient to explain the
significantly smaller size of RelA' producing cells, and/or to explain the ability of
the spheres to propagate under these conditions, is presently unclear. Either
way, these results further emphasize the similarities between mecillinam-induced
(PBP2'), and Mre' spheres.

We conclude that the growth requirements of genetically unsuppressed Mre-
and Mrd' spheres are quite similar. Both are viable at low growth rates, and their
death at high growth rates can be prevented by an increase in ppGpp levels
and/or an extra supply of just the FtsZ division protein.

**Conditional lethality of Mre' cells is associated with a division defect
and abberant assembly of FtsZ.**

The finding that ftsZ overexpression allows propagation of Mre' cells under
nonpermissive growth conditions suggested that, as what was inferred for
spherical mrd mutants (177), the lethality associated with loss of mre might be
primarily caused by a division defect. The phenotype of Mre' cells supported this
possibility. Strains completely lacking one or more of the Mre proteins, but carrying an sdiA plasmid, grew as spheres of various sizes. Many of these appeared to be in the process of constriction, and immunostaining with anti-FtsZ antibodies showed the protein associated with these sites. In most of the smaller spheres, FtsZ had accumulated in well defined rings, although some rings showed atypical branches (Fig. 2-7A). About 10-20% of these populations consisted of distinctly larger cells, likely due to unequal inheritance of the suppressing sdiA plasmid. In these cells, FtsZ invariably appeared assembled in more complex patterns that often included isolated patches and foci as well as more extended structures that failed to span the girth of the cell, but appeared branched and/or folded back on themselves (Fig. 2-7B).

Depletion of each (or all) of the three Mre proteins under non-suppressing conditions resulted in a uniform giant-sphere phenotype (Table 2-5). For example, when cells of the MreB-depletion strain FB17/pFB118/pFB124 [mreB<>frt / P_{lac}::mreB / P_{λR}::mreCD] were shifted from LB medium containing IPTG to medium lacking the inducer, cells initially grew and divided as rods, but then lost rod-shape and ultimately formed very large spherical cells. FtsZ assembled in typical rings early during depletion (Fig. 2-8A,B), but the non-dividing large cells that formed later on again contained the protein in more complex patterns as described above (Fig. 2-8C-E).

To ensure that the odd FtsZ assembly patterns were not an immunostaining artifact, we also localized FtsZ in live Mre− spheres, using a GFP-fusion to the cytoplasmic FtsZ-binding protein ZapA as a convenient marker for FtsZ
assembly. Figure 2-9 shows cells of the MreBCD-depletion strain FB30(λCH268)/pFB174 [ΔmreBCD(P_{lac}::gfp-zapA)/P_{BAD}::mreBCD] inoculated in M9-maltose medium at 37°C. The medium lacked arabinose to shut down mreBCD expression and contained IPTG to induce expression of gfp-zapA. In addition, cytoplasmic membrane was visualized by treatment with the membrane dye FM4-64 immediately before microscopy. Early during depletion, cells appeared as fat rods, and GFP-ZapA (i.e. FtsZ) formed a ring or arc across the long axis of the rods (Fig. 2-9A,B). Later on, cells again had become large spheres with complex patterns of FtsZ accumulation (Fig. 2-9C,D), corroborating the results obtained with immunostaining.

Fig. 2-9D highlights features of a large Mre^- spheroid that appears in the process of division. It contains a shallow constriction perpendicular to the middle of its long axis and the bulk of FtsZ appears to have assembled in a zone around the constriction. The constriction is asymmetric, however, in that the bottom part of the cell shows a clear invagination that is associated with a fairly well-defined arc of FtsZ (panels D7,D8), whereas invagination is less obvious in the top part where FtsZ seems present in ill-defined clusters scattered about midcell (panels D5,D6). As Mre^- cells grow into very large spheres under these non-permissive conditions, we imagine that many such constriction attempts eventually abort.

One possible explanation for the failure of Mre^- spheres to divide properly under non-permissive growth conditions, and for the fact that extra FtsZ can restore division, is that the absence of the Mre proteins somehow caused a drop in the level of FtsZ. This was not supported by Western analyses, however, as
we detected no significant change in the level of FtsZ in non-dividing MreB-depleted spheres (Fig. 2-8F, lane 6) when compared to dividing rod-shaped control cells (lanes 4 and 5).

**Vesicle-like bodies in *E. coli* spheres.**

The cell in Fig.2-9D also shows another striking feature of Mre⁻ spheres under nonpermissive conditions, which is the presence of vesicle-like bodies in their interior. Imaging by DIC indicated the presence of a large vesicle-like body in the left-hand half of the cell and that of smaller ones elsewhere (panel D1). Most of these were not stained by the membrane-impermeable FM4-64 dye, suggesting that if these compartments were surrounded by membrane, it was discontinuous with the externally accessible CM. One of the optical slices shows a clear small circle of FM4-64 staining near the cell center, however, suggesting the presence of a finger-like involution of the CM at this site that reaches well into the body of the cell. Some FtsZ clusters surrounding this FM4-64 stained material can be seen as well (panel D5). Another projection of FM4-64 stain that appears continuous with the CM is visible in a plane near the cell bottom (panel D8).

Vacuole-like inclusions were previously noted upon inactivation of PBP2 (MrdA) by mecillinam in *E. coli* (113) and in a *S. typhimurium* rodA (*mrdB*) mutant grown on soft agar (36). In addition, they were observed in a number of *E. coli* shape mutants with ill-characterized lesions (2, 3, 82, 113, 140). Two of these older studies included thin section TEM analyses of the mutant cells. Allison (3)
performed these studies on a *mon* (*envB*) mutant (2), that may have been allelic with one of the *mre* genes (113, 181), while Henning et al (82) studied a temperature sensitive shape mutant (*lss12*) that may have been allelic to *mrdA* as it produced a thermolabile PBP2 (162). Both studies showed the presence of CM involutions, stacked cisternae, and vesicle-like compartments traversing the cytoplasmic space of large spherical cells. These compartments appeared lined by a unit membrane and their lumens lacked ribosomes, suggesting they formed by involutions of the CM. Whether all intracytoplasmic membrane was continuous with the CM was not assessed (3, 82).

To better define the genetic requirements for vacuolization in shape mutants, we depleted each of the Mre and Mrd proteins separately under non-suppressing conditions and observed cells by both membrane staining and DIC. In each case, cells formed large spheres with readily apparent vesicle-like inclusions (Table 2-5). Therefore, the phenomenon is not provoked by the absence of any of the shape proteins specifically, but is more likely a general consequence of growth as a non-dividing sphere per se. As this phenotype is inherently interesting, and correlates with the failure of spheres to divide properly, we studied the formation of vesicle-like inclusions in Mre− spheres in more detail.

**Involution of the cytoplasmic membrane and endocytosis in *E.coli* spheres.**

To ensure that the vesicle-like bodies we observed in spheres were bounded by cytoplasmic membrane we visualized the latter in live MreBCD-depleted cells...
with a fusion of GFP to the N-terminal transmembrane domain of the bitopic CM protein ZipA (TM-GFP) (96). The fluorescent fusion accumulated around each vesicle that was visible by DIC, suggesting they were indeed surrounded by CM (Fig. 2-10 B and C). Topologically, the lumen of these bodies is expected to correspond to extracytoplasmic space. If they are bounded by CM only, this space should correspond to the periplasm. Though unlikely, it is also conceivable that they are lined with both CM and OM, in which case the lumen is expected to be compartmentalized further. To probe these possibilities, we used a GFP fusion that is targeted to the periplasm via the twin arginine transport system (TT-GFP) (15). Panels G-I of figure 2-10 illustrate that TT-GFP indeed accumulated in the lumen of each vesicle. In addition, the fusion distributed evenly within vesicular space, indicating it was not compartmentalized further.

In most MreBCD-depleted spheres both TM-GFP and TT-GFP also accumulated in intracytoplasmic patterns that were not or poorly distinguishable by DIC (e.g. panels B2 and F2). These patterns likely corresponded to membrane compartments, such as small vesicles and/or stacked forms, with lumens too small to detect by DIC.

As mentioned above, only a subset of vesicle-like bodies showed accumulation of fluorescence at their periphery when the membrane-impermeable CM dye FM4-64 was added to Mre⁻ spheres immediately before microscopy (Fig. 2-11A-C). In contrast, when spheres were pulse-labeled with the dye 30 min prior to observation, virtually all bodies that were visible by DIC were now also clearly outlined by a fluorescent border (Fig. 2-11D-G). In
addition, as was observed with the \textsuperscript{TM}GFP and \textsuperscript{TT}GFP probes, the dye accumulated in various other patterns that traversed the sphere’s interior and that were often quite extensive. At the time of observation, the spheres in this experiment (Fig. 2-11D-G) had an average diameter of 3.7 µm (n=102) and 90% contained vesicle-like and/or other fluorescent patterns within their interior. Thus, the interior of the majority of Mre\textsuperscript{-} spheres contained a considerable amount of membrane originating, at least in part, from externally accessible CM. Moreover, the fact that a substantial subset of vesicular bodies were not immediately accessible to FM4-64 (e.g. Fig. 2-11A and B) suggested that they were discontinuous with the external CM and periplasm and, thus, represented ‘true’ vesicles.

The latter inference was confirmed by three additional experiments. In the first, spheres were incubated with the membrane-permeable membrane dye CellTrace BODIPY TR methyl ester (BTME) for 15 min, and then mixed with the membrane-impermeable CM dye FM1-43 just prior to microscopy. As expected, BTME stained the membrane of each vacuole that was visible by DIC as well as additional internal membrane structures, whereas FM1-43 only stained a subset of these structures (Fig. 2-11H,I). In the second, spheres producing \textsuperscript{TT}GFP were resuspended in isotonic buffer and observed by time-lapse microscopy immediately after addition of lysozyme. Upon disintegration of the murein wall, a subset of vesicle-like bodies released \textsuperscript{TT}GFP into the medium, implying their lumen was continuous with the exterior periplasm. Over half remained intact however, showing they were topologically separate from the cell’s exterior CM.
and periplasm (Fig. 2-10J, and data not shown). In the third experiment, WT rods or Mre^- spheres were grown in the presence of lucifer yellow (LY), a water-soluble, membrane-impermeant fluorescent compound that is excluded from the cytoplasm by the CM barrier, but should have access to the periplasm as it is sufficiently small (522 D) to pass OM porins (136). Cells were then washed in medium lacking LY and immediately imaged live. Cells of the wt parent TB28 completely failed to retain LY, indicating that if the dye entered the periplasm it readily washed out again (Fig. 2-11J). Similarly, the dye appeared absent from the external periplasm of Mre^- spheres, as well as from a subset of vesicle-like bodies, suggesting that these were still continuous compartments. However, the dye remained trapped in the majority of vesicle-like bodies that were visible by DIC, indicating that this subset had become topologically separate from the external CM and periplasm and, thus, represented ‘true’ endosomes (Fig. 2-11K and L).

We conclude that under non-permissive growth conditions spherical Mre^- cells form extensive involutions of the CM into the cytoplasm, resulting in elaborate membraneous compartments in the cell interior. Moreover, many of the involutions must be subject to an endocytic-like membrane fission event that releases them as a closed compartment into the cytoplasm.

**Membrane involution and scission do not require FtsZ assembly.**
What causes the involution of CM and the generation of internal vesicles in spherical cells? We considered the possibility that unsuccessful attempts at cell division by spherical cells under non-permissive conditions might be a primary cause of CM invasion into the cytoplasm. Therefore, we examined the ability of Δmre cells to accumulate internal membrane while FtsZ polymerization in the cells was inhibited by the SfiA (SulA) protein. Strain FB30/pTB63/pDR144 [ΔmreBCD / P_QAZ::ftsQAZ/ P_{lac}::sfiA] was grown in the absence or presence of IPTG, and cells were stained with BTME to visualize membrane. In the absence of inducer (SfiA^-), the extra supply of FtsZ from pTB63 allowed these ΔmreBCD cells to grow and divide as small spheres (Fig. 2-12A,C). Over 50% of the spheres were in the process of constriction (Fig. 2-12D), and no obvious internal membrane accumulations were detectible by DIC or fluorescence microscopy under these conditions (Fig. 2-12A). In the presence of IPTG (SfiA^+), cells ceased constriction and increased in size as expected. After only about 2.6 mass doublings, over 60% of these non-dividing spheres contained internal membrane systems that were readily detectible by DIC and/or fluorescence microscopy (Fig. 2-12B-D). In addition, double staining with permeable (BTME) and non-permeable (FM1-43) membrane dyes (Fig. 2-12E), as well as LY-internalization assays (Fig. 2-12F), again indicated that many of the internal membrane structures in the non-dividing FB30/pTB63/pDR144 spheres were discontinuous with the external CM.

We conclude that FtsZ polymerization is not required for generation of the internal membrane compartments in spherical cells of E.coli.
Spherical cells fail to adjust the rate of phospholipid synthesis.

Though no data were shown and no implications were discussed, Henning et al mentioned that lss12 [mrd(ts)?] spheres synthesized phospholipids at the same rate as wt rods (82). This is notable as a rod-to-sphere conversion is accompanied by an increase in the volume to surface ratio (V/S). Hence, a failure to properly reduce the net rate of phospholipid synthesis after conversion to spheres would lead to excess membrane, which, in turn, could well be the primary cause of the extensive internal membrane systems that develop in *E. coli* *mre* and *mrd* mutants under non-permissive growth conditions. To address this possibility, we compared the incorporation of radiolabeled orthophosphate (³²P) into total phospholipid in MreBCD⁺ rods and MreBCD-depleted spheres of strain FB21/pFB149 [mreb<>aphl Pₗac::mrebCD]. Cells were inoculated in low-phosphate medium at 37°C in the presence (MreBCD⁺) or absence (MreBCD⁻) of 1 mM IPTG. Relative rates of phospholipid synthesis were determined using aliquots of cells taken at both mid- (T1) and late-logarithmic (T2) growth (Fig. 2-13). At both time-points the cells that were growing without IPTG were both distinctly spherical and maintained a mass doubling rate similar to that of their rod-shaped counterparts (Fig. 2-13A, C). Figure 2-13B shows that whereas the rate of phospholipid synthesis declined as cells of either shape entered stationary phase, the rates were virtually identical for both cell types at both T1 and T2.
It thus appears that upon a rod-to-sphere conversion, *E. coli* cells indeed fail to respond to their new surface requirements and continue to produce phospholipid membrane at a rate suitable for rod-shaped cells.

**Mislocalization of division proteins to internal membrane systems in Mre<sup>−</sup> spheres.**

The finding that depletion of any of the five shape proteins from fast-growing cells resulted in large non-dividing spheres with internal membrane systems raised the possibility that the division block in the spheres is causally related to the invasion of CM into their interior (Table 2-5). This idea was supported by the distribution of GFP-ZapA in Mre-depleted spheres. The fusion not only accumulated in various pattern on the exterior CM, but also in clusters and patches surrounding interior membrane compartments, indicating non-productive assembly of FtsZ on the latter (Fig. 2-14B-E).

The oscillating Min system normally controls placement of the division machinery by antagonizing FtsZ assembly on the CM at off-center sites. In *E. coli*, proper Min function relies on alternating bulk assembly and disassembly phases of the MinD ATPase on the CM at either cell end (Fig. 2-14F) (147, 149). We observed that MinD readily assembles on the intracytoplasmic membrane systems in spheroids as well. GFP-MinD and MinE were produced from a lysogenic phage during depletion of MreBCD in strain FB30(λ.DR122)/pFB174 [Δ*mreBCD*(P<sub>lac</sub>::*gfp*-minDE)/P<sub>BAD</sub>::*mreBCD*]. Consistent with other studies in which Min proteins were observed in spheroids (35, 155, 166), GFP-MinD was
seen to oscillate between opposite sites of the exterior CM, most commonly along the longest axis of the spheroids. In addition, however, GFP-MInD also transiently decorated interior vesicles that were visible by DIC. Decoration by the fusion was most obvious on any vesicle that resided near one of the MinD landing sites on the exterior CM (Fig. 2-14G). The fusion also associated transiently with internal structures not readily resolveable by DIC (Fig. 2-14H).

Panels H2 to H4 show oscillation of the fusion in a large spheroid that contained extensive internal membrane systems as visualized with the BTME dye. GFP-MinD moved from assembly sites in the spheroid’s interior (H2) to two opposite landing sites on the external CM (H3), and back again to interior sites (H4), and so on. Overlays of the GFP and BTME channels indicated that GFP-MinD accumulation sites in the sphere’s interior invariably coincided with the presence of internal membrane at these sites (H5-H8).

In a previous study, a YFP-MinD fusion was observed to sometimes assemble into subsurface loops and filaments in spheres of an mreB mutant (155). The membrane was not visualized in this study, and the significance of these puzzling MinD patterns remained unclear. Our results suggest that the ability of MinD to assemble on intracytoplasmic vesicles and membrane systems of more complicated shapes may also have been responsible for the subsurface loops and filaments, respectively, observed in that study.

We propose that the assembly of FtsZ and other division proteins on interior membrane systems contributes significantly to the lethal division defect in rapidly growing Mre⁻ and Mrd⁻ spheres. Non-productive assemblies on interior
membrane will compete with potentially productive ones on the exterior CM for pools of unassembled FtsZ and other septal ring components. As the amount of FtsZ, in particular, is normally limiting the number of functional septal rings that a cell produces (186), this competition must lower the division rate. Moreover, disturbances of Min dynamics by interior membrane is expected to compound the problem by allowing FtsZ assembly at too many inappropriate sites.

Discussion

Avoiding selective pressure for secondary genetic alterations, we created comprehensive sets of mre and mrd cell shape mutants of E.coli, and used these to study the importance of rod-shape to the viability of cells under various conditions. One notable general conclusion of our results is that a lack of one or more of each of the five Mre and Mrd shape proteins results in a very similar phenotype. In each case, mutant cells: i) grew and divided as small spheres that appeared genetically stable, under conditions of slow mass increase, ii) grew into giant non-dividing and non-propagating spheres with elaborate intracytoplasmic membrane systems, under more common conditions favoring moderate to fast mass increase, and iii) could be rescued by a modest increase in the production of FtsZ, which allowed them to grow and divide as small spheres at any growth rate. It is evident that, like the Mrd proteins (10, 12, 45, 98, 177), MreB, C and D are only conditionally essential. This result helps explain incongruous conclusions in the literature on the matter of mre essentiality in E.coli (104, 105,
155, 180, 181, 183). In addition, these similarities in mutant phenotypes lend further support to the notion that the Mre and Mrd proteins cooperate in the same complex and/or pathway to effect maintenance of rod-shape (29).

One striking feature of all shape mutants examined was the presence of elaborate membrane systems in the interior of the large non-dividing spheroids that formed under non-permissive conditions. Although de novo formation cannot be excluded, our results indicate that many, if not all, of these interior membrane systems originated from FtsZ-independent involution and elaboration of the CM. Importantly, MreBCD-depleted spheres failed to adjust phospholipid synthesis to their new surface requirements, but rather continued synthesis at about the same rate as rod-shaped control cells. This has interesting implications for the regulation of phospholipid synthesis (38, 145), as it suggests the absence of a mechanism that couples membrane synthesis to actual cell surface requirements. One intriguing possibility is that allowing or mediating such coupling is actually one of the functions of the shape proteins. If so, the mechanism seems to require all five Mre and Mrd proteins, as depletion of each resulted in excess internal membrane. Alternatively, a mechanism that 'senses' the surface and transmits its state to the phospholipid synthesis machinery does not exists in E.coli. Rather, the rate of phospholipid synthesis may be primarily, or solely, coupled to the rate of increase in cell mass (142), and being rod-shaped is somehow hardwired in the system to arrive at a proper membrane/mass ratio. In any event, the failure to reduce phospholipid synthesis
upon becoming spherical provides a ready rationale for the ‘excess’ membrane in the interior of Mre\(^{-}\) and Mrd\(^{-}\) spheroids under non-permissive conditions.

It is also remarkable that while some of the internal membrane systems in non-dividing spheroids were continuous with the external CM, others were clearly not. In addition, several assays indicated that many, if not all, of the discontinuous systems had at some point been continuous with externally accessible CM. This implies that the discontinuous intracytoplasmic compartments resulted from involution of the CM followed by a membrane scission event that released them in the cytoplasm. We are unaware of any other well-documented example of membrane endocytosis in *E.coli* or related bacteria. Though the present example concerns cells that are not in a physiologically ‘normal’ state, it does raise the question of what is responsible for the scission event that separates the ‘endosomes’ from the external CM. Membrane fusion or fission rarely occur spontaneously (33, 122), suggesting the possibility that some enzymatic activity in *E.coli* is capable of stimulating endosome formation.

Given the failure to adjust phospholipid synthesis rate, one might also expect an excess of outer membrane (OM) in MreBCD-depleted spheroids under non-permissive conditions. Indeed, we did observe an occasional TTGFP-filled bleb emanating from the surface of FB30/pFB174/pTB6 [ΔmreBCD / P\(_{BAD}\)::mreBCD / P\(_{lac}\)::\(\text{ss} torA-gfp\)] spheroids grown in the absence of arabinose and presence of IPTG. However, the occurrence of such blebs seemed rather low (about one out of every ten spheres), and the culture medium seemed similarly barren of free fluorescent vesicles (data not shown). It is possible that excess OM was shed as
vesicles that were too small to readily detect by fluorescence microscopy in this fashion (< 300 nm (71)). Alternatively, perhaps phospholipid transport to the OM does slow down to a rate that is more appropriate to the surface requirement of a spheroid, and almost all of the excess phospholipid the cell produces is incorporated in the intracytoplasmic systems. More work will be needed to distinguish between these possibilities.

This and previous studies indicate that the inability of shape mutants to propagate under non-permissive conditions is primarily due to a cell division defect. A popular explanation for this is that the amount of FtsZ that is normally available is insufficient to accomodate the increased circumference of a cell after it converts from rod to sphere (104, 177). However, following through on this geometrical argument indicates that it is incomplete, at best. For example (Fig. 2-15), consider that FtsZ concentration remains constant and, thus, that its amount increases with cell mass (volume). Further assume a rod-shaped cell with a diameter (D) of 1.4 μ that is about ready to begin Z-ring assembly/constriction when it reaches 3.6 μ from tip to tip. This cell then has a volume (V) of 4.82 μ³ (line b in Fig. 2-15), a surface (S) of 15.83 μ², a circumference (C) of 4.40 μ, a volume to surface ratio (V/S) of 0.31 μ, and a volume to circumference ratio (V/C) of 1.10 μ² (line a in Fig. 2-15). If this cell were to morph instantly to a sphere with D= 2.10 μ, V remains at 4.82 μ³, but V/C decreases to 0.73 μ² as C increases to 6.59 μ. Thus, to divide at the same mass as a rod, this sphere might indeed require an additional [100x(6.59-4.40)/4.40]= 50% FtsZ molecules to be able to assemble a complete ring. As mass increases, however, the V/C ratio rises...
quickly (Fig. 2-15, upper panel), and we calculate that if the sphere increased in volume by only a factor of 1.83 to 8.84 $\mu^3$, it would once again attain $V/C=1.10 \mu^2$ (intersection of lines a and c in Fig. 2-15). In other words, the sphere would once again contain a sufficient amount of FtsZ to form a complete ring after less than one additional mass doubling. Why then do unsuppressed \textit{mre} and \textit{mrd} mutants not simply propagate as dividing spheres with a moderately increased average mass at any growth rate?

Based on the results of this study, we now think it likely that the presence of intracytoplasmic membrane in fast-growing spheres contributes directly to the accompanying division block by diverting FtsZ and other division proteins into non-productive assemblies on their surfaces. The failure of spheres to adjust phospholipid synthesis to their shape can lead to large excesses of membrane, and this problem becomes worse as spheres increase in mass (Fig. 2-15, lower panel). As an example, we consider the same cell as above. Upon instantly morphing into a sphere of $V=4.82 \mu^3$, this cell would face only a moderate excess of CM of $15.83-13.81=2.02 \mu^2$ ($\sim15\%$). In principle, it would again accumulate a sufficient amount of FtsZ ($V/C=1.10 \mu^2$) upon further growth to $V=8.84 \mu^3$ (line c in Fig. 2-15). However, it would now also have accumulated an excess of $27.30-20.67=6.63 \mu^2$ membrane, which is over 30% more than needed to cover its surface, and by itself sufficient to cover a sphere with a diameter of $1.45 \mu$. Note that this amount of excess intracytoplasmic membrane would be even higher if spheres were to reduce the fraction of total phospholipid ($\sim1/3$) that is normally transported to the OM (see above). In our model, this excess intracytoplasmic
membrane competes effectively with the external CM for FtsZ, delaying division further. As a result, cell mass keeps increasing, but so does the proportion of excess membrane (Fig. 2-15, lower panel). Meanwhile, the chance of non-canonical patterning of the Min system increases, and the cell circumference becomes multiples that of a normal rod. This combination of factors is likely to render assembly of a functional division apparatus increasingly rare as spheroids increase in mass, which is consistent with the dearth of typical Z ‘rings’ in large spheroids (Fig. 2-8, 2-9, 2-14 and 2-7).

Assuming that membrane synthesis is indeed solely coupled to mass increase and that excess intracytoplasmic membrane interferes with division, small spheres might have the best chance to divide because they contain the least excess membrane. In fact, a sphere with a diameter of 1.83 \( \mu \) \( (V=3.21 \mu^3, S=10.52 \mu^2, C=5.75 \mu) \) would have no excess membrane at all in our example, as its volume to surface ratio of 0.31 \( \mu \) matches that of our sample rod-shaped cell above. However, as both its mass is smaller (by 33 %) and its circumference larger (by 31%), its V/C ratio of 0.56 \( \mu^2 \) is only about half that required for division. Put differently, this sphere has not yet accumulated a sufficient amount of FtsZ to accommodate its circumference. It is important to note, however, that a mere doubling in FtsZ concentration would provide this small sphere with enough FtsZ to assemble a complete ring before excess membrane becomes a problem. The absence of discernible intracytoplasmic membrane in FtsZ-overproducing \( \Delta mreBCD \) spheres (e.g. Fig. 2-12A) is consistent with this scenario.
Although this model renders it easy to appreciate why a modest increase in FtsZ production would allow *mre* and *mrd* mutants to propagate as small spheres under otherwise non-permissive conditions, the innate ability of the mutants to propagate as spheres under permissive conditions cannot be readily explained by geometrical arguments alone. About half of MreBCD-depleted spheres of strain FB30/pFB149 [Δ*mreBCD*/*P*\_lac::*mreBCD*] propagating with native levels of FtsZ under permissive conditions (in liquid M9 at 22°C) still appeared to contain some amount of excess intracytoplasmic membrane, as judged by the presence of small BTME-stained foci that were mostly distributed along their periphery (Fig. 2-16). This suggests that, at least under slow growth conditions, cells can tolerate a certain amount of excess CM. We imagine that cells manage to build up sufficient pools of FtsZ and other division proteins to assemble a functional division apparatus as long as the ratio of ‘excess’ to total available membrane surface remains below some critical value at the time of assembly. Based on the division phenotypes of *min slmA* double mutants, which are similarly medium-dependent, we previously suggested that assembly of a constriction-competent septal ring in a slow growing cell may be accomplished at a lower minimum concentration of FtsZ than in a fast growing one (16). The same suggestion can be offered here, but what might be responsible for a more efficient use of available FtsZ during slow mass increase remains unclear.

**Acknowledgements**
We thank Thomas Bernhardt, Cynthia Hale, and Yu-Ting Su for plasmid construction and advice, David McDonald, Patrick Viollier, and Masaaki Wachi for materials, Patricia Conrad and Minh Lam for advice on confocal and deconvolution microscopy, and Arne Rietsch and Patrick Viollier for comments on the manuscript. Supported by a Human Frontiers Science Program award (RGP0001/2003) and NIH GM57059 (to PdB), and NIH NRSA Institutional Training Grant T32GM08056 (to FB). Some of the images were prepared at the Confocal Microscopy Core Facility of the Comprehensive Cancer Center of CWRU/UHC, supported by NIH P30 CA43703.
Table 2-1. *E.coli* strains, plasmids, and phages used in this study.

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**Construct** | **Relevant genotype** | ori | **Source or Reference** |
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pFB194  aadA cI857(ts) P_{\text{JX}}::mrdB(rodA)  pSC101  This work
pGB2    aadA                                  pSC101 (34)
pMLB1112 bla lac^a \ P_{\text{lac}}::lacZ      ColE1 (43)
pTB6    bla lac^a \ P_{\text{lac}}::\text{torA-gfp-t} ColE1 (15)
pTB63   tet fisQ fisA fisZ                    pSC101 (15)
pTB182  aadA fisQ fisA fisZ                  pSC101 This work
pTB188  aadA P_{\text{JX}}::fisZ              pSC101 This work
pYT11   bla lac^a \ P_{\text{lac}}::relA(1-454)-le ColE1 This work

Phages:
\lambda CH178 imm^{21} bla lac^a \ P_{\text{lac}}::zipA(1-183)-gfp   \lambda   (96)
\lambda CH221 imm^{21} bla lac^a \ P_{\text{lac}}::gfp-t-mrdB        \lambda   This work
\lambda CH235 imm^{21} bla lac^a \ P_{\text{lac}}::mreD-le           \lambda   This work
\lambda CH268 imm^{21} bla lac^a \ P_{\text{lac}}::gfp-t-zapA        \lambda   This work
\lambda DR122 imm^{21} bla lac^a \ P_{\text{lac}}::gfp-t-minD minE   \lambda   (147)
\lambda FB120 imm^{21} bla lac^a \ P_{\text{lac}}::mreC-le           \lambda   This work
\lambda FB185 imm^{21} bla lac^a \ P_{\text{lac}}::mrdB(rodA)       \lambda   This work
\lambda FB190 imm^{21} bla lac^a \ P_{\text{lac}}::mrdA(pbpa)       \lambda   This work
\lambda TB59 imm^{21} bla lac^a \ P_{\text{lac}}::mrdA mrdB         \lambda   This work

^a Note that strains marked with * required an appropriate plasmid or phage for survival under common growth conditions.
^b Genotypes indicate when constructs encode in-frame Gfpmut2 (gfp), T7.tag, (t), hexahistidine (h), or the dipeptide LE (le) sequences. <> denotes DNA replacement by \lambda red recombineering, and frt a scar sequence remaining after eviction of aph with FLP recombinase (41, 190).
^c Strain harbors an undefined suppressor (sup?) of mreB<>aph-associated lethality.
Table 2-2. Complementation analyses of \textit{mre<>ft} mutants.

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<tr>
<td>FB19/ pFB128</td>
<td>\textit{mreCD&lt;&gt;ft}/ cl(ts) P}<em>{\text{P}</em>{\text{Pr;:mreD}}</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>MreC</td>
</tr>
<tr>
<td>FB17</td>
<td>\textit{mreBCD&lt;&gt;ft}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>MreBCD</td>
</tr>
<tr>
<td>FB17/ pFB124</td>
<td>\textit{mreBCD&lt;&gt;ft}/ cl(ts) P}<em>{\text{P}</em>{\text{Pr;:mreCD}}</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>MreB</td>
</tr>
<tr>
<td>FB17/ pFB128</td>
<td>\textit{mreBCD&lt;&gt;ft}/ cl(ts) P}<em>{\text{P}</em>{\text{Pr;:mreD}}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>MreB</td>
</tr>
</tbody>
</table>

\textsuperscript{a}At the start of the experiment all strains also contained plasmid pFB112 [\textit{tet sdA}].

\textsuperscript{b}The P}_{\text{lac;:mre}} plasmids were introduced separately into each strain and transformation mixtures were spread on LB-Amp plates that lacked tetracycline, but contained 100 \textmu M (pFB120, pCH235, and pFB121), or 250 \textmu M (pFB118, pFE142, pCH244) IPTG.

\textsuperscript{c}Plates were incubated at 37\textdegree C, and 20 colonies from each transformation were examined for cell morphology and tetracycline resistance. All 20 showed the same phenotype in each case. +, rod-shaped; and Tet sensitive (pFB112 lost); -, sphere-shaped and Tet resistant (pFB112 retained).
Table 2-3. Relative FtsZ levels in viable *mre* mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>IPTG (µM)</th>
<th>Relative FtsZ level</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA340</td>
<td><em>wt</em></td>
<td>0</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>PA340-129</td>
<td><em>mre-129</em></td>
<td>0</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>PA340-678</td>
<td><em>mre-678</em></td>
<td>0</td>
<td>2.4</td>
<td>0.8</td>
</tr>
<tr>
<td>PB103</td>
<td><em>wt</em></td>
<td>0</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>PB103/pCX16</td>
<td><em>wt</em>/<em>sdiA</em></td>
<td>0</td>
<td>4.2</td>
<td>1.6</td>
</tr>
<tr>
<td>FB2/pCX16</td>
<td><em>mreB&lt;&gt;aph</em>/<em>sdiA</em></td>
<td>0</td>
<td>3.0</td>
<td>0.7</td>
</tr>
<tr>
<td>FB2/pCH244</td>
<td><em>mreB&lt;&gt;aph</em>/*P&lt;sub&gt;lac&lt;/sub&gt;::<em>mreBCD</em></td>
<td>500</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>FB2sup/pGB2</td>
<td><em>mreB&lt;&gt;aph sup?</em> /vector</td>
<td>0</td>
<td>2.6</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Cells were grown at 37°C in LB medium, supplemented with IPTG as indicated, to an OD<sub>600</sub> of 0.55-0.65, and prepared for quantitative Western analyses.

*b* Values were normalized to the level of FtsZ in the appropriate *wt* strain. Values given are an average of 3 measurements, except for FB2sup/pGB2, which was measured once.

*c* SD, standard deviation.
Table 2-4. Suppression of \textit{mre}^\textsuperscript{-} and \textit{mrdB}^\textsuperscript{-} lethality by multiple copies of \textit{sdiA}.

<table>
<thead>
<tr>
<th>\textsuperscript{a}P1 lysate</th>
<th>PB103/pGB2 [\textit{wt} /vector]</th>
<th>PB103/pCX16 [\textit{wt} /\textit{sdiA}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{lacIZYA} &lt;\textit{aph}</td>
<td>326</td>
<td>496</td>
</tr>
<tr>
<td>\textit{mreBCD} &lt;\textit{aph}</td>
<td>1</td>
<td>149</td>
</tr>
<tr>
<td>\textit{mreB} &lt;\textit{aph}</td>
<td>\textsuperscript{b}1</td>
<td>133</td>
</tr>
<tr>
<td>\textit{mreC} &lt;\textit{aph}</td>
<td>1</td>
<td>123</td>
</tr>
<tr>
<td>\textit{mreD} &lt;\textit{aph}</td>
<td>0</td>
<td>110</td>
</tr>
<tr>
<td>\textit{mrdB} &lt;\textit{aph}</td>
<td>0</td>
<td>77</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Equal aliquots of saturated cultures were mixed with equal aliquots of each transducing lysate, the mixtures were plated on LB-Kan-Spec, and the number of transductant colonies that appeared after incubation at 37\textdegree C were counted.
\textsuperscript{b}This transductant yielded strain FB2sup/pGB2.
Table 2-5. Morphology of Mre- and Mrd-depleted spheres.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Depletion strain</th>
<th>Parent</th>
<th>Genotype</th>
<th>¹No glucose</th>
<th>²0.1% glucose, no inducer</th>
<th>Inducer</th>
<th>Depleted protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Large non-dividing spheroids</td>
<td>Internal membrane systems</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Long non-dividing spheroids</td>
<td>Internal membrane systems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>FB17 /pFB118 /pFB124</td>
<td>PB103</td>
<td>mreBCD&lt;&lt;frt /P lac::mreB /P Ir::mreCD</td>
<td>+</td>
<td>+</td>
<td>250 mM IPTG</td>
<td>MreB</td>
</tr>
<tr>
<td>2</td>
<td>FB10 (AFB120) /pFB128</td>
<td>PB103</td>
<td>mreC&lt;&lt;aph (P lac::mreC) /P Ir::mreD</td>
<td>+</td>
<td>+</td>
<td>10 mM IPTG</td>
<td>MreC</td>
</tr>
<tr>
<td>3</td>
<td>FB11 (λCH235)</td>
<td>PB103</td>
<td>mreD&lt;&lt;aph (P lac::mreD)</td>
<td>+</td>
<td>+</td>
<td>10 mM IPTG</td>
<td>MreD</td>
</tr>
<tr>
<td>4</td>
<td>FB17 /pFB149</td>
<td>PB103</td>
<td>mreBCD&lt;&lt;frt /P lac::mreBCD</td>
<td>+</td>
<td>+</td>
<td>250 mM IPTG</td>
<td>MreBCD</td>
</tr>
<tr>
<td>5</td>
<td>FB30 /pFB174</td>
<td>TB28</td>
<td>mreBCD&lt;&lt;aph /P BAD::mreBCD</td>
<td>0.5% Arabinose</td>
<td>+</td>
<td>+</td>
<td>MreBCD</td>
</tr>
<tr>
<td>6</td>
<td>FB38 (AFB190) /pFB194</td>
<td>TB28</td>
<td>mrdAB&lt;&lt;aph /P lac::mrdA /P Ir::mrdB</td>
<td>0 mM IPTG</td>
<td>+</td>
<td>+</td>
<td>MrdA (PBP2)</td>
</tr>
<tr>
<td>7</td>
<td>FB22 (AFB185)</td>
<td>TB28</td>
<td>mrdB&lt;&lt;aph /P lac::mrdB</td>
<td>0 mM IPTG</td>
<td>+</td>
<td>+</td>
<td>MrdB (RodA)</td>
</tr>
<tr>
<td>8</td>
<td>FB40 (λTB59)</td>
<td>TB28</td>
<td>mrdAB&lt;&lt;frt /P lac::mrdAB</td>
<td>25 mM IPTG</td>
<td>+</td>
<td>+</td>
<td>MrdAB</td>
</tr>
</tbody>
</table>

¹All strains displayed a normal rod-shaped morphology when grown at 37°C in LB supplemented with appropriate antibiotics, and IPTG or arabinose as indicated.
²Strains were grown overnight as above, save for exp. 6 and 7 where the presence of 0.1% glucose in the ON culture helped to ensure an efficient specific depletion of MrdA and B, respectively. Cells were then washed once in LB, and diluted 4000-fold in LB with antibiotics and 0.1% glucose to (further) deplete the indicated shape protein(s).
³Incubation was continued at 37°C for at least six hours, and cell morphology and the presence of internal vesicles/vacuoles and other membrane systems was determined by both DIC and fluorescence microscopy after addition of the membrane-permeable membrane dye BODIPY TR methyl ester (BTME, CellTrace).
Table 2-6. Growth rates, FtsZ levels, and viability of spherical derivatives.

<table>
<thead>
<tr>
<th></th>
<th>PB103</th>
<th></th>
<th>TB28</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Td (min)(^a)</td>
<td>[FtsZ](^b)</td>
<td>- MreBCD(^c)</td>
<td>- MrdAB</td>
</tr>
<tr>
<td>LB 37°C</td>
<td>34</td>
<td>1.0</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>30°C</td>
<td>60</td>
<td>1.0</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>25°C</td>
<td>100</td>
<td>nd</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>M9 37°C</td>
<td>151</td>
<td>nd</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>30°C</td>
<td>302</td>
<td>0.9</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>25°C</td>
<td>&gt;500</td>
<td>nd</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

\(^a\)Td, mass doubling time as measured by optical density of cultures at 600 nm.

\(^b\)FtsZ levels in PB103 and TB28 as measured by quantitative Western analyses. Values were normalized to the level in cells grown in LB at 30°C; nd, not determined.

\(^c\)No (---), poor (+--), or good (+++), growth on solid media of Mre- and Mrd-depletion derivatives. Also, see figures S1 and S3.
Table 2-7. Recovery of \textit{mreBCD}\textless\textgreater \textit{aph} and \textit{mrdAB}\textless\textgreater \textit{aph} transductants on minimal medium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>20°C</th>
<th>30°C</th>
<th>37°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P1 lysate: \textit{mreBCD}\textless\textgreater \textit{aph}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB103</td>
<td>\textit{wt}</td>
<td>nd</td>
<td>103</td>
<td>nd</td>
<td>4</td>
</tr>
<tr>
<td>PB103/pFB149</td>
<td>\textit{wt}/P\textsubscript{lac}::\textit{mreBCD}</td>
<td>nd</td>
<td>134</td>
<td>149</td>
<td>0</td>
</tr>
<tr>
<td>TB28</td>
<td>\textit{wt}</td>
<td>92</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>P1 lysate: \textit{mrdAB}\textless\textgreater \textit{aph}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB103</td>
<td>\textit{wt}</td>
<td>nd</td>
<td>96</td>
<td>nd</td>
<td>6</td>
</tr>
<tr>
<td>PB103(\lambda TB59)</td>
<td>\textit{wt}/P\textsubscript{lac}::\textit{mrdAB}</td>
<td>nd</td>
<td>112</td>
<td>98</td>
<td>3</td>
</tr>
<tr>
<td>TB28</td>
<td>\textit{wt}</td>
<td>84</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Equal aliquots of saturated cultures were mixed with equal aliquots of each transducing lysate and the mixtures were plated on M9-maltose supplemented with 25 \(\mu\)g/ml Kan and either no (-) or 250 \(\mu\)M IPTG (+). Plates were incubated at the indicated temperature, and transductant colonies were counted. nd, not done.
Table 2-8. Effects of RelA’ production on doubling times, FtsZ levels, and cell size.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>IPTG (µM)</th>
<th>Td (min)</th>
<th>[FtsZ]</th>
<th>Long axis (µm)</th>
<th>Short axis (µm)</th>
<th>Volume (µm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB28/pJF118EH</td>
<td>wt/ vector</td>
<td>0</td>
<td>61</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>TB28/pJF118EH</td>
<td>wt/ P_tac::relA’</td>
<td>50</td>
<td>59</td>
<td>1.0</td>
<td>4.1 (0.9)</td>
<td>1.1 (0.1)</td>
<td>3.45 (1.1)</td>
</tr>
<tr>
<td>TB28/pYT11</td>
<td>wt/ P_tac::relA’</td>
<td>0</td>
<td>62</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>TB28/pYT11</td>
<td>wt/ P_tac::relA’</td>
<td>50</td>
<td>103</td>
<td>1.3 (0.0)</td>
<td>1.9 (0.4)</td>
<td>0.9 (0.1)</td>
<td>1.0 (0.3)</td>
</tr>
<tr>
<td>FB30/pFB174/pJF118EH</td>
<td>ΔmreBCD/ P_BAD::mreBCD/ vector</td>
<td>50</td>
<td>76</td>
<td>1.1 (0.1)</td>
<td>4.0 (0.1)</td>
<td>3.0 (0.6)</td>
<td>20.2 (10.9)</td>
</tr>
<tr>
<td>FB30/pFB174/pYT11</td>
<td>ΔmreBCD/ P_BAD::mreBCD/ P_tac::relA’</td>
<td>0</td>
<td>68</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>FB30/pFB174/pYT11</td>
<td>ΔmreBCD/ P_BAD::mreBCD/ P_tac::relA’</td>
<td>50</td>
<td>108</td>
<td>1.6 (0.1)</td>
<td>2.3 (0.6)</td>
<td>1.7 (0.3)</td>
<td>3.9 (2.6)</td>
</tr>
</tbody>
</table>

A Strains were first grown to density in M9-maltose plus 0.5% arabinose. They were then diluted in LB lacking arabinose and containing IPTG as indicated, and incubated at 30°C.
B Td, mass doubling time as determined by OD₆₀₀ measurements.
C Values were normalized to that in TB28/pJF118EH with 50 µM IPTG.
D,E,F Average values for 100 cells at OD₆₀₀=0.5.
F Considering TB28 and FB30 cells as perfect capsules and prolate spheroids, respectively.
C,D,E,F Standard deviation shown in parenthesis; nd, not determined.
Fig. 2-1. Genetic constructs.

Shown are the *E. coli* mre (A) and mrd (B) loci, chromosomal deletion-replacements, and inserts present on plasmids and phages. Portions of the chromosome that were replaced with an *aph* cassette, or a *frt* scar sequence remaining after eviction of the cassette, are indicated by brackets. Numbers next to brackets refer to the base pairs replaced, counting from the start of *mreB* (A) or *mrdA* (B). Inserts were placed downstream $P_{\lambda R}$ (pFB124 and pFB128), $P_{BAD}$ (pFB174), or $P_{lac}$ (all others) control regions of appropriate plasmid/phage vectors (see Table 1). LE*, in-frame CTCGAGTAA sequence appended to end of gene; M, start codon of *mreD* was changed from GTG to ATG.
Fig. 2-2. Mre− lethality and suppression of MreBCD− lethality by extra SdiA.

Even numbered rows show that depletion of MreB (row 2), MreC (4), MreD (6), or MreBCD (8) causes a severe growth defect, and that the latter can be suppressed by extra SdiA (10). Odd numbered rows show appropriate wildtype controls.

Cultures were grown to density at 37°C in LB supplemented with appropriate antibiotics and IPTG, and were diluted $10^4$ (columns A and D), $10^5$ (B and E), or $10^6$-fold (C and F). 10 µl aliquots were spotted on LB plates containing either 0.1% glucose (D to F), or IPTG (A to C) at 100 µM (rows 3 to 6) or 250 µM (other rows). Plates were incubated overnight at 37°C, and photographed.

Strains used were PB103/pFB118/pFB124 and FB17/pFB118/pFB124 (rows 1 and 2), PB103(λFB120)/pFB128 and FB10(λFB120)/pFB128 (3 and 4), PB103(λCH235) and FB11(λCH235) (5 and 6), PB103/pCH244 and FB17/pCH244 (7 and 8), and PB103/pCH244/pCX16 and FB17/pCH244/pCX16 (9 and 10).
Fig. 2-3. Conditional viability of Mre− cells.

(A) The MreBCD-depletion strain FB30/pFB174 [ΔmreBCD/PBAD::mreBCD] carrying either plasmid pDR3 [Plac::ftsZ] (row 2) or a vector control (1) was grown to density at 37°C in LB with 0.5% arabinose. Cultures were diluted and spotted on LB plates containing 0.5% arabinose (columns A–C), 0.1% glucose (D–F), or 0.1% glucose plus 100 µM IPTG (G–I). Plates were incubated at 37°C.
(B) Overnight cultures of strain TB28 [wt] carrying either pDR3 [P\textsubscript{lac}::\textit{ftsZ}] (2) or a vector control (1) were diluted and spotted on LB plates containing the A22-stock solvent methanol (A-C), 10 µg/ml A22 (D-F), or 10 µg/ml A22 plus 50 µM IPTG (G-I). Plates were incubated at 37°C.

(C) The MreB(CD)-depletion strain FB21/pFB149 [Δ\textit{mreB}/P\textsubscript{lac}::\textit{mreBCD}] (2 and 4) and its wt parent TB28/pFB149 (1 and 3) were grown overnight at 37°C in LB with 250 µM IPTG. Cultures were diluted and spotted on M9-maltose plates containing 250 µM IPTG (A-C) or 0.1% glucose (D-F). Plates were incubated at 30°C (rows 1 and 2) or 20°C (RT, 3 and 4).

(D) An overnight culture of strain TB28 [wt] was diluted and spotted on M9-maltose plates containing methanol (1) or 5 µg/ml A22 (2). Plates were incubated at RT or 37°C, as indicated.

(E) The MreBCD-depletion strain FB30/pFB174 [Δ\textit{mreBCD}/P\textsubscript{BAD}::\textit{mreBCD}] (1 and 2) and its wt parent TB28/pFB174 (3 and 4) carrying either plasmid pYT11 [P\textsubscript{lac}::\textit{relA}] (2 and 4) or pJF118EH [vector] (1 and 3) were grown to density at 30°C in M9-maltose with 0.5% arabinose. Cultures were diluted in LB and spotted on an LB plate with 50 µM IPTG, which was incubated at 30°C.

Overnight cultures were serially diluted 10\textsuperscript{4} (columns A, D, and G), 10\textsuperscript{5} (B, E, and H), and 10\textsuperscript{6}-fold (C, F, and I) (panels A-D), or 10\textsuperscript{3} (A), 10\textsuperscript{4} (B), 10\textsuperscript{5} (C), and 10\textsuperscript{6}-fold (D) (panel E) in LB, and 10 µl aliquots were spotted in each case. Plates were incubated for two days (M9 at RT) or overnight (all others).
Fig. 2-4. Conditional lethality of MreBΔ cells.

MreBΔ-depletion strains (even row numbers) FB17/pFB149 (panel A) and FB21/pFB149 (panel B), and their respective wt parents (odd row numbers) PB103/pFB149 (panel A) and TB28/pFB149 (panel B), were grown to density at 37°C in LB supplemented with Amp and 250 µM IPTG. Cultures were diluted 10^5 (columns A and D), 10^6 (B and E), or 10^7-fold (C and F), and 10 µl aliquots were spotted on LB (rows 1-6) and M9-maltose (rows 7-12) plates containing either 250 µM IPTG (A-C) or 0.1% glucose (D-F). Plates were incubated at room temperature, 30°C, or 37°C, and photographed.
Fig. 2-5. Suppression of MrdAB\(^{+}\) and MreBCD\(^{+}\) lethality by overexpression of FtsZ.

Suppression of lethality caused by depletion of MrdAB (rows 4-6), or MreBCD (10-12), by pTB182 [ftsQAZ] (5 and 11) and pTB188 [P\(_{\lambda}\)R::ftsZ] (6 and 12), but not by pGB2 [vector] (4 and 10). Rows 1-3 and 7-9 show the appropriate wildtype controls.

 Cultures were grown to density at 30°C in LB supplemented with appropriate antibiotics and 25 \(\mu\)M IPTG (1-6) or 0.5% arabinose (7-12), and were diluted \(10^4\) (columns A and D), \(10^5\) (B and E), or \(10^5\)-fold (C and F). 10 \(\mu\)l aliquots were spotted on LB plates containing either 100 \(\mu\)M IPTG (columns A-C), 0.5% arabinose (Columns G-I), or 0.1% glucose (Columns D-F and J-L). Plates were incubated overnight at 37°C, and photographed.

Strains used were TB28(\(\lambda\).TB59)/pGB2 and FB40(\(\lambda\).TB59)/pGB2 (rows 1 and 4), TB28(\(\lambda\).TB59)/pTB182 and FB40(\(\lambda\).TB59)/pTB182 (2 and 5), TB28(\(\lambda\).TB59)/pTB188 and FB40(\(\lambda\).TB59)/pTB188 (3 and 6), TB28/pFB174/pGB2 and FB30/pFB174/pGB2 (7 and 10), TB28/pFB174/pTB182 and FB30/pFB174/pTB182 (8 and 11), and TB28/pFB174/pTB188 and FB30/pFB174/pTB188 (9 and 12).
Fig. 2-6. Conditional lethality of MrdAB− cells.

MrdAB-depletion strains (even row numbers) FB39(λ.TB59) (panel A) and FB40(λ.TB59) (panel B), and their respective wt parents (odd row numbers) PB103(λ.TB59) (panel A) and TB28(λ.TB59) (panel B), were grown to density at 37°C in LB supplemented with Amp and 25 μM IPTG. Cultures were diluted 10^5 (columns A and D), 10^6 (B and E), or 10^7-fold (C and F), and 10 ul aliquots were spotted on LB (rows 1-6) and M9-maltose (rows 7-12) plates containing either 100 μM IPTG (A-C) or 0.1% glucose (D-F). Plates were incubated at room temperature, 30°C, or 37°C, and photographed.
Cells of strain FB2/pCX16 [ΔmreB/sdiA] were grown at 37°C in LB to OD₆₀₀=0.5 and processed for immunofluorescence confocal microscopy of FtsZ. Maximum projection fluorescence (panels 3 and 4), DIC (panels 1), and merged (panels 2) images are shown. For A4, the image in A3 was rotated 48° about the x axis. A majority of the population consisted of small spheres as in panels A, while a minority consisted of large spheroids as in B. Bar equals 2µ.
Fig. 2-8. MreB-depleted cells contain normal levels of FtsZ but form aberrant FtsZ structures.

(A-E) α-FtsZ immunofluorescence confocal microscopy of MreB-depleted cells. Strain FB17/pFB124/pFB118 [ΔmreBCD/ P_{αR}::mreCD/ P_{λac}::mreB] was grown in the presence of either 250 μM IPTG (A), or 0.1% glucose (B-E). Samples for staining were taken both early (B, OD$_{600}$=0.2) and late (A, C-E, OD$_{600}$=0.6) during growth/depletion. Maximum projection fluorescence (A1, B1, C1, D1, E2), DIC (A3, B3, C4), and merged (A2, B2, C3, D2, and E1) images are shown. In C2, the image in C1 is rotated 90° about the y axis. Panels E3, E4, and E5 show y axis rotations of the left-hand (168°), middle (60°), and right-hand (60°) cell in E2, respectively. Bar equals 2μ.
(F) α-FtsZ immunoblot of whole cell extracts of MreB-depleted spheres (lanes 3 and 6) and rod-shaped controls (other lanes). Extracts were prepared on strain PB103 [wt] (lanes 1 and 4), and its MreB-depletion derivative FB17/pFB124/pFB118 grown in the presence of either 250 μM IPTG (2 and 5), or 0.1% glucose (3 and 6). Cells were harvested both early (OD$_{600}$=0.2, lanes 1-3) and late (OD$_{600}$=0.4-0.5, lanes 4-6) during growth/depletion, and each lane received 10 μg total protein. Measured intensity values of FtsZ bands in lanes 2 and 3 were normalized to that in lane 1, and those in lanes 5 and 6 to that in 4. Resulting relative values are shown below the lanes. Cells were grown in LB at 37°C in each case (A-F).
Fig. 2-9. Aberrant FtsZ assemblies in live MreBCD-depleted spheres.

The MreBCD-depletion strain FB30(λ.CH268)/pFB174 [ΔmreBCD(P_{lac}::gfp-zapA)/
P_{BAD}::mreBCD] was grown at 37°C in M9-maltose medium lacking arabinose and containing 50 µM IPTG. Live cells were imaged early (A and B, OD_{600}=0.2) and later (C and D, OD_{600}=0.4) during depletion of MreBCD. Cells were mixed with FM4-64 (0.5 µg/ml) immediately prior to imaging. Maximum projection GFP (panels 2), FM4-64 (3), and merged (4) fluorescence, as well as corresponding DIC (1) images are shown. Panels D5-D8 show merged fluorescence images of individual optical slices from the top to the bottom of the cell. The arrows in C4 highlight some odd-looking GFP-ZapA accumulations. The arrow in D1 and D5 points at a small vesicle-like body that is both visible by DIC and outlined with FM4-64 fluorescence, while the arrowhead points at a much larger body that failed to be outlined by the dye. The arrow in D8 points at a membrane involution near the bottom of the cell. Bar equals 2 µ. 
Fig. 2-10. Intracytoplasmic membrane compartments in MreBCD-depleted spheroids. Shown are live cells of the MreBCD-depletion strain FB30/pFB174 [ΔmreBCD / \text{P}_{\text{BAD}}::mreBCD] producing either trans-membrane \textsuperscript{TM}GFP from lysogenic phage \textlambda CH178 [\text{P}_{\text{lac}}::\text{zipA}(1-183)-\text{gfp}] (panels A-C), or periplasmic \textsuperscript{TT}GFP from plasmid pTB6 [\text{P}_{\text{lac}}::\text{sstorA}-\text{gfp}] (D-J). Cells were grown to OD_{600}=0.2 at 37°C in LB with 50 μM IPTG and either with 0.5% arabinose (panels A and D), or without (other panels). Both DIC (1) and GFP fluorescence (2) images are shown in panels A-I.

Panels J show a time-lapse series of a spheroid exposed to lysozyme. For this experiment, 2 μl of culture was applied to a slide and covered with a coverslip. A 1 μl aliquot of egg white lysozyme (100 μg/ml in GTE) was then pipetted against the edge of the coverslip, where it was drawn under by capillary action. GFP fluorescence was recorded immediately at 1 sec intervals (J1-6). The arrowhead in J1 points to a compartment that quickly released \textsuperscript{TT}GFP into the medium, in contrast to other compartments that retained the fusion throughout the procedure (arrows). J7 shows a DIC image of the lysozyme-treated sphere a few seconds after the image in J6 was taken. Bar equals 2 μ. 

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Fig. 2-11. Topological separation of internal membrane systems from the external cell membrane.

Shown are live MreBCD-depleted spheroids of strain FB30/pFB174 [ΔmreBCD/P_{BAD}::mreBCD] that were grown at 37°C in LB to OD_{600}=0.2.

(A-C) Cells were mixed with FM4-64 and imaged immediately. Panels show DIC (1) and FM4-64 fluorescence (2) images. Arrowheads in A and B point to large vesicles that were not outlined by FM4-64, while the arrow in C points out a vesicle that was.

(D-G) Cells were pulse-labeled with FM4-64 for 5 min, 30 min prior to imaging. Shown are both DIC (1) and FM4-64 fluorescence (2-6) images. Note that all vesicles visible by DIC are now also outlined by FM4-64 fluorescence. In addition, FM4-64 stains structures not readily resolveable by DIC (e.g. arrows in D and F). For the cell in (G), individual z-slices from top to bottom (G2-G5) as well as a maximum projection image (G6) are shown. Note that FM4-64 stained material is present throughout the interior of the cell.
(H and I) CellTrace BODIPY TR methyl ester (BTME) was added 15 min, and FM1-43 immediately, before imaging. DIC (1), FM1-43 (2), and BTME (3). images are shown. Arrow heads point to material labeled with BTME but not with FM1-43.

(J-L) The growth medium was supplemented with Lucifer Yellow (LY). Cells were gently washed in prewarmed medium lacking LY, and imaged immediately. Many vesicles visible by DIC contained trapped LY (e.g. arrowhead in K). Some bodies that appeared as a vesicle by DIC did not retain the dye, suggesting they were still continuous with the external CM and periplasm (arrow in L). Panel J illustrates that cells of the wt parent control (TB28) completely failed to retain the dye. Bar equals 2µ.
Fig. 2-12. Vesicle formation does not require FtsZ polymerization.

Shown are spheroids of strain FB30/pTB63/pDR144 [ΔmreBCD/ftsQAZ/P_Lac::sfiA].

(A-D) Cells were inoculated to OD$_{600}$=0.05 in LP-maltose containing no (A) or 0.5 mM (B) IPTG, and then grown at 37°C to OD$_{600}$=0.3, fixed, and labeled with BTME. Shown are DIC (1) and BTME fluorescence (2). Of each culture, 100 cells were further analyzed to determine the average length of their long and short axes (C), and the percentages of cells containing internal membrane and/or showing signs of constriction (D). Arrowheads in B point to examples of vesicles visible by both DIC and BTME fluorescence.

(E-F) Cells were diluted to OD$_{600}$=0.025 in LB supplemented with 0.5 mM IPTG and either no (E) or 50 μg/ml (F) LY, and grown at 37°C to OD$_{600}$=0.2-0.3. For panel E, cells were incubated with BTME for 15 minutes at 37°C and then treated with FM 1-43 immediately before imaging live. Shown are DIC (1), FM1-43 (2), and BTME (3) fluorescence. Note the intracytoplasmic membrane stained by BTME that was inaccessible to FM1-43. For panel F, cells were gently washed in pre-warmed growth media prior to imaging. Shown are DIC (1) and LY fluorescence (2) images. Note the trapped LY in intracytoplasmic vesicles. Bar equals 2 μm.
The MreBCD-depletion strain FB21/pFB149 \([\Delta mreB/P_{lac::mreBCD}]\) was grown at 37°C in LP-glucose with no or 1 mM IPTG, and the increase in optical density was monitored over time (A). At time points T1 and T2, aliquots were removed to determine phospholipid synthesis rates (B) and cell shape parameters (C).
Fig. 2-14. Assembly of division proteins on intracytoplasmic membrane.

(A-E) Shown are cells of the MreBCD-depletion strain FB30(λ.CH268)/pFB174 [ΔmreBCD (P_lac::gfp-zapA)/P_BAD::mreBCD]. Cells were grown at 37°C to OD_600=0.2 in M9-maltose supplemented with 50 µM IPTG and either no (B-E) or 0.5% arabinose (A). DIC (1) and GFP-ZapA fluorescence (2) images are shown. Note the accumulation of fluorescence in foci/patches at the cell exterior as well as surrounding internal vesicles in B-E.

(F-H) λ.DR122 [P_lac::gfp-minDE] lysogens of TB28 [wt] (F) and the MreBCD-depletion strain FB30/pFB174 [ΔmreBCD! P_BAD::mreBCD] (G and H) were grown at 37°C to OD_600=0.2-0.4 in LB supplemented with 50 µM IPTG but lacking arabinose. DIC (1) and GFP-MinD time-lapse fluorescence (2-4) images at ten sec intervals are shown in panels F and G. Note the transient assembly of GFP-MinD on both the CM and the surface of nearby internal vesicles as it oscillates about the short axis of the speroid in panel G.

The large sphere in panel H was treated with BTME prior to imaging. Shown are DIC (1), BTME fluorescence (5), GFP-MinD time-lapse fluorescence (2-4) at 15 sec intervals, and merged GFP-MinD and BTME images (6-8). Note the periodic movement of GFP-MinD between BTME-stained sites in the interior of the sphere (H2,4,6, and 8) and sites on the CM (H3 and 7).
Fig. 2-15. Relationships between volume, surface and circumference in rods and spheres.

The upper panel shows the increase of the volume (V) to circumference (C) ratio with increasing volume of rod-shaped (stippled line) and spherical (solid line) cells. The lower panel shows the increase in surface of rod-shaped and spherical cells with increasing volume. The hatched area highlights the difference in surface requirements between the two cell shapes. See text for details.
Strain FB30/pFB149 [ΔmreBCD/ Plac::mreBCD] was grown to density at 30°C in LB supplemented with Amp and 250 µM IPTG. The culture was diluted 2000-fold in M9-maltose supplemented with Amp and either 250 µM IPTG (A and C) or 0.1% glucose (B and D). Cells were grown at 37°C (A and B) or 22°C (C and D) to OD_{600}=0.6, fixed, and stained with BTME. Shown are DIC (1) and BTME fluorescence (2). Of each culture, 50 cells were further analyzed to determine the average length of their long and short axes (E), and the percentages of cells showing signs of intracytoplasmic membrane and/or showing cell constriction (F). Arrows point to intracytoplasmic BTME fluorescence in spheroids grown under permissive (D) and non-permissive (B) conditions. Note the difference in cell size under these conditions. Also note that the dominant intracytoplasmic BTME pattern under permissive conditions consisted of one or more small peripheral foci (presumably small CM involutions and/or CM-derived vesicles) as indicated by the arrow in D2. Bar equals 2µ. 

Fig. 2-16. Membrane staining of MreBCD-depleted cells grown under permissive and non-permissive conditions.
Chapter 3

Identification and properties of RodZ, a novel cell shape protein required for proper MreB actin assembly in *Escherichia coli*.
Summary

Rod-shape determination in *Escherichia coli* requires five proteins, MreB, MreC, MreD, PBP2 and RodA. We identified an additional and as yet uncharacterized shape factor we named RodZ (YfgA). By analyzing growth characteristics and cell morphology, cells lacking RodZ were found to have a pleiotropic phenotype that can be quite different from cells lacking one or more of the previously known shape proteins. Sub-cellular localization of RodZ, a bi-topic membrane protein, revealed that it is distributed throughout the cell in a spiraled, or banded pattern similar to other shape proteins. Furthermore, using the first fully functional fluorescent MreB-fusion protein in *E. coli*, we were able to show that RodZ co-localized with MreB, even in the absence of the other shape proteins. RodZ lost the ability to localize properly when MreB was absent, however, implying RodZ’s localization is determined by that of MreB. MreB’s localization was likewise found to be dependent on RodZ, indicating that the proper positioning of both proteins is mutually dependent. In the absence of RodZ, MreB appeared to accumulate into patches, demonstrating that RodZ is required for maintaining the proper localization of the bacterial actin.

Interestingly, RodZ contains a predicted Helix-turn-Helix (HTH) DNA-binding domain at its N terminus, which, we show is dispensable for rod-shape but sufficient for proper localization if anchored to the cell membrane. Further domain analyses showed a small cytoplasmic juxta-membrane domain to be absolutely necessary for rod-shape, provided it is preceded by the HTH or followed by the
periplasmic domain. We propose a model in which RodZ contributes to rod-shape determination by using either the HTH or periplasmic domain to position the juxta-membrane domain alongside MreB to ensure proper assembly of the bacterial actin.
Introduction

In many bacterial species, the particular shape a cell adopts is dictated by the exoskeleton composed of the sugar-peptide murein (peptidoglycan). The life cycle of the bacterium *Escherichia coli* involves two phases of murein growth, cylindrical murein synthesis (CMS) and septal murein synthesis (SMS). Each phase appears to be coordinated by two separate systems, with the tubulin-like FtsZ protein coordinating SMS and the actin-like MreB protein coordinating CMS. For SMS, FtsZ polymerizes into a ring at mid cell and recruits over a dozen proteins that work together to build and constrict the three layers of the cell envelope, including septal murein (63).

To date only five proteins, encoded in two different operons, are known to be specifically required for CMS. These are PBP2 and RodA, encoded by the *mrd* operon and MreB, MreC and MreD encoded by the *mre* operon (13, 27, 162, 163, 181). PBP2 is a bi-topic membrane protein with D,D transpeptidase activity and RodA a poly-topic membrane protein of unknown function but suggested to play a role in transport of the lipid-anchored murein precursor lipid II (87). The functions of MreC and MreD are unclear, but they are believed to be necessary for stabilizing the proposed complex of shape proteins (173). Affinity purification and bacterial two-hybrid assays have indicated that MreC interacts with MreD and a number of high-molecular weight PBPs, including PBP2 (55, 104, 173). Consequently, all five shape proteins are proposed to function as a multi-protein
complex. This is further supported by the localization of several shape proteins into a helical fashion in a number of different organisms (51, 59, 67, 108, 158, 159). The sub-cellular localization of nascent murein, imaged with fluorescent analogues of the lipid II-binding antibiotics vancomycin and ramoplanin, is likewise arranged in a helical pattern (40, 167). Taken together, these observations have led to the following model for rod-shape determination in bacteria, here termed the MreB-scaffold model. This model states that MreB serves as a scaffold, or track, to direct murein synthases and/or hydrolases into a similar spiral-like pattern along the cell cylinder, and this pattern in turn ensures proper cylindrical growth. Although this model is attractive, there is little direct evidence supporting it.

We recently showed that all five shape proteins are conditionally essential for life in *E. coli* (13). Loss of one or more shape proteins cause cells to grow and propagate as small spheroids, provided they grow under conditions of slow mass increase. However, when grown under conditions of fast mass increase, cells grow into giant non-dividing spheres that eventually die. Importantly, spheres fail to adjust phospholipid synthesis rates to their new surface requirements and consequently accumulate elaborate membrane systems in their interior. We showed that these internal membrane systems are effective at competing with the cell membrane for essential division proteins, which is likely to significantly contribute to the division defect of spheres. Interestingly, spheroids grown under otherwise lethal conditions of fast mass increase can be rescued by over-expression of *ftsZ*. Here, we used this common feature of the known shape
proteins to search for additional shape factors. Using a screen for mutants that require extra FtsZ for survival on rich medium, we identified a new shape factor in *E. coli*, a bi-topic membrane protein we named RodZ (YfgA). We show that cells lacking RodZ have a pleiotropic phenotype that is partially distinct to those of *mrd* and *mre* mutants. Sub-localization of RodZ revealed that this protein co-localizes with, and importantly, is required for proper assembly of the bacterial actin MreB. We therefore propose that RodZ's contribution to rod-shape determination is to ensure proper MreB assembly. Additionally, we provide direct evidence in support of the MreB-scaffold model of rod-shape determination by showing that nascent murein insertion occurs in a pattern overlapping that of MreB, even in the absence of RodZ.
Materials and Methods

_E. coli plasmids and phages._ Plasmids pJE42 (95), pDB311 (79), pET21a (Novagen), pDR144 (16), pFB124, pFB174, pCH233, pCH235 and pTB188 (13), pRC7 (43), pZC100 (184), pCAH63 (78), pCP20, pKD3 and pKD13 (41), pCH151 and pTB29 (14), pTB6 (15), and pALA2705 (111) were described previously. Plasmids pTB146, pTB183, pCH310 and pMG12 will be described elsewhere.

To create pFB105 [P<sub>T7</sub>::_t-mreB-h], we performed a PCR with primers 5'-CTTGGATCCCTTTGAAAAAATTTCGTGGCATGTTTTC-3' and 5'-CGCTTCTCGAGCTTTCGCTGAACAGGTCGCCG-3'. The fragment was treated with _BamH_I and _Xho_I (sites underlined), and the 1044 bp fragment was inserted into similarly treated pET21a. Plasmid pFB105 encodes a 39.3 kD derivative of MreB (T-MreB-H) in which the starting methionine has been replaced with the T7.tag (MASMTGGQQMGRGS, T), and the hexahistidine tag (LEH<sub>6</sub>, H) has been appended to its C-terminal residue.

To construct pFB184 [P<sub>lac</sub>::T7<sup>rb</sup>sdiA lacZ], pCX16 [sdiA] was used as template to amplify _sdiA_ with primers 5'-GGAATTCAAGAAGGAGATTTTACTATGCGAGGATAAGG-3' and 5'-GAGCGTGCTCGACTCAAATTAAGCCAGTAGCGG-3'. The product was treated with _EcoR_I and _Sall_ (sites underlined) and the 746 bp fragment was ligated to the 13027 bp _EcoR_I-Sall_ fragment of pRC7. The strong T7 _gene_10 ribosome binding site (rbs) is in bold italics.
For plasmid pFB206 [pACYC P\textsubscript{BAD}::\textit{mreCD}], the 1616 bp fragment of \textit{Xba}l and \textit{Xho}l treated pFB124 was ligated to \textit{Xba}l-\textit{Xho}l treated pFB174.

Plasmid pFB214 [P\textsubscript{lac}::\textit{mreB}] was made in two steps. First, PCR was done with MG1655 chromosomal DNA template and primers 5’-CGACTCTAGACAGCTTTCCAGGATTATCCCTTAGTATG-3’ and 5’-GCAAAAGCTTACTCTTGCCTGAACAGGTGC-3’. The product was treated with \textit{Xba}l and \textit{Hind}III (sites underlined) and the 1072 bp fragment was ligated to the 7639 bp fragment of similarly treated pDR144, yielding pFB209. Then, the 1148 bp \textit{Eco}R1 and \textit{Hind}III generated fragment of pFB209 was ligated to the 5242 bp \textit{Eco}R1 and \textit{Hind}III fragment of pJF188EH, yielding pFB214.

Plasmid pFB216 was made by ligating the 2688 bp \textit{Nru}-\textit{Hind}III fragment of pFB214 with the 4711 bp \textit{Nru}-\textit{Hind}III fragment of pFB174.

For plasmids pFB233 [P\textsubscript{lac}::\textit{rodZ}(1-319)], pFB234 [P\textsubscript{lac}::\textit{ispG}], and pFB235 [P\textsubscript{lac}::\textit{rodZ} \textit{ispG}], the appropriate PCR product (MG1655 DNA template) was treated with \textit{Xba}l and \textit{Hind}III (1076, 1165, and 2221 bp, respectively) and used to replace the 1294 bp \textit{mreD} fragment from pCH233. Primers used were (\textit{Xba}l and \textit{Hind}III sites underlined): 5’-GCTCTAGAGGTGAGCATGATGGTTACCCGGC-3’ [primer \textit{rodZ5’A}] and 5’-CCCAAGCTTTTACTGCGCCGTTGATTTGTCGCC-3’ [\textit{rodZ3’A}] (pFB233), 5’-GCTCTAGACACCGGCGCAACAGACGGG-3’ and 5’-CCCAAGCTTTTATTTTCAACCTGCTGAACGTCATTCGACGCG-3’ [\textit{ispG3’A}] (pFB234), and \textit{rodZ5’A} and \textit{ispG3’A} (pFB235).
For plasmid pFB237 [P_{lac}::gfp-t-rodZ], PCR was done to amplify rodZ from MG1655 DNA with primers \texttt{5'-CGCGGGATCCATGAATACTGAAGCCACGCACGACC-3'} and \texttt{5'-CCCAAGCTTTTACTGCGGTTGATTGTTCGGC-3'}. The product was treated with \texttt{BamHI} and \texttt{HindIII} (sites underlined) and the resulting 1020 bp product was ligated to the similarly treated 8412 bp fragment of pFB114.

Plasmid pFB244 [P_{lac}::rodZ(1-319)] was made by replacing the 1072 bp \texttt{XbaI-HindIII} fragment of pFB181 with the 1076 bp \texttt{XbaI-HindIII} fragment from pFB233. A mutation in pFB233 and pFB244 that resulted in an early stop codon after codon 319 was repaired by replacing the 511 bp \texttt{SalI-HindIII} from each plasmid with the 511 bp \texttt{SalI-HindIII} fragment from pFB237. This yielded plasmids pFB290 [P_{lac}::rodZ] and pFB291 [P_{lac}::rodZ], respectively.

For plasmid pFB254 [P_{T7}::h6-SUMO-rodZ(1-319)], PCR was done with pFB233 template DNA and primers \texttt{5'-GGTGGTTGCTTTCCGGTGATGAATACTGAAGCCACGCACGACC-3'} and \texttt{5'-CCGCTCGAGTTACTGCGGTTGATTGTTCGGC-3'}. The product was digested with \texttt{SalI} and \texttt{XhoI} (sites underlined) and the resulting product was ligated to \texttt{SalI} and \texttt{XhoI} treated pTB146.

Plasmid pFB259 [P_{lac}::mreB-linker-mreB] was made as follows. First, PCR was done to amplify \texttt{mreB} codons 1 through 228 from plasmid pFB209 with primers \texttt{5'-TCACAAAGCTTACAGCTATGACCATGATTACG-3'} and \texttt{5'-CCGCTCGAGTTACTGCGGTTGATTGTTCGGC-3'}. The product was digested with \texttt{XbaI} and \texttt{SphI} (site underlined) to yield a 730 bp product. PCR was also done to amplify \texttt{mreB} codons 229 through 367 with primers \texttt{5'-TCACAAAGCTTACAGCTATGACCATGATTACG-3'} and \texttt{5'-CCGCTCGAGTTACTGCGGTTGATTGTTCGGC-3'}. The product was digested with \texttt{XbaI} and \texttt{SphI} (site underlined) to yield a 730 bp product.
TCGAGGCGCATGCTCTGGCGCGCCGGCGATGAAGTCCCCTGAAATCG-3’ and 5’-GACGTTGTAACACGACGGCAGTG-3’. The product was digested with SphI (site underlined) and HindIII to yield a 375 bp product. Both digestion products were ligated to the 7639 bp XbaI-HindIII fragment of pFB209 to yield plasmid pFB259. The linker encodes a -SGSSACSGAPG- insertion sequence that lies in between amino acids glycine 228 and aspartate 229.

For plasmid pFB262 [P_{lac}::mreB’-RFP-’mreB], PCR was done to amplify ecRFP from plasmid pCH314 with primers 5’-
GGTTCTGGCTCGAGCATGGTTTCCAAGGGCGAGGAGGATAAC-3’ and 5’-
CATCGCCCCGCCGCCAGATTGTACAGCTCATCCATGCCACC-3’. The product was treated with XhoI and Ascl (sites underlined) and the resulting 719 bp fragment was ligated to the similarly treated 17 bp fragment of pFB259 to yield pFB262. This plasmid encodes a –SGSS-(RFP)-SGAPG- insertion sequence that lies between amino acids glycine 228 and aspartate 229.

For plasmid pFB273 [oriR6K attHK022 P_{lac}::gfp-t-rodZ], the 1020 bp BamHI-HindIII fragment of pFB237 was ligated to the 4257 bp BamHI-HindIII fragment of pTB183.

For plasmid pFB274 [oriR6K attHK022 P_{lac}::gfp-30-parB], the 1147 bp MfeI-HindIII fragment of pALA2705 was ligated to the 4069 bp fragment of similarly treated pTB183.

Plasmid pFB285 [oriR6K attHK022 P_{lac}::gfp-t-rodZ(1-111)-r] was made in two steps. First, plasmid pFB252 was made [P_{lac}::gfp-t-rodZ(1-111)-r]. PCR was done with template pFB237 and primers 5’-
TTAGGCAACCCAGGCTTTACAC-3’ and 5’-
CCGCTCGAGTTATCGGCGTGCGTTTTGTCGCGCTTTACCGAGGG-3’
and the resulting product was treated with XbaI and XhoI (site underlined). The resulting 1118 bp product was ligated to the 7662 bp XbaI-XhoI fragment from similarly treated pCH233. The 1141 bp fragment of XbaI and HindIII treated pFB252 was ligated to the 3484 bp fragment of similarly treated pTB183, yielding pFB285.

Plasmid pFB289 [oriR6K attHK022 _P_lac::gfp-t-rodZ(1-111)-r-malF(17-39)-rfp] was made in multiple steps. The 1346 bp BamHI-PstI fragment of pET21a was ligated to the 4213 bp BamHI-PstI fragment of pDB311, yielding pDB328. Then, PCR was done with MG1655 chromosomal DNA template and primers 5'-CGCGGATCCATCCGTACTTATATGGATACC-3' and 5'-CCCGAAGCTTCTATTGCCCTCCGGCGC-3'. The product was digested with BamHI and HindIII (sites underlined) and the resulting 999 bp fragment was ligated to the 5534 bp fragment of similarly treated pDB328. This yielded pFB212, which was then treated with XbaI and XhoI and the 1208 bp product was ligated to the 7762 bp XbaI-XhoI fragment of pCH235 to yield pFB221. PCR was then done with MG1655 chromosomal DNA template and primers 5'-GCTCTAGAGAAAGCCTTATCCGTCCTGG-3' and 5'-CGGGATCTTCCCCTTGTGCGTAC-3'. The product was digested with XbaI and BamHI and the 159 bp product was ligated to the 8676 bp fragment of similarly treated pFB221. This yielded plasmid pFB225, which was subsequently digested with HindIII. The 8797 bp backbone was then ligated to remove a EagI site, yielding plasmid pFB240. An EagI site was then engineered into plasmid pFB240 by the Quichchange method using oligo 5'-
CATTGGTGCGAAAGCGACGC
CGGCCG
ATGGTCAGTGCTAGGTCTGCTCGG
3’ and its reverse compliment (Eag site underlined). This yielded plasmid
pFB243. For plasmid pFB250, PCR was done with template pFB243 and primers
5’-GCTCTAGAGAAAGCCTATCCGTCTGG-3’ and 5’-
CCGGATCTCCCCTTGTGCGTAC-3’ and the product was treated with XbaI
and BamHI (sites underlined). The resulting 158 bp product was ligated to the
8375 bp fragment of similarly treated pCH311 yielding pFB250. Next, plasmid
pFB264 [P_{lac}::gfp-t-rodZ(1-111)-r-malF(17-39)-rfp] was made. PCR was done
with template pFB237 and primers 5’-TTAGGCACCCCAGGCTTTTACAC-3’ and
5’-CCGCTCGAGTTATCGGCGTCGCTGGTTTTTTCGGCGGCTTTACCGAGGG-3’
and the product was treated with XbaI and Eagl (site underlined). The resulting
1108 bp product was ligated to the 8450 bp fragment of similarly treated pFB250,
yielding pFB264. Finally, plasmid pFB289 was made by treating pFB264 with
XbaI and SalI and ligating the resulting 1907 bp fragment with the 3490 bp
fragment of similarly treated pTB183.

Plasmid pFB293 [oriR6K attHK022 P_{lac}::gfp-t-malF(2-14)-gr-rodZ(111-337)]
was made in several steps. Plasmid pFB261 was made by PCR with template
pFB233 and primers 5’-
GCGACGCAGCCGGCAGGCTGGTGTGACCTTCACTTGGC-3’ and 5’-
CCGCTCGAGTTACTCGCCGGTGATTGTGTCGCG-3’ followed by treatment of
the product with Eagl and Xhol (sites underlined). The resulting 691 bp product
was ligated to the similarly treated 8479 bp fragment of pCH310. The last step in
the construction of pFB293 was to replace the 1282 bp XbaI-Sall fragment of pFB273 with the 1997 bp XbaI-Sall fragment of pFB261.

For plasmid pFB299 [P_{lac}::mreB rodZ] PCR was done with template pFB214 and primers 5'-TCATCGGCTCGTATAATGTGTTAA-3' and 5'-CACCTCTAGATTACTCTTCGCTGAACAGGTCGCC-3'. The product was treated with XbaI (site underlined) and the resulting 1074 bp product was ligated to the 6394 bp fragment of similarly treated pFB291.

Plasmid pFB309 [P_{syn135}::gfp-t-rodZ] was made in several steps. Plasmid pEZ1 was made by treating plasmid pTB97 with XbaI and SalI and ligating the resulting 1109 bp fragment to the 2631 bp XbaI-SalI treated fragment of pCAH63. To generate plasmid pEZ4, the -35 promoter element of pEZ1 modified by the QuickChange procedure (Stratagene), using primer 5'-GAATTCTAGGGTTTAAGGTTATGCTTCCGTCG-3' and its reverse compliment, resulting in a T to C mutation (underlined). For pCH362, the 1163 bp EcoRI-SalI insert from pEZ4 was ligated to the similarly treated pZC100. For pFB309, the 1793 bp XbaI-HindIII fragment of pFB237 was ligated to the similarly treated pCH362.

Plasmid pFB310 [P_{λR}::mreB-rfp^{SW}] was made in two steps. First, the 1105 bp XbaI-HindIII fragment from pFB259 was ligated to the 4164 bp XbaI-HindIII fragment of pTB188 to yield plasmid pFB305. Then, the 719 bp XhoI-AciI fragment of pFB262 was ligated to the 5252 bp XhoI-AciI fragment of pFB305 to yield pFB310.
For plasmid pFB312 [P<sub>lac</sub>::gfp-t-rodZ(83-138-rfp)] PCR was done with pYT27 template and primers 5’-TTAGGCACCCAGGCTTTACAC-3’ and 5’-CGCGAGATCTGGAGCTTTGCGGTCTTGCCACCACC-3’. The product was treated with XbaI and BglII (site underlined) and ligated to the 4214 bp fragment of XbaI-BamHI treated pYT22.

Plasmid pFB319 [P<sub>lac</sub>::gfp-t-rodZ(1-84)-malF(2-39)-rfp] was made in several steps. First, PCR was done with template pFB290 and primers 5’-TTAGGCACCCAGGCTTTACAC-3’ and 5’-CGCGCCATGGTTTCCAGCCCTGGCAGCAGTTCTTC-3’. The product was treated with XbaI and NcoI (site underlined), and the resulting 315 bp fragment was ligated to the similarly treated 8493 bp fragment of pFB250. This resulted in plasmid pFB306, which was then treated with XbaI and NruI. The 8679 bp product was then ligated to the 846 bp fragment of similarly treated pFB237, yielding plasmid pFB311. Finally, the 1874 bp XbaI-Sall fragment of pFB311 was ligated to the similarly treated 3490 bp fragment of pTB183 to yield pFB319.

Plasmid pFB321 [P<sub>lac</sub>::gfp-t-rodZ(1-84)-malF(2-14)-rodZ(111-337)] was made by ligating the 1807 bp Apal-NgoMIV fragment from pFB311 with the 3443 bp fragment of Apal-NgoMIV treated pFB293.

Plasmid pYT22 [oriR6K attHK022 P<sub>lac</sub>::gfp-t-rodZ(1-138)-rfp] was made in several steps. Plasmid pCH311 [Plac::zipA-rfp] was constructed first. PCR was done to amplify an E. coli codon-optimized mCherry RFP gene from template pJ1:G01080 (gift from K. Marians) using primers 5’-
AGGGGATCCCGGAATTCAATGTTTCCAAGGGCAGGAGGATAAC-3' and 5'-TTACGGCATGCCTCGACTTATTTGTACAGCTCATCCATGCC-3' and the product was treated with BamHI and SphI (sites underlined). The resulting 734 bp product was ligated to the 8672 bp fragment of similarly treated pCH151. For plasmid pFB249 [P_{lac::}rodZ(1-138)-rfp], PCR was done with template pFB233 and primers 5'-GCTCTAGAGGTGAGCATGATGGTTCACCGGC-3' and 5'-CGGGATCCGGAGCTTTGCGGTCTTGCCACCACC-3' and the product was treated with XbaI and BamHI (sites underlined). The resulting 478 bp product was ligated to the 8375 bp XbaI-BamHI fragment of pCH311. Plasmid pYT17 [oriR6K attHK022 P_{lac::}rodZ(1-138)-rfp] was made by replacing the 1109 bp XbaI-Sall insert of pTB183 with the 1202 bp XbaI-Sall fragment of pFB249. Plasmid pYT22 was made by replacing the 129 bp XbaI-Nrul insert of pYT17 with the 846 bp XbaI-Nrul fragment of pFB237.

Plasmid pYT27 [oriR6K attHK022 P_{lac::}gfp-t-rodZ(83-337)] was made in several steps. First, plasmid pYT1 [P_{lac::}rodZ(83-337)] was made. PCR was done with template MG1655 DNA and primers 5'-CGGGATCCTCGAGAAGCAGGCTCCACTTCCGGA-3’ and 5’-CCCAAGCTTTTACTGCGCCGCTTTAATGTTCGTCG-3’ and the product was digested with BamHI and HindIII. The resulting 774 bp product was ligated to the 7717 bp fragment of similarly treated pJE42. The XhoI-HindIII fragment from pYT1 was used to replace the 7719 bp XhoI-HindIII fragment of pFB266. This yielded plasmid pYT9 [P_{lac::}t-rodZ(83-337)]. Next, the 773 bp XbaI-BamHI fragment of pTB183 was ligated to the 8409 bp fragment of similarly treated
pYT9 to yield plasmid pYT24 [P\textsubscript{lac}::gfp-t-rodZ(83-337)]. Finally, pYT24 was treated with XbaI and HindIII, and the 1549 bp fragment was ligated with the 3484 bp fragment of similarly treated pTB183, yielding plasmid pYT27 [oriR6K \textit{att}HK022 P\textsubscript{lac}::gfp-t-rodZ(83-337)].

Phages \(\lambda\)FB234 and \(\lambda\)FB237 were generated by crossing \(\lambda\)NT5 with pFB234 and pFB237, respectively, as described previously (43).

\textbf{E. coli strains.} Strains BL21(\(\lambda\)DE3), Rosetta(\(\lambda\)DE3) (Novagen), FB1, FB30, FB38, FB48 (13), TB10 (96), TB28 (14), CC4711 and CC4713 (111), and NR693 (150) have been described.

Knockout strains were constructed by \(\lambda\) red recombineering. Plasmid pKD13 was used as a template for amplification of an \textit{aph} cassette consisting of \textit{aph} flanked by FLP recombinase substrate sites (\textit{frt}) (41, 190). Knockout alleles on linear fragments were recombined with the chromosome of strain TB10. For construction of the \textit{rodZ<>aph} (FB58) and \textit{rodZ155<>aph} (FB59) strains, TB10 was harboring plasmid pTB63. For construction of the \textit{ispG<>aph} (FB62) strain, TB10 was harboring pFB234 and plated with IPTG.

The following primer sets were used (chromosomal sites underlined):

For \textit{rodZ<>aph}, 5’-

\begin{verbatim}
CATGATGGTTCACCGGCATCTCAATTCTCATTAAACGTACCTGCAGCGAGTG
GTAAGCTGGAGCTGCTTC-3’ [primer rodZ(KO)5’] and 5’-
CCGCTAAACAATTTTTACCGGTAGCATCAGTGACCTCCAGCCAGCAATCAT
TCCGGGGATCCGTCGACC-3’ [rodZ(KO)3’]; for \textit{rodZ155<>aph}, 5’-
\end{verbatim}
AGCAGGAAGAGATCACCACATGGCCGATCAATCTTCGCGGAACTGAGCT
AAGTGTAGGCTGGAGCTGGCCGTCTGACCCTCAATGCGAACAAATCA
CCGCCGCAAGTAAGTAGGACTGGAGCTGCTTC-3’ [ispG(KO)5’]
and 5’-
TTTATTCCATGCTGACGTCAATTGCACCGCCTTCGACCAGCTGACAT
TCCGGGGATCCGTCGACC-3’ [ispG(KO)3’]; and for rodZ ispG<>aph,
rodZ(KO)5’ and ispG(KO)3’.

For construction of FB80 [P<sub>rodZ</sub><>(aph araC P<sub>BAD</sub>::)] the aph araC P<sub>BAD</sub>:: locus
from pTB29 was amplified by PCR with primers 5’-
AAGTTAGCCTTAGTAATGTTGCTGATTGTCACGAGACACAGCATCGCC
GGCTGAGCTTGCTTC-3’ and 5’-
AGGTACGTTTTAAATGAGATGATGCCGATGACAACAGATCGCCG
AATTTCGCTAGCCC-3’ and recombined onto the chromosome of strain TB10 in
the presence of 0.2% arabinose (chromosomal sequences underlined). A P1
lysate on strain FB80 was used to transduce TB28 to kanamycin resistance in
the presence of 0.2% arabinose to yield strain FB81 [P<sub>rodZ</sub><>(aph araC P<sub>BAD</sub>::)].

Strain FB72/pCX16 [mreB’-rfp-’mreB / sdiA] was essentially made by allelic
replacement of the mreB<>aph mutation of strain FB1/pCX16/pFB149 with the
mreB’-rfp-’mreB gene from pFB262 by λ red recombineering. First, mreB’-rfp-
’mreB gene from plasmid pFB262 was amplified with primers 5’-
CGACTCTAGACAGCTTTACGGATTATCCCCTAGTATG-3’ and 5’-
GCAAAAGCTTTACTCTCTCGCTGAACAGGTCGCC-3’. The resulting product was electroporated into competent FB1/pCX16/pFB149 cells grown in LB supplemented with Spec, Ap and 250 µM IPTG. Cells were outgrown in SOC media at room temperature for 20 minutes and 1 hour at 30°C. Outgrown cells were plated onto LB agar supplemented with 1% glucose and 1% sodium dodecyl sulfate (SDS), which is lethal for spherical cells, and incubated at 30°C. 90% of the transformants were kanamycin sensitive, rod-shaped, and showed no fluorescent signal in the red channel and most likely resulted from recombination of the mreB gene from pFB149. The remaining 10% were kanamycin sensitive rods that showed fluorescent signal in the red channel and represented good candidates for the desired recombination events. Two isolates that had lost plasmid pFB149 but still harbored pCX16 were named FB72 and analyzed further. The chromosomal DNA was purified from wt strain TB28 and FB72/pCX16 (Masterpure kit from Epicentre) and PCR was done to verify the desired recombination event using primer pairs designated A (5’-
GGTGGATCCTTGAAAAATTCGTCATTTTCAATGAC-3’ [A1] and 5’-
GCAAAAGCTTTACTCTCTCGCTGAACAGGTCGCC-3’ [A2]) and B (5’-
AGGGGCATATGGTTTCCAAGGCGAGGATAACATGGC-3’ [B1] and 5’-
CGTTCTCGAGTTGCCCTCCCGCGCGACGCAGGC-3’ [B2]).

Strain FB74/pCX16 [mreB’-rfp-mreB yhdE<>cat / sdiA] was constructed by λ. red recombineering as follows. Plasmid pKD3 was used as a template for PCR with primers 5’-
ATGACTTCTCTGTATTTAGCTTCCGGTTCTCCGCGTCGTCAGGAGTTACTGTGTAG
GCTGGAGCTGCTTCG-3' and 5'-
CATAGCCCGCGATGTCTTCGTCTGTTAAACGTGGAAAGTCACATCGGTGTAGTT
CCTATTCCGAAGTTCC-3'. The product was electroporated into competent
FB72/pCX16 cells (yhdE homology underlined). A P1 lysate was made on the resulting
strain, FB74/pCX16, and used to transfer the mre locus containing the internal fusion to
strain TB28 by P1 transduction by selecting for chloramphenicol resistance. This yielded
strain FB76 [mreB'-rfp-'mreB yhdE<>cat].

Transposon mutagenesis, screening for rod mutants, and mapping of
transposon insertion sites. Strain TB28/pFB184 [ΔlacIZYA / bla P_{lac}::sdiA lacZ]
was grown overnight at 30°C in LB supplemented with Ap and 0.1% glucose and
diluted to a ratio of 1:400 into LB supplemented with Ap and 50 \( \mu \)M IPTG. Cells
were grown with vigorous shaking at 37°C to \( \text{OD}_{600} = 0.46 \). Cells were prepared
for electroporation using 10% ice cold glycerol as described previously (58). Cell
aliquots (40 \( \mu l \)) were mixed with 1 \( \mu l \) (20 \( \mu g \)) of EZTnKan-2 transposome
(Epicentre) and immediately electroporated. Electroporated cells were
resuspended in LB supplemented with Ap and 50 \( \mu \)M IPTG and incubated at
room temperature for 10 minutes. Cells were outgrown at 30°C for 1.5 hours and
plated onto 4 plates of M9-maltose minimal agar containing Kan, Ap and 100 \( \mu \)M
IPTG and incubated for 44 hours at room temperature. The plates were
estimated to contain about 220,000 colonies. The colonies were resuspended in
3.5 ml LB, to which 2.5 ml of 40% glycerol was added. Aliquots were made of the library and frozen at -80°C.

The rod screen was done as follows. An aliquot of the library was thawed, a $10^{-7}$ dilution was made in LB, and 100 µl was plated onto LB supplemented with Xgal (40µg/ml) and 100 µM IPTG. The plates were incubated at 30°C for 2 days to allow for easy selection of solid blue colonies. Each plate contained about 150 colonies. Solid blue colonies were purified on LB 100 µM IPTG and LB 0.1% glucose agar. The location of the transposon insertion was mapped as described previously (15, 138).

**Growth conditions.** Cells were routinely grown in LB (0.5% NaCl) or M9 minimal media supplemented with 0.2% maltose, 0.2% casamino acids and 50 µg/ml L-tryptophan. Unless stated otherwise, ΔrodZ knockout strains were grown in M9-maltose media at 30°C. When appropriate, medium was supplemented with 50 µg/ml ampicillin, 50 µg/ml spectinomycin, 25 µg/ml kanamycin, 25 µg/ml chloramphenicol, or 12.5 µg/ml tetracycline. In the instances when resistance markers were present in single copy or for strains FB108 and FB109, medium was supplemented with 15 µg/ml ampicillin or 10 µg/ml chloramphenicol. Other supplements are specified in the text.

**Protein purification and αMreB and αRodZ antibodies.** Anti-MreB antiserum was raised against a 39.3 kDa fusion [T-MreB(2-347)-H] in which the starting residue of MreB has been substituted with the T7.tag
(MASMTGGQQMGRGS), and the peptide LE(H)_6 has been appended to its C-terminus. A culture of strain BL21(λDE3)/plysS/pFB105 was grown overnight in LB supplemented with Amp, Cam, and 0.1% glucose. Cells were diluted 150-fold in 2 liters of LB Amp with 0.04% glucose to OD_{600} of 0.5. IPTG was added to 840 µM, and growth was continued for 2 hrs. Cells were harvested by centrifugation, washed in 60 ml saline, resuspended in 20 ml NiB(70/50) [20 mM Tris-Cl, 70 mM NaCl, 50 mM imidazole, pH=8.0], and broken by freeze-thawing, and mild sonication (107). The suspension was subjected to centrifugation at 5,800 x g for 15 min. at 4°C. Virtually all the fusion protein was present in inclusion bodies in the pellet fraction. This fraction was washed once in 20 ml of NiB(70/50) containing 0.1 % Triton X-100, and twice in 10 ml of NiB(70/50) without detergent. Using mild sonication, the washed inclusion bodies were solubilized by resuspension in 12 ml of 8M urea in NiB(70/50), followed by incubation at 65°C for 2 h. The cleared suspension was subjected to centrifugation at 16,000 x g for 15 min at 4°C, and the supernatant was diluted two-fold by addition of 8M urea in NiB(70/50). Per run, a 2 ml aliquot was loaded on a 0.5 ml fast-flow chelating Sepharose column (Pharmacia) that had been charged with NiCl₂ and equilibrated with 6 M urea in NiB(70/50). The column was washed with 6 M urea in NiB(70/50), and bound protein eluted in the same buffer, but containing 200 mM imidazole. Appropriate fractions were pooled, and dialyzed to 4 M urea in buffer A (20 mM Tris-Cl, 25 mM NaCl, 2 mM EDTA, pH=8.0). The dialysate was further fractionated on a MonoQ column, using a 25-500 mM NaCl gradient in buffer A containing 4 M urea. Purified T-MreB(2-347)-H was dialyzed to 2 M urea.
in buffer B (20 mM Tris-Cl, 100 mM NaCl, pH=8.0), and then to buffer B without urea. Once solubilized, virtually all of the protein remained soluble during the purification steps. The protein was used to immunize Rabbits.

Anti-RodZ antibody was raised against a 46.9 kDa RodZ(1-319) protein that was generated from the cleavage of a purified H-SUMO-RodZ(1-319) fusion. An overnight culture of strain Rosetta(λDE3)/plysSRARE/pFB254 in LB-Ap-Cam supplemented with 0.1% glucose at 30°C was diluted 1:50 into 1 liter (2 x 500 ml cultures) of LB-Ap-Cam with 0.04% glucose and grown to OD_{600}=0.75. IPTG was added to 840 μM, and growth was continued for 2.5 hrs to OD_{600}=1.42. Cells were harvested by centrifugation at 2,600 x g for 20 minutes at 4°C. Cells were resuspended in 20 ml ice cold 0.9% NaCl and centrifuged as before. The pellet was resuspended in 10 ml cell lysis buffer (CL buffer; 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8), flash frozen in dry ice-acetone and stored at -80°C. Cells were broken by freeze-thawing, and mild sonication (107). Broken cells were centrifuged at 6,000 x g for 15 minutes at 4°C and the pellet, which was found to contain the majority of H-SUMO-RodZ(1-319), was resuspended in 10 ml lysis buffer. 500 μl of 10% triton X-100 was added and mixed thoroughly. The suspension was chilled on ice for 2 hours and gently shaken overnight at 4°C. The suspension was centrifuged at 6,000 x g for 15 minutes at 4°C. The resulting supernatant was enriched in H-SUMO-RodZ(1-319) protein and was set aside on ice. The pellet was resuspended in 10 ml lysis buffer followed by addition of 500 μl 10% Triton X-100 and centrifuged again (6,000 x g, 15
minutes, 4°C). The supernatant was found to contain additional H-SUMO-RodZ(1-319) protein and was combined with the previous supernatant. The combined fractions were loaded onto 2 x 0.5 ml columns of NiNTA-agarose (Qiagen) pre-equilibrated in CL buffer with 0.5% Triton X-100. The columns were then washed with 4 ml column buffer (C buffer; 50 mM NaH₂PO₄, 300 mM NaCl, 0.05% Triton X-100, pH 8) containing 20 mM imidazole followed by 1 ml C buffer containing 50 mM imidazole. The H-SUMO-RodZ(1-319) protein was then eluted with three 0.5 ml fractions of C buffer containing 100 mM imidazole, three 0.5 ml fractions of C buffer with 250 mM imidazole and 2 0.5 ml fractions of C buffer with 500 mM imidazole. Peak fractions were collected and dialyzed extensively into UlpI protease buffer (P buffer; 50 mM Tris-Cl, 150 mM NaCl, 10% glycerol, 1mM DTT, 0.05 % Triton X-100, pH 8). H-UlpI protease was added to a final molar ratio of 1:500 and incubated overnight on ice to cleave off the H-SUMO tag. Digested protein was passed over a 0.5 ml NiNTA-agarose column equilibrated in P buffer without glycerol to remove the His-tagged protease and H-SUMO tag. The flowthrough containing RodZ(1-319) protein was collected and dialyzed against 20 mM Tris-Cl(pH 8), 25 mM NaCl, 1 mM EDTA, 0.05% Triton X-100. The protein was further purified by fast protein liquid chromatography on a Mono-Q column (Pharmacia) with a linear 25 to 1 M gradient of NaCl in the same buffer. The majority of RodZ(1-319) eluted in two close peaks between 200 and 300 mM NaCl. Pooled fractions were stored at -80°C. The protein was used to immunize rabbits.
Microscopy and fluorescent vancomycin staining. The cells in Fig. 3-3E-I and Fig. 3-4G-J were imaged on a Nikon 90i microscope equipped with a 100X (1.45 numerical aperture) oil objective. Fluorescence imaging was done using an X-Cite 120 fluorescence illuminator (EXFO) and RFP-specific (532- to 587-nm excitation filter, 595-nm dichroic mirror, and 608- to 683-nm barrier filter) and/or GFP-specific (450- to 490-nm excitation filter, 495-nm dichroic mirror, and 500- to 550-nm barrier filter) filter sets. For cells in Fig. 3-3E-I, optical sections were collected with a CoolSNAP HQ camera (Photometrics). As indicated, optical slices from either the top (3-3F-G2-4 and H-I-3) or middle (3-3E-2-4 and H-I4) of the cell are shown. For cells in Fig. 3-4G-J images were collected using a 512C QuantEM camera (Photometrics). Images were captured using MetaMorph 7.1.2 software (Molecular Devices). All other imaging was performed on a Zeiss Axioplan-2 microscope as previously described (95). Live cells were imaged on 1.2% agarose pads made with either 0.5% NaCl (for LB cultures) or M9 salts (for M9 cultures). When indicated, cells were chemically fixed as previously described (13) and DAPI was added to fixed cells at a concentration of 0.25 \( \mu \text{g/ml} \) 2 minutes prior to imaging.

Fluorescent vancomycin staining was performed with a BODIPY FL derivative of vancomycin (Invitrogen). A working stock consisting of 50 \( \mu \text{g/ml} \) of a 1:1 ratio of labeled to unlabeled vancomycin in 5% DMSO was prepared by mixing unlabeled vancomycin (in dH\(_2\)O) with labeled vancomycin (in DMSO). To label lipid II, 100 \( \mu \text{l} \) of culture was added to 2 \( \mu \text{l} \) 50 \( \mu \text{g/ml} \) stock solution and incubated at room temperature for 2-4 minutes. Cells were pelleted by centrifugation in a
tabletop micro-centrifuge for 20 seconds, washed once in 1 ml PBS and resuspended in 50 µl PBS and immediately imaged with GFP-specific filter sets.

**Other methods.** Whole cell extracts were prepared as previously described (80). Measurements of protein concentration and quantitative Western analysis were done as previously described (13, 96).
Results

A screen for cell shape mutants.

We previously found that all five cell shape maintenance proteins MreBCD and MrdAB are conditionally essential for growth (13). Cells lacking any of these proteins propagate stably as small spheres at low mass doubling rates, but form giant non-dividing spheroids at higher ones. An extra supply of the FtsZ division protein, however, suppresses lethality by allowing the shape mutants to propagate as small dividing spheres at higher rates as well. We made use of this latter property in a screen for additional cell shape factors, by asking for mutants that: i) required additional FtsZ for survival or good growth on rich medium, and ii) showed a distinct cell shape defect. The screen was similar to synthetic lethality screens we developed before (15, 16), and relied on enhanced retention of an unstable F plasmid derivative (pFB184 [bla lacI P_{lac-}$\cdot$sdiA lacZ]) encoding an operon fusion of sdiA and lacZ under control of the lac regulatory region. SdiA stimulates transcription of the ftsQAZ genes (184), and cells harboring pFB184 produce extra FtsZ in the presence of IPTG. The plasmid was introduced in TB28 [ΔlacIZYA], which lacks the chromosomal lac operon, and TB28/pFB184 cells were subjected to random mutagenesis with EZTnkan-2 (Epicentre). Aliquots of the resulting transposome library were plated on LB-agar supplemented with 40 µg/ml Xgal and 100 µM IPTG (LB-IX), but lacking antibiotics, and incubated at 30°C for 48hr. The vast majority of transposon mutants formed white or sectored-blue colonies, reflecting rapid loss of the plasmid, but some formed solid-blue
colonies, suggesting that retention of the plasmid provided a selective advantage. The latter were purified on LB-Xgal with either 100 µM IPTG or 0.1% glucose, to test for plasmid-dependent growth, and examined by microscopy for cell morphology. Transposon insertion sites were determined as before (15, 138). As expected, the screen yielded insertions in genes/operons known to affect cell division and/or cell shape, including zipA, ftsEX, ddlB, mraZ, min, mre and mrd, as well as in previously uncharacterized genes.

**RodZ(YfgA) is a new cell shape factor in *E. coli***

Mutant Rod2352/pFB184 formed mostly solid blue colonies on LB-IX, as well as some small white ones, suggesting that occasional loss of the plasmid was detrimental to growth. The mutant was especially interesting as it propagated as spheroids and EZTnkan-2 had inserted in yfgA, a gene of unknown function that we renamed *rodZ*. The gene lies immediately upstream of *ispG* (*gcpE*) whose product catalyses an essential step in isoprenoid biosynthesis (9, 81, 84). We next created strains in which most of the *rodZ* ORF was removed from the chromosome (Fig.3-1, Table 3-1), and analyzed their phenotypes in more detail. Like the original transposon mutant, Δ*rodZ* cells failed to maintain rod-shape. The growth properties of Δ*rodZ* cells appeared complex, however, and cell shapes depended to some extent on growth conditions as well (Fig.3-2B). Compared to the wt parent TB28, the mass doubling time of the Δ*rodZ* mutant in M9 minimal medium increased markedly (from ~ 64 to ~ 270 min at 37°C, Table 3-2) and the mutant also plated relatively poorly on M9-agar, especially at or below 25°C.
Cells propagated mostly as spheroids or ellipsoids in M9 medium, though some cells had more complicated shapes (Fig.3-2B). Mass doubling time was less reduced in LB medium (~ 54 versus ~ 35 min at 37°C, Table 3-2), but the relative plating efficiency on LB-agar was even worse than on M9 (Fig.3-2A). Thus, while the mutant still formed colonies on LB-agar at 42°C and 37°C, it failed to do so at or below 30°C. Cell shapes in LB at these lower temperatures were the most complex, with many cells growing into large spheroids bearing one or more cone-like protrusions (Fig.3-2B). In contrast, although virtually all cells were clearly still misshapen on LB at 42°C with many resembling ellipsoids and/or very wide rods, cell shape appeared the closest to normal under these conditions (Fig.3-2B).

Both the growth and shape defects of ΔrodZ cells were fully corrected by pFB290 [P\text{lac}::rodZ], showing that these defects were not due to polar effects of the rodZ lesions on ispG or other nearby genes.

Moderate overexpression of ftsZ is sufficient to suppress the lethality of mre and mrd shape mutants under non-permissive conditions. We found the same to be true for rodZ mutants as illustrated by spot titer analysis of strain FB60/pDR3 [ΔrodZ/ P\text{lac}::ftsZ] on LB-agar at low temperature. While cells failed to form colonies at 30°C in the absence of IPTG, they did do so at a frequency close to the wt control in the presence of inducer, although the colonies were significantly smaller (Fig.3-2C). As with mre and mrd cells, extra FtsZ allowed rodZ cells to propagate as smaller cells, without restoring their shape defects (Fig.3-2D). In M9 medium this resulted in the formation of smaller spheroids (not shown), while in LB medium, under otherwise non-permissive conditions, extra FtsZ gave rise
to heterogeneous populations of severely mis-shaped cells, that frequently displayed branches, bulges, and oddly placed and angled constrictions (Fig.3-2D).

We conclude that rodZ is critical in maintaining the rod shape of *E. coli*. Like the *mreBCD* and *mrdAB* genes, rodZ is conditionally essential, and RodZ lethality under non-permissive conditions can be suppressed by an elevated level of FtsZ.

**RodZ is a conserved trans-membrane protein with a putative DNA-binding domain.**

The RodZ protein is predicted to contain 337 residues and to have several interesting features. The N-terminus resembles a Cro/Cl-type HTH DNA-binding domain (~10-85), and is followed by a highly basic region (~86-110), a trans-membrane domain (~111-133), and a substantial periplasmic domain consisting of a region rich in proline and threonine (~134-253) that may be largely unstructured, and a C-terminal domain that may be relatively rich in β-strands (~254-337) (Fig.3-1). Database searches indicate that RodZ-related trans-membrane proteins with a cytoplasmic Cro/Cl (Xre)-type DNA-binding domain are present in many Gram negative as well as Gram positive organisms from most bacterial phyla (not shown). The function of none of these has yet been established.
The prediction that RodZ is a type II (N-in) bitopic membrane species is supported by previous work with alkaline phosphatase fusions (135), as well as by cell fractionation and microscopy (below, and not shown).

We considered the possibility that RodZ might be a transcription factor needed for expression of one or more of the other shape proteins. Plasmids pFB174 [pBAD::mreBCD] and pTB59 [P_lac::mrdAB] are compatible and express the mre and mrd operons under control of the ara and lac regulatory regions, respectively. In addition, the plasmids restore rod-shape to ΔmreBCD and ΔmrdAB cells, respectively, when cells are grown in the presence of the appropriate inducer. However, neither plasmid by itself, or in combination, affected the shape defect of ΔrodZ cells, suggesting this defect was not due to a lack of any of the other known shape proteins (not shown). Consistent with this result, MreB levels in ΔrodZ cells were close to that in wt cells as judged by quantitative western analyses (not shown). Together with results discussed below, these observations render it unlikely that RodZ controls cell shape by acting merely as a transcription factor of other shape factors.

**RodZ localizes in a spiral-type fashion along the membrane.**

We studied the cellular distribution of RodZ using a fusion of GFP to the N-terminus of the protein. Strain FB60(iFB273) [ΔrodZ(P_lac::gfp-rodZ)] expresses the GFP-RodZ fusion in an IPTG-dependent fashion from a single copy construct that was integrated in the chromosome at attHK022 using the CRIM system (78). Cells of this strain showed the typical spheroidal RodZ^{−} morphology upon growth
in M9-maltose medium without inducer (Fig.3-2E). In the presence of 250 µM IPTG, however, cell morphology was indistinguishable from wt cells, indicating that the fusion of GFP to full-length RodZ is fully capable of restoring rod-shape. Fluorescence microscopy showed a spiral-like distribution of the fusion along the length of cells, reminiscent of that previously seen for the MreB cytoskeleton (Fig.3-2E).

**Construction of a functional fluorescent MreB sandwich fusion.**

Although previously described GFP fusions to *E. coli* MreB localized in expected spiral-like patterns, they failed to correct the shape defect of *mreB* mutants. We created a series of fusions to either terminus of MreB using various fluorescent proteins and linker peptides. Again, while some of these localized in spiral-like patterns in wt (MreB⁺) cells, none retained the ability to restore rod-shape to Δ*mreB* or MreB-depleted cells (not shown).

We then considered the possibility of a functional sandwich fusion and inspected the crystal structure of *Thermotoga maritima* MreB for internal sites that might be permissive for insertion of extraneous domains. S216 and adjacent residues seemed attractive as they are poorly conserved and reside in a solvent-exposed loop between helices 6 and 7 that lies some distance away from residues involved in MreB polymerization as well as from the termini (172). Hence, we created constructs encoding a MreB’-RFP-‘MreB sandwich (henceforth indicated with MreB-RFP<sub>SW</sub>) in which mCherry RFP and flanking linker peptides are inserted at the corresponding location of *E. coli* MreB, in
between G228 and D229. Notably, plasmid pFB262 [P_{lac}:: mreB-rfp^{sw}], which encodes this sandwich fusion downstream of the lac regulatory region, was capable of fully restoring rod-shape to ΔmreB cells in an IPTG-dependent manner (not shown). We next used recombineering to replace native chromosomal mreB with the mreB-rfp^{sw} allele, yielding strains that produce the MreB-RFP^{SW} sandwich under native regulatory control and as the sole actin in the cell. The substitution of mreB with mreB-rfp^{sw} in these strains was verified by PCR and Western analyses (Fig.3-3A and C). Cell morphology and growth rates of mreB-rfp^{sw} strains were indistinguishable from wt controls, and MreB-RFP^{SW} localized in a banded/spiral-like fashion along the long axis of cells (Fig.3-3B and D). Thus, the presence of an extra protein domain between H6 and H7 is remarkably well-tolerated by E. coli MreB, and it seems worth exploring if this is true for other (bacterial) actins as well.

**RodZ is part of the spiral-like MreB cytoskeleton and required for its assembly**

Strain FB83(iFB273) [mreB-rfp^{sw}(P_{lac}::gfp-rodZ)] was next used to visualize MreB-RFP^{SW} and GFP-RodZ in the same cells. The two fluorescent fusions appeared to co-localize perfectly at all stages of the cell cycle and in all cells examined (Fig.3-3E, and not shown). This suggested that the MreB cytoskeleton might act as a cytoplasmic scaffold for proper localization of RodZ. As this scaffold is thought to determine the sub-localization of one or more of the other four trans-membrane shape factors as well, we explored the possibility that the
latter might mediate the co-localization of RodZ and MreB. To this end, we examined derivatives of FB83(iFB273) that completely lack MreC and MreD [ΔmreCD], or PBP2 and RodA [ΔmrdAB]. These strains also carried pTB63 [ftsQAZ], allowing cells to propagate as small spheres (13). MreB-RFPsw and GFP-RodZ still co-localized perfectly in either strain, although both proteins accumulated in mostly peripheral clusters rather than in obvious spiral-like patterns (Fig.3-3F and G). To examine if formation of such GFP-RodZ membrane clusters depends on MreB, we examined cells of strain FB30(λFB273)/pTB63 [ΔmreBCD(Plac::gfp-rodZ)/ftsQAZ], which lacks all three Mre proteins, as well as in a MreB⁺ derivative of this strain that produced MreC and MreD from a resident plasmid. As illustrated in Fig.3-3H and I, GFP-RodZ distributed evenly along the membrane in spheroids of either strain, showing that MreB is indeed involved in directing the cellular location of RodZ.

To assess how RodZ affects MreB localization, we compared the distribution of MreB-RFPsw in spheroids of strains that lacked RodZ [ΔrodZ] with those lacking PBP2 and RodA [ΔmrdAB]. In the latter, MreB-RFPsw accumulated in numerous small patches/foci of comparable intensity along the cell membrane (Fig.3-4A). In RodZ⁺ spheroids, by contrast, MreB-RFPsw concentrated in notably fewer and larger peripheral clusters (Fig.3-4B and C). These results suggest that, by preventing MreB from forming large aggregates, RodZ helps to distribute MreB polymers more evenly along the membrane, and that it plays an important role in formation of the extended spiral-like configuration of the MreB cytoskeleton typically seen in rod-shaped cells. Importantly, depletion of RodZ
from strain FB81 \([\text{mreB-rfp}^{SW}\ P_{B\text{AD}}::\text{rodZ}}\) showed that MreB clustering was evident early in the depletion process, well before severe shape defects are evident, suggesting MreB mis-localization is the cause of shape-loss (Fig.3-4E and F).

**Cytological evidence for MreB-directed murein synthesis in the presence or absence of RodZ.**

The membrane-impermeable glycopeptide antibiotic vancomycin is effective against many Gram positive, but few Gram negative organisms. It binds to the D-Ala-DAla moiety of nascent murein strands, and of their lipid II precursor, on the external face of the cytoplasmic membrane, and fluorescent derivatives of the drug (Van-FL) have been used to visualize sites of murein synthesis in organisms that are sensitive to the drug (40, 50, 54, 167). The outer membrane of *E. coli* normally occludes access of the drug to its target, and wt cells failed to stain with Van-FL, as might be expected (data not shown). However, certain alleles of the essential OM assembly factor Imp render *E. coli* sensitive to the drug (60, 150, 151), suggesting they might allow the use of Van-FL in probing for sites of active murein synthesis in live *E. coli* cells. Indeed, incubation of *imp4213* cells with Van-FL resulted in staining patterns reminiscent of those first seen with *Bacillus subtilis* (40). In normally dividing cells, the Van-FL signal was particularly strong at sites of constriction in strain FB109 \([\text{mreB-rfp}^{SW}\ yhdE<>\text{frt imp4213}}\), forming a relatively bright fluorescent ring (Fig.3-4G). In addition, weakly fluorescent spots could be detected along the cylindrical parts of cells (Fig.3-4H).
arrow heads). The latter could be part of a larger helical arrangement, as is apparent in Van-FL stained *B. subtilis* cells (40, 167), but the fluorescent intensity of Van-FL along the cell cylinder was by itself too low to suggest this with confidence. We reasoned that cylindrical staining could be enhanced by preventing septal targeting of Van-FL in cells inhibited for division. To this end, simultaneous imaging of MreB-RFP<sup>SW</sup> and Van-FL in FB109 [*mreB-rfp<sup>sw</sup> yhdE<>ft imp4213*] cells harboring plasmid pJE80 [P<sub>BAD</sub>::sfiA] was done following induction of the cell division inhibitor SfiA. This showed an appreciable degree of overlap in signal along the cylindrical portions of cells (Fig.3-4I and J arrow heads).

To examine the effect MreB patches in RodZ- cells might have on the pattern of murein synthesis we stained strain FB110/pFB310 [*imp4213 rodZ<>aph / P<sub>λR</sub>::mreB-rfp<sup>sw</sup>*) with Van-FL. Plasmid pFB310 constitutively expresses MreB-RFP<sup>SW</sup> from the strong *λ*<sub>R</sub> promoter. Van-FL staining of strain FB110/pFB310 grown in LB at 30°C often revealed several fluorescent patches. Some of these clearly overlapped with MreB-RFP<sup>SW</sup> clusters (arrow head, Fig.3-4K and L), while others did not (Fig.3-4L large arrow). We assume that the latter type of fluorescent patches, not associated with MreB, correspond to FtsZ directed sites of murein insertion. These data support the scaffold model of MreB-directed murein growth and also indicate RodZ is not necessary for this function of MreB.

**Rod-shape depends on a proper MreB to RodZ ratio**
Over-expression of MreB was previously reported to cause a cell division defect (105, 182). We noticed, however, that the effects of MreB over-expression depend strongly on the growth medium. When FB83/pFB216 [mreB-rfp<sup>sw</sup>/P<sub>tac</sub>::mreB] was grown in LB medium with 250 μM IPTG, cells became filamentous as expected. The filaments were notably wider (average diameter D=1.7μ) than normal rods (D=1.0μ), however, and MreB-RFP<sup>SW</sup> localized at the cell periphery in small foci or spiral-like patterns as well as in atypical large aggregates (not shown). In contrast, when MreB was over-expressed in M9 medium, cells lost shape and eventually formed large non-dividing spheroids (Fig.3-5C). Similarly, over-expression of RodZ in FB83/pFB291 [mreB-rfp<sup>sw</sup>/P<sub>tac</sub>::rodZ] caused some widening (D=1.3μ) and elongation of cells in LB (not shown), but a complete loss of rod-shape in M9 (Fig.3-5B). In the latter spheroids, MreB-RFP<sup>SW</sup> accumulated in large patches, a distribution not unlike that observed in cells lacking RodZ altogether (arrows in Fig.3-5B2). This result indicates that both the lack and over-abundance of RodZ leads to the assembly of MreB into large structures that are ineffective in directing proper cell elongation.

Interestingly, co-overexpression of MreB and RodZ in FB83/pFB216/pFB291 prevented the loss of cell shape seen when either protein is over-expressed alone. Instead, cells formed very long, relatively narrow (D=0.8μ), filaments in either medium (Fig.3-5D). This result implies that maintenance of a cylindrical cell shape depends critically on a proper MreB to RodZ ratio. In addition, it appears that MreB and RodZ significantly reinforce each other’s ability to block
cell division when present at elevated levels. We also found, quite unexpectedly, that MreB-\text{RFP}^{Sw} was enriched in a banded pattern throughout the filaments, and primarily accumulated in extended DNA-free regions in these filaments (arrow heads in Fig.3-5D and E). Interestingly, when a local enrichment of MreB-RFP^{Sw} did overlap with DNA, the surrounding cell wall frequently showed swelling, or bulging (large arrow in Fig.3-5D).

The cause of these unusual phenotypes when MreB and RodZ were co-overexpressed was not intuitively obvious to us. Specifically, we were intrigued by the four phenotypes: i) narrowing of cells, ii) division inhibition, iii) aberrant chromosome localization, and iv) bulges in the cell wall where MreB enriched zones coincided with DNA. In attempts to shed some light on the cause of these phenotypes, we asked where RodZ was localized in the filaments relative to MreB. We reasoned that these phenotypes could be modulated by this putative DNA binding protein. We therefore constructed a plasmid that encoded an operon fusion of \textit{mreB} and \textit{rodZ} (pFB299) as well as a plasmid encoding the \textit{gfp-rodZ} fusion expressed constitutively from the P_{syn35} promoter (pFB309). We grew strain FB83/pFB299/pFB309 [\textit{mreB-rfp}^{Sw}/\text{P}_{\text{lac}}::\textit{mreB} \textit{rodZ}/\text{P}_{\text{syn35}}::\textit{gfp-rodZ}] in M9 with 250 \muM IPTG and imaged cells during the early stages of filamentation to get a better sense of the progression of these phenotypes. This clearly showed that MreB-RFP^{Sw} accumulated at the cell poles, which, similar to the longer filaments, were also void of DNA (Fig.3-5E2). The distribution of GFP-RodZ in these filaments appeared to follow the same general pattern as that of MreB-RFP^{Sw} (enriched in the polar regions) (Fig.3-5E5). We found it difficult to discern
a sharp co-localization, however, as the narrowing of the filaments may have made imaging of the spirals more difficult. Nevertheless, we conclude MreB and RodZ still co-localize in the co-overexpression filaments.

What then causes MreB and RodZ to accumulate in DNA free regions when co-overexpressed, and how does this relate to the phenotypes? We considered several possible scenarios. It is possible that RodZ promotes MreB localization in DNA-free regions, and in doing so stimulates murein synthesis there. This could have the effect of forming large DNA-free regions as the cell grows away from the nucleoid. This scenario is attractive because it could also provide a possible explanation for the division defect. By stimulating zones of murein synthesis, MreB and RodZ could effectively compete with FtsZ for the murein precursor lipid II and halt SMS. Or, MreB and RodZ could interfere with FtsZ ring formation in DNA-free regions, which, is where it normally would “prefer” to assemble (due to nucleoid occlusion by SlmA). Alternatively, MreB and RodZ enriched zones could shuttle DNA away from themselves, thus causing DNA-free regions. If so, this may implicate RodZ as playing a role in DNA segregation.

**Depletion of RodZ does not greatly affect chromosome segregation.**

The MreB cytoskeleton has been implicated in chromosome segregation (48, 74, 103, 105, 160). Given its properties described above, RodZ would be an attractive candidate for mediating MreB-directed nucleoid dynamics. Inspection of DAPI-treated FB60 [ΔrodZ] cells failed to reveal DNA-less cells or any other gross nucleoid segregation defects (Fig.3-6D and E). However, nucleoid staining
patterns in spherical cells are a bit difficult to interpret with confidence. Moreover, even if RodZ were not required for bulk segregation, it might still be more specifically involved in the directed poleward movements of newly replicated origin regions, which are proposed to depend on the MreB cytoskeleton (103, 105). To test this, we examined RodZ-depletion strains in which the location of the oriC or ter regions of the chromosome can be visualized by the P1 parS/GFP-Δ30ParB system (111). We slightly modified this system in that GFP-Δ30ParB is encoded on the chromosome rather than on a multicopy plasmid, affording better control over its production. Thus, TB99(iFB274) and the isogenic RodZ-depletion strain FB88(iFB274) harbor a P lac::gfp::Δ30parB cassette at attHK022 and a single copy of parS at 84.2’ near oriC. In addition, FB88(iFB274) contains a chromosomal P Bad::rodZ allele in which the native rodZ promotor was replaced with the ara regulatory region. Cells were inoculated in M9-maltose medium with IPTG (100µM), but lacking arabinose, and then monitored for RodZ levels, cell morphology, and nucleoid and oriC separation after 3.3 (T1) and 5.3 (T2) mass doublings. RodZ was barely detectible in FB88(iFB274) cells at either time point, but, while cells had begun to round up at T2, most still had a distinct rod-shape at T1 (Fig.3-6H, J and O). Measurements of the number of nucleoids and origin foci and of distances between origin sisters in these cells failed to reveal significant differences with that of the wt control (Fig.3-6P-R). Moreover, we did not detect any obvious effects of RodZ-depletion on localization of the ter region, using a similar set of strains that contain a single parS site near ter (at
33.8’) (Fig.3-6L, N, O and R). These results indicate that a lack of RodZ has little if any direct effect on chromosome dynamics.

**The periplasmic domain of RodZ is dispensable for cell growth, shape maintenance and RodZ localization.**

In a genomic analysis of essential genes by genetic footprinting, rodZ (yfgA) was classified as a non-essential gene because transposon insertions in codons 156, 182, 216 and 280 were apparently well-tolerated by cells growing in rich medium (70). This suggested that the C-terminal portion of RodZ might be dispensable for good growth and shape maintenance. To test this, we first engineered and studied a strain (FB61) which is identical to FB60 [ΔrodZ] except that the 5’ portion of chromosomal rodZ is intact, and a translation stop codon was introduced after codon 155. Notably, FB61 [rodZ1-155] cells grew well and were still rod-shaped in both minimal and rich growth medium, though they were slightly shorter and wider on average than cells of the wt parent TB28, particularly in M9 at 42°C (Table 3-2 and not shown).

To verify the implication that the periplasmic domain is largely dispensable for the roles of RodZ in proper growth and shape maintenance, we tested the properties of a GFP-RodZ fusion derivative in which almost the complete periplasmic domain (139-329) is replaced with cherry mRFP. The fusion was encoded on a CRIM construct (pYT22), and expressed under control of the lac regulatory region upon integration in the chromosome at attHK of both a ΔrodZ (FB60) and corresponding wt (TB28) strain. Production of the GFP-RodZ1-138-
RFP fusion was indeed sufficient to restore both rod-shape and doubling times of FB60(iYT22) cells (Table 3-3, Fig.3-7B). Moreover, the fusion still localized in a spiral-like fashion in the resulting rods, and the same localization pattern was evident upon expression of the fusion in cells of TB28(iYT22).

Even though RodZ(1-138) still localized in a spiral fashion, it was conceivable that the periplasmic domain could do so by itself as well. Therefore, we similarly studied the properties of a fusion in which the entire cytoplasmic domain of RodZ is replaced with GFP and a small cytoplasmic portion of the MalF protein. This fusion was incapable of inducing rod-shape in FB60(iFB293) cells, or of alleviating their slow growth rate (Table 3-3). Moreover, the fusion appeared evenly distributed along the membrane of both ΔrodZ and wt cells, suggesting that the periplasmic domain of RodZ does not contribute much, if anything, to the spiral-like localization pattern of the full-length protein (Fig.3-7G). We conclude that the periplasmic domain of RodZ is by and large dispensable for normal viability and maintenance of rod-shape, although it may help to fine-tune the precise diameter of wt cells. On the other hand, the cytoplasmic domain of RodZ is required for its critical roles in cell growth, shape maintenance, and its typical spiral-like localization.

**The HTH domain of RodZ is also dispensable for shape maintenance and good growth, but contributes to localization of the protein.**

As the results above showed that the cytoplasmic domain of RodZ is required for its major functions, we next studied the relative contributions of the HTH and
basic domains to the properties of RodZ. Interestingly, a fusion lacking the complete HTH domain (GFP-RodZ^{83-337}) was still capable of restoring good growth to FB60(iYT27) [ΔrodZ(P lac::gfp-rodZ^{83-337})] cells as well as to restore rod shape, though many rods appeared wider than normal (Table 3-3, Fig.3-7F). However, the distribution of the fusion showed a clear distinction with that of the full-length fusion. Thus, whereas GFP-RodZ showed a clear spiral-like distribution in virtually all cells producing it, GFP-RodZ^{83-337} appeared distributed more homogenously along the membrane in both corrected ΔrodZ rods and wt cells. The distribution was not completely homogenous, however, as some small spots of relatively high signal could still be detected along the periphery of cells, suggesting that some fraction of the fusion molecules still accumulated in a more ordered fashion (Fig.3-7F3-4).

This result showed that the HTH domain of RodZ is not absolutely required for good growth or a rod shape of cells, but that it does contribute significantly to the typically sharp spiral-like distribution of the protein. To assess if the HTH domain might also be sufficient for such spiral-like distribution, we studied additional derivatives of GFP-RodZ. A soluble fusion of GFP to the complete cytoplasmic domain of RodZ (GFP-RodZ^{1-111}), appeared completely unable to correct the growth and shape defects of ΔrodZ cells, and distributed throughout the cytoplasm of both ΔrodZ spheroids and wt cells (Table 3-3, Fig.3-7C). Because the GFP-RodZ^{1-138} fusion described above appeared, by contrast, completely functional, these results indicated that association of its cytoplasmic portion with the membrane was critical to RodZ functions. To assess if
membrane-tethering of just the HTH domain would restore any functionality, we studied a membrane-bound fusion (GFP-RodZ$^{1-84}$-MalF$^{1-39}$-RFP) in which the HTH domain of RodZ is appended to the first TM helix (TM1) of the MalF protein. This fusion failed to restore the growth and shape defects of ΔrodZ cells (Table 3-3). However, the fusion localized in distinct small fluorescent spots along the periphery of FB60(iFB319) [ΔrodZ(Plac::gfp-rodZ$^{1-84}$-malF$^{1-39}$-rfp)] spheres, and its distribution was clearly spiral-like when the fusion was produced in wt rods of strain TB28(iFB319) (Fig.3-7E). These results support the notion that, as long as it is membrane-tethered, the HTH domain of RodZ is a dominant determinant of the normal spiral-like distribution of the protein.

**Role of the trans-membrane domain of RodZ.**

The fact that the growth and shape defects of ΔrodZ cells were suppressible by both the GFP-RodZ$^{1-138}$ and GFP-RodZ$^{83-337}$ fusions pointed to potentially important roles for the charged juxta-membrane and the trans-membrane domains of RodZ in allowing normal cell growth and shape. To assess if the TM domain is specifically required for these functions, we studied a fusion (GFP-RodZ$^{1-111}$-MalF$^{17-39}$-RFP) in which the entire cytoplasmic domain of RodZ is appended to TM1 of MalF. Production of this fusion in ΔrodZ cells allowed them to grow at an almost normal rate, and the fusion localized in spiral-like patterns in both ΔrodZ and wt cells (Table 3-3, Fig.3-7D). The fusion also restored rod-shape to ΔrodZ cells to an appreciable extent, although it was clearly less effective in doing so than either the full-length GFP-RodZ, or the more
comparable GFP-RodZ\textsuperscript{1-138}-RFP fusion, which contains the natural RodZ TM rather than the heterologous one from MalF. Thus, the shape of FB60(iFB289) cells ranged from wide rods to small spheres, and the majority had an intermediate ellipsoid appearance. Combined with the finding that the cytoplasmic GFP-RodZ\textsuperscript{1-111} fusion appeared to have no RodZ function at all, these results implied that there is a strict requirement for RodZ\textsuperscript{1-111} to be tethered to the membrane in order for it to support normal cell growth and shape. In addition, while there is no strict requirement for RodZ\textsuperscript{1-111} to be tethered via its native TM, the latter does seem to contribute to the efficacy of the former to some degree.

The basic juxta-membrane domain of RodZ is required for function.

To assess the importance of its basic domain to RodZ function, we studied a fusion (GFP-RodZ\textsuperscript{1-84}-MalF\textsuperscript{1-16}-RodZ\textsuperscript{111-337}) in which this domain is replaced with the N-terminal juxta-membrane portion of MalF (also basic, +3). Consistent with a crucial role of the basic domain in RodZ function, this fusion was incapable of correcting the growth and shape defects of \(\Delta\text{rodZ}\) cells.

To test the distinct possibility that the charged and TM domains might in fact be sufficient for RodZ function, we next studied a GFP-RodZ\textsuperscript{83-138}-RFP fusion containing just these domains. Production of this fusion in FB60(iFB312) cells, however, completely failed to suppress their growth and shape defects, and the fusion was distributed homogenously along the membrane(s) of the spheroids (Fig.3-7H). Note that this included those surrounding internal compartments that
form in all shape mutants under non-permissive conditions (13). To evaluate the possibility that the absence of function of any of the fusions we studied was due to excessive degradation, we performed western analyses with GFP-specific antibodies and concluded that degradation did not contribute significantly to loss of function (not shown).

In toto, our RodZ domain analyses indicate that both normal growth and shape of *E. coli* cells is strictly dependent on the presence of a membrane-anchored juxta-membrane charged domain that is either preceded by its cytoplasmic HTH domain, or followed by its periplasmic domain.
Discussion

To identify additional shape proteins in *E. coli*, we carried out a genetic screen designed to isolate mutants that, similar to the known shape mutants (13), require additional copies of the division protein FtsZ for survival on rich medium. The screen led us to identify an uncharacterized protein we named RodZ (YfgA) as being required for normal rod-shaped morphology.

**RodZ- cells have a unique and pleiotropic phenotype.**

Although we showed that RodZ is a bona fide shape protein, the phenotypes of RodZ- cells are more complex than those of cells lacking the other known shape proteins. Loss of either of the Mrd or Mre proteins results in loss of rod-shape and conditional lethality (13). Under conditions that support life, these mutants propagate as spheroidal cells. In contrast, cells lacking RodZ grew in a complex set of shapes that depended, to some extent, on growth media and temperature. These shapes ranged from viable spheroids when grown in minimal media, to large severely misshapen cells that failed to propagate when grown in rich media at and below 30°C (Fig.3-2). Notably, ΔrodZ cells grown at 42°C in both rich and minimal media appeared to adopt rod-like shapes. This suggests that a factor that is differentially expressed at elevated temperatures can partially suppress the aberrant shape phenotype. However, it is conceivable the mechanism responsible is indirect.
In addition, $\Delta ro\text{d}Z$ cells suffered drastic media-dependent reductions in growth rate that were not noted for $\Delta m\text{rd}$ and $\Delta m\text{re}$ cells. In minimal medium at 37°C, the mass doubling time of $\Delta ro\text{d}Z$ cells increase to over 298 minutes compared to just 64 minutes for RodZ+ cells. This increase in growth rate may be sufficient to explain why $\Delta ro\text{d}Z$ cells survive in minimal media even at high temperatures, as this doubling time is within the range at which $\Delta m\text{rd}$ and $\Delta m\text{re}$ mutants survive without the need for additional FtsZ (13). The lethality of RodZ-cells in rich media at or below 30°C can be suppressed by over-expression of FtsZ, suggesting that cell death under these conditions results from a failure to divide (Fig.3-2). As in $\Delta m\text{rd}$ and $\Delta m\text{re}$ cells grown under non-permissive conditions, the non-dividing RodZ- spheroids accumulated elaborate internal membrane systems that likely contribute to the division defect (13).

Thus, the loss of RodZ leads to unique pleiotropic phenotype that is only partially similar to those of the other shape mutants, all of which share a similar set of null phenotypes. One possible explanation for these phenotypic differences is that the proposed complex of shape proteins may still be partially active under certain conditions even in the absence of RodZ. In other words, RodZ may not be essential for function of the shape complex and, therefore, the set of phenotypes are the result of diminished complex function. The causes of the conditional differences in cell shape and growth phenotypes of RodZ- cells is unclear at this time. It is possible that M9 minimal media lacks some factor that is present in LB and that affects both cell shape and growth rate. Alternatively, a differentially
regulated gene product between the two media could be responsible for the phenotypic differences.

We note that divD mutations in the closely related bacterium *Salmonella enterica* may be allelic to rodZ (4, 5, 37, 188). Although the exact divD gene was not identified, corresponding mutations mapped close to hisS, and caused cells to grow as spheres. (5, 37). Further work would have to be done to establish if divD mutants are, in fact, functionally RodZ-.

**RodZ is part of the MreB cytoskeleton.**

An important contribution of the work described here is the construction and localization of a functional MreB fluorescent fusion protein, the MreB-RFP<sup>SW</sup> sandwich fusion. It is significant because it allows us to make conclusions about the localization and properties of the bacterial actin while minimizing the possibilities of anomalous or incorrect results. Furthermore, it allowed us the opportunity to co-localize it with a functional GFP-RodZ fusion and conclude this new shape protein is part of the MreB cytoskeleton (Fig.3-3E). In fact, co-localization of MreB-RFP<sup>SW</sup> and GFP-RodZ held true even in the absence of the other shape proteins suggesting the association is not bridged by one of these proteins (Fig.3-3F-G). The localization of GFP-RodZ was also found to be dependent on the presence of MreB, implying RodZ’s localization is determined by that of MreB (Fig.3-3H-I). These data suggest the co-localization of RodZ and MreB occurs directly, or alternatively, through an unknown intermediary. Interestingly, in the absence of RodZ, MreB appeared to accumulate into
patches, demonstrating that RodZ is required for maintaining the proper localization of the bacterial actin (Fig.3-4B,C and E). To better understand how RodZ ensures proper localization of MreB, we set out to define the sub-domains required for its function in the hope it would provide possible mechanisms of action.

The HTH domain is dispensable for rod-shape.

How does RodZ ensure proper MreB assembly and maintain rod-shape? A glance at the domain structure offered several interesting mechanistic possibilities. Perhaps the most striking feature of RodZ is the Cro/CI-type HTH DNA-binding domain (aa ~10-85) situated in the cytoplasm of this bi-topic membrane protein (Fig.3-1). We consequently considered the possibility that RodZ contributes to morphogenesis by acting as a transcriptional regulator. Several findings argue against this possibility, however. The most convincing argument against morpho-gene transcriptional regulation by RodZ is the fact that deletion of the HTH domain does not greatly affect rod-shape (Fig.3-6). Also, rod-shape cannot be corrected in ΔrodZ cells by expression of mrd and/or mre. Moreover, the loss or over-expression of RodZ does not greatly affect MreB levels (Fig.3-5 and not shown). These results indicate RodZ’s contribution to morphogenesis is most likely not that of a transcriptional regulator. However, a role of RodZ in transcriptional regulation of genes not necessary for rod-shape cannot be excluded at the present time. It should also be noted that RodZ does not appear to play a meaningful role in the segregation of DNA. We could not
detect significant numbers of DNA-less cells in the ΔrodZ strain FB60 and depletion of RodZ from strain FB88(iFB274) [ori-parS P_{BAD}::rodZ (P_{lac}::gfp-Δ30parB)] or FB89(iFB274) [dif-parS P_{BAD}::rodZ (P_{lac}::gfp-Δ30parB)] did not reveal significant segregation defects of the origins or termini of replication, respectively (Fig.3-6).

**Defining the functional domain of RodZ.**

If the HTH domain of RodZ is not necessary for function, then what domain(s) are? And furthermore, what are they doing? The dispensable nature of the HTH domain suggests that at least one of the other domains is necessary for rod-shape growth. Interestingly we found that both the trans-membrane and periplasmic domain are largely dispensable for good growth and rod-shape. In fact, the only domain absolutely required for rod-shape and robust growth is the basic juxta-membrane domain, which interestingly, needs to be membrane associated and either preceded by the HTH domain or followed by the periplasmic domain. One interpretation from these results is that the basic juxta-membrane domain is the true functional domain and the HTH and periplasmic domains are accessory domains with overlapping functions. If so, this overlapping function could be to co-localize the functional domain with MreB. This idea is supported by the observation that the HTH domain, when anchored to the membrane, shows robust spiral-like localization (GFP-RodZ\textsuperscript{1-84-MalF\textsuperscript{1-39}-RFP) and a construct lacking the HTH but with the periplasmic domain (GFP-RodZ\textsuperscript{83-337}) shows notably diminished localization (Fig.3-7). Therefore, we
propose that RodZ carries out its function with the juxta-membrane domain with the aid of two localization domains, the HTH and periplasmic domains. Accordingly, the HTH being a stronger localization domain is capable of maintaining better rods that the periplasmic domain which is noticeable deficient at localizing the functional domain (GFP-RodZ\textsuperscript{1-138}-RFP vs. GFP-RodZ\textsuperscript{83-337}, Fig.3-7B vs. F).

We predict the localization of the HTH domain is mediated by its direct association with MreB, or at the very least through an intermediary that is present in sufficient amounts to associate all of the RodZ protein in the cell with MreB. In contrast, the periplasmic domain may localize the functional domain indirectly through one of the other shape proteins. If so, this interaction is most likely weak, or else we would expect a sharper localization of the GFP-RodZ\textsuperscript{83-337} construct.

**How does RodZ contribute to rod-shape determination?**

How then does the properly localized juxta-membrane domain ensure rod-shape? Our data suggest the primary function of this domain is to prevent the formation of MreB patches. Evidence of patch inhibition comes from the localization data of the functional MreB-RFP\textsuperscript{SW} fusion protein in the absence of RodZ. Importantly, depletion of RodZ from strain FB81 [\textit{mreB-rfp}\textsuperscript{SW} \textit{P}_{BAD}::rodZ] leads to rapid patch formation of MreB-RFP\textsuperscript{SW} well before shape-loss, suggesting it is the cause of the aberrant morphological phenotype (Fig.3-4). Also, the poor localization of GFP-RodZ\textsuperscript{83-337} suggests that the juxta-membrane domain may
associate with MreB in a very transient manner, or else a sharper spiraled localization pattern would be expected.

Rod-shape, and importantly MreB spiral assembly, depends on a proper ratio of MreB to RodZ (Fig.3-5). Indeed, MreB patches are formed when RodZ is depleted (Fig.3-4F2) as well as when RodZ is over-expressed (Fig.3-5B2). In the latter situation, however, over-expression of MreB alleviates patch formation and restores rod-shape (Fig.3-5D). We find these observations to be further evidence that RodZ plays a role in regulating MreB assembly.

Evidence to support the MreB-scaffold model for rod-shape determination.

The MreB-scaffold model for rod-shape determination states that MreB provides a track to direct the synthesis of new murein material. Our work here provides evidence that RodZ associates with this putative shape complex in E. coli. However, it appears that RodZ is not required for MreB to direct murein synthesis. Using the fluorescent Vancomycin (Van-FL) staining technique to label nascent murein, we show murein material can still be incorporated in a pattern overlapping that of MreB even in the absence of RodZ (Fig.3-4). It thus appears that RodZ- cells are shape defective not due to the inability of MreB to direct murein synthesis, but rather due to the fact that MreB assembly is perturbed. Our ability to co-localize Van-FL with MreB was undoubtedly aided by the fact that in the absence of RodZ, MreB-RFP^SW accumulated in patches serving to concentrate the Van-FL label. Importantly, this observation, along with weakly
corresponding Vancomycin-MreB staining in wt rods and division-inhibited filaments, helps to provide the first direct evidence supporting the MreB-scaffold model for rod-shape growth.

**Intriguing observations from co-overexpression of MreB and RodZ.**

Co-overexpression experiments of MreB and RodZ, in addition to establishing that a proper ratio of the two proteins is required for rod-shape, also resulted in several intriguing and unexpected phenotypes. These phenotypes include i) narrowing of cells, ii) division inhibition, iii) aberrant chromosome localization, and iv) banded patterns of MreB and RodZ assembly and bulges in the cell wall where these zones coincide with DNA. Aside from division inhibition and aberrant chromosome localization, the causes of which are still unclear, the other phenotypes are novel. We therefore find it difficult to determine the primary defect that could lead to such a wide array of phenotypes. For example, it is conceivable that the narrowing of cells could cause the other phenotypes to develop, albeit by some unknown mechanism. Or, it is possible that RodZ shuttles DNA away from the enriched zones of MreB/RodZ, and this somehow interferes with division.

Whatever the cause of these phenotypes is, several of them represent the only instance in which a relationship between RodZ and DNA was found. At this time we cannot rule out the possibility that the nucleoid influences the positioning of MreB via RodZ, or, that the converse is true. In either case, high levels of MreB and RodZ, when coincident with DNA, leads to cell wall bulging. This is
particularly intriguing because it resembles a localized example of the phenotype that develops when only one of these proteins is over-expressed. Perhaps DNA competes with MreB for a common interaction domain on RodZ, namely the HTH. By doing so, it could titrate away the pool of RodZ molecules needed to suppress the effects of elevated MreB. This could have the effect of mimicking the over-expression of MreB alone, and could therefore suggest that DNA inhibits the shape maintaining function of RodZ. This scenario is attractive because we conclude that the HTH domain is a localization domain. We therefore believe it is possible that the HTH domain differentially interacts with both DNA and MreB, and that this somehow regulates the function of the juxta-membrane domain.

This type of reasoning can be used to propose any number of possible scenarios in which RodZ and DNA could work together to modulate cell wall metabolism, nucleoid segregation and division. We therefore believe these types of co-overexpression studies merit further analysis. For example, it may be of interest to determine if the HTH domain of RodZ, and thus possible DNA binding, is necessary or sufficient for developing these phenotypes. This may shed light on the cause of the phenotypes and contribute to our understanding of RodZ’s role in the cell.

**Remaining questions.**

There are a number of remaining questions that, when answered, should clarify the mechanism by which RodZ contributes to morphogenesis. Does RodZ directly interact with the other shape proteins? If so, what sub-domains of RodZ
are necessary for these interactions? A future goal will likely be to establish a network of interactions with all of the shape proteins and their sub-domains. Our model predicts that the RodZ HTH domain interacts directly with MreB and the TM and/or periplasmic domain interacts with one or more of the other shape proteins. Our model further predicts the juxta-membrane domain contacts MreB and prevents patch formation. However, we do not known the exact state of the MreB patches. These could include bundles of protofilaments, or perhaps aggregates of partially or fully unfolded protein. It would therefore be of interest to define the biochemical and ultra-structural nature of the patches and study the effects that RodZ may have on them.

Perhaps the most intriguing question that remains from this work is what purpose the HTH domain of RodZ plays, as it is clearly dispensable for rod-shape. It is conceivable this domain shares an essential function with that of another unknown protein. If so, it may be worthwhile to look for such a protein. Nevertheless, it would be beneficial to determine if the HTH domain even binds DNA, or if it mediates a direct interaction with MreB.

**Acknowledgements**

I thank Yu-Ting Su for plasmid construction, Thomas Bernhardt for developing the SUMO-based protein purification system for our laboratory, and Matthew Gerding for contributing to Van-FL staining. Supported by a Human Frontiers Science Program award (RGP0001/2003) and NIH GM57059 (to PdB), and NIH NRSA Institutional Training Grant T32GM08056 (to FB).
Table 3-1. *E. coli* strains, plasmids, and phages used in this study.

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<td>(111)</td>
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Phages:

\[ \lambda.FB234 \quad \text{imm}^{21} \quad \text{bla lac}\hat{\beta} \quad \text{P}_{\text{lac}}::\text{ispG} \]

\[ \lambda.FB237 \quad \text{imm}^{21} \quad \text{bla lac}\hat{\beta} \quad \text{P}_{\text{lac}}::\text{gfp-t-rodZ} \]

This work

Genotypes indicate when constructs encode in-frame Gfpmut2 (gfp), T7.tag, (t), hexahistidine (h), or the dipeptide LE (le) sequences. <> denotes DNA replacement by \( \lambda \) red recombineering, and frt a scar sequence remaining after eviction of an antibiotic resistance cassette with FLP recombinase (41, 190). Note that strains marked with * required an appropriate plasmid, phage, inducer, or media for survival.

\(^a\)h encodes His\(_6\) and t the T7.tag
Table 3-2. Growth and shape of rodZ cells.

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<th>Td (min)</th>
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*a*Cells were first grown to density at 30°C in M9-maltose plus IPTG when appropriate. They were then diluted in either M9-maltose or LB with or without IPTG, as indicated.

*b*Td, mass doubling time, as measured by culture OD$_{600}$. 

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<th>Integrated construct</th>
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<th>Corrects division</th>
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$^a$Td, mass doubling time, as determined by OD$_{600}$. 
Figure 3-1. Mutations in the rodZ locus and predicted RodZ domain structure.

A, Shown are the *E. coli* rodZ locus, the insertion site of the rod2352 EZ::TN<KAN-2> transposon (open triangle), the insertion sites of transposons previously recovered in a genomic analysis of essential genes (70) (solid black triangles), the positions of predicted promotors for downstream genes (P?), the positions of chromosomal deletion-replacements and corresponding strain designations, chromosomal inserts in plasmids used for initial complementation assays (thick lines), and the results of these assays. Portions of the chromosome that were replaced with an *aph* cassette, an *frt* scar sequence, or a heterologous transcription regulatory cassette (*aph araC* P<sub>BAD</sub>) are indicated by brackets. Numbers next to brackets refer to the base pairs replaced, counting from the start of rodZ. A translation stop (TAA) was placed immediately following codon 155 of rodZ in strain FB61. Plasmids carried the indicated inserts downstream the lac regulatory region. +, plasmid capable of correcting RodZ- and/or IspG- phenotypes; -, plasmid incapable of correcting phenotype; nd, not done.
B, Predicted domain organization and membrane topology of RodZ. HTH, Cro/CI-type helix-turn-helix motif; +++, basic juxta-membrane domain, Tm, trans-membrane domain; P, periplasmic domain consisting of a region rich in prolines and threonines followed by a region that may form several β strands.

C, Comparison of the cytoplasmic portion of RodZ with the N-terminus of λ repressor. Basic residues are in blue and acidic ones in red. Helices 1-5 of λ.cI, and the corresponding predicted helices in RodZ are boxed. Identical (*) and similar (.) residues are indicated. The basic juxta-membrane domain (residues 85-111), containing nine basic and one acidic residue, is underlined.
Figure 3-2. Growth and shape phenotypes of ΔrodZ cells, and correction by a functional GFP-RodZ fusion.

A, Spot-titer analyses of wt and ΔrodZ cells on minimal (M9) and rich (LB) medium at different temperatures. Strains FB60 [ΔrodZ] (uneven rows) and its parent TB28 [wt] (even rows) were grown to density overnight in M9-maltose at 37°C. Cultures were diluted in the same medium to an optical density at 600 nm (OD₆₀₀) of 2.4 x 10⁻² (columns A and E), 10⁻³ (B and F), 10⁻⁴ (C and G), and 10⁻⁵ (D and H), and 10 µl aliquots were spotted on M9-maltose (left panel) and LB (right panel) agar. The plates were incubated for 24 (LB) or 48 (M9) at the temperature indicated, and photographed.

B, Cell phenotypes of wt and ΔrodZ cells grown in liquid minimal (M9) or rich (LB) medium at different temperatures. Aliquots of the same overnight cultures of TB28 and FB60 described above were diluted to OD₆₀₀=0.01 in M9-maltose or LB and grown to OD₆₀₀=0.1-0.3 at the indicated temperatures. Cells were chemically fixed and imaged by DIC microscopy. Bar equals 5 µ. 
C, Suppression of ΔrodZ–associated lethality by overexpression of ftsZ. Overnight cultures of strains TB28/pDR3 [wt/P\textsubscript{lac}::ftsZ] (even rows) and FB60/pDR3 [ΔrodZ /P\textsubscript{lac}::ftsZ] (uneven rows) were grown to density at 30°C in LB supplemented with ampicillin and 50 μM IPTG. Cultures were diluted 10\textsuperscript{4} (columns A and D), 10\textsuperscript{5} (B and E), and 10\textsuperscript{6} (C and F) times in LB, and 10 μl aliquots were spotted on LB plates containing ampicillin and either no (A-C) or 50 μM (D-F) IPTG.

D, Phenotype of ΔrodZ cells producing extra FtsZ. FB60/pDR3 [ΔrodZ /P\textsubscript{lac}::ftsZ] cells were grown at 30°C in liquid LB supplemented with ampicillin and 50 μM IPTG to OD\textsubscript{600} = 0.3, chemically fixed, and imaged by DIC microscopy. Note the branching, bulges, and oddly placed constrictions. Bar equals 2μ.

E, Spiral-like localization of functional GFP-RodZ. Strain FB60(iFB273) [ΔrodZ (P\textsubscript{lac}::gfp-rodZ)] was grown to density in M9-maltose supplemented with no (left panel) or 250 μM (right panel) IPTG. Cells were diluted into the same media, grown to OD\textsubscript{600} = 0.4-0.5, and imaged live. Bar equals 2μ.
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<table>
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<tbody>
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**Figure:**
- **E1-E4:** Images showing different stages of bacterial growth in DIC and RodZ views.
- **Merge:** Combined images showing the merged view of bacterial growth.
- **MreB:** Images highlighting the MreB protein localization.
- **MreCD:** Images showing the MreCD protein localization.
- **MreAB:** Images showing the MreAB protein localization.
- **MreBCD:** Images showing the MreBCD protein localization.
- **MreB:** Images showing the MreB protein localization.
Figure 3-3. Construction of a functional MreB-RFP<sub>SW</sub> sandwich fusion, and co-localization of MreB and RodZ.

A-D, Construction and analyses of a chromosomally-encoded MreB-RFP<sub>SW</sub> sandwich fusion. Panel A shows the chromosomal mre loci of wt and mreB-rfp<sub>SW</sub> strains, illustrating the location of the rfp ORF within that of mreB in strain FB72 and its derivatives. The annealing sites and orientations of primers A1 and A2 (pair A) and B1 and B2 (pair B) are indicated. These pairs were used to amplify chromosomal DNA of TB28 [wt] (lanes 2 and 3) or FB72/pCX16 [mreB-rfp<sub>SW</sub>/sdiA] (lanes 4 and 5) by PCR, and the products were analyzed by agarose gel electrophoresis. Size standards (in kb) are shown in lane 1.

For panels B and C, overnight cultures of strains TB28 [wt], FB66 [yhdE<>cat], and FB76 [mreB-rfp<sub>SW</sub> yhdE<>cat] were diluted in LB to OD<sub>600</sub>=0.01 and grown at 30°C. Mass doubling rates were determined by measuring OD<sub>600</sub> at 1 h intervals. At OD<sub>600</sub>=0.5, aliquots from each culture were used for the determination of cell shape parameters by DIC microscopy or for preparation of whole cell extracts. Note that the yhdE<>cat mutation was engineered to serve as a selectable marker for transduction of the closely linked mreB-rfp<sub>SW</sub> allele into various strain backgrounds. Panel B lists the cell doubling times and the average cell length and width (n=200) of the three strains. Panel C shows a Western blot of the corresponding whole cell extracts from strains TB28 (lane 1), FB66 (lane 2), and FB76 (lane 2). Each lane received 10 µg total protein, and MreB (lanes 1 and 2) and MreB-RFP<sub>SW</sub> (lane 3) were detected using affinity-purified α-MreB antibodies. The positions of 66, 45, and 36 kDa (top to bottom) molecular mass standards are indicated to the left of the panel. The predicted masses of native MreB and MreB-RFP<sub>SW</sub> are 37.0 and 64.3 kDa, respectively. Panel D shows DIC (D1) and fluorescent (D2) images of FB83 [mreB-rfp<sub>SW</sub> yhdE<>frt] cells that were grown at 30°C to OD<sub>600</sub>=0.5 in M9-maltose medium. Note the normal rod shape of cells, and the spiral-like distribution of MreB-RFP<sub>SW</sub>.

E-G, Co-localization of MreB and RodZ. Corresponding DIC (1), RFP (2) and GFP(3) fluorescent, and merged fluorescent (4) images are shown. Panel E shows the co-localization of MreB-RFP<sub>SW</sub> and GFP-RodZ in rod-shaped cells in which these functional fusions are the only MreB and RodZ proteins present. Note the perfect co-localization in the typical spiral-like cytoskeleton. Panels F and G illustrate that MreB-RFP<sub>SW</sub> and GFP-RodZ still co-localize in more disorganized patterns at the periphery of spheroids that are
completely devoid of MreC and MreD (F), or of MrdA (PBP2) and MrdB (RodA). The focal plane was near the top of cells in panels F and G.

H-I, Location of RodZ in the absence of MreB. Corresponding DIC (1), RFP (2) and GFP (3 and 4) fluorescent images are shown. The panels show the distribution of GFP-RodZ in spheroids that either lack all three Mre proteins (H), or that produce MreC and MreD, but lack MreB specifically. The focal plane was near the top (panels 3) or through the interior (panels 4) of the spheroids. Note the even distribution of GFP-RodZ along the cell membrane, including along that of an intracytoplasmic vesicle visible in I (arrow).

Strains used for panels E-I were: FB101(iFB273) [mreB-rfpSW ΔrodZ (P\lac::gfp-rodZ)] (E), FB95(iFB273)/pTB63 [mreB-rfpSW ΔmreCD (P\lac::gfp-rodZ) /ftsQAZ] (F), FB90(iFB273)/pTB63 [mreB-rfpSW ΔmrdAB (P\lac::gfp-rodZ) /ftsQAZ] (G), FB30(λ.FB237)/pTB63 [ΔmreBCD (P\lac::gfp-rodZ) /ftsQAZ] (H), and FB30(λ.FB237)/pTB63/pFB206 [ΔmreBCD (P\lac::gfp-rodZ) /ftsQAZ /P\BAD::mreCD] (I). Cells were grown to density at 30°C in M9-maltose supplemented with appropriate antibiotics and 250 μM IPTG, and with either no (E-H) or 0.05% (I) arabinose. After dilution to OD\text{600}=0.1, growth was continued under the same conditions to OD\text{600}=0.4-0.6, and cells were imaged live. Note that under the same conditions, pFB206 directs the production of sufficient MreC and MreD to correct the shape defect of a ΔmreCD strain (not shown). Bar equals 2μ.
Figure 3-4. RodZ-dependent localization of MreB and MreB-directed murein synthesis.

A-F, Formation of large aberrant MreB patches in RodZ- cells. Shown are corresponding DIC (panels 1) and RFP fluorescence (panels 2) images of live cells.
Panels A-C show a comparison of the localization of MreB-RFP<sup>SW</sup> in spheroids that either completely lack MrdA (PBP2) and MrdB (RodA) (A), or RodZ (B and C). Note the numerous small fluorescent spots along the periphery of ΔmrdAB cells versus the distinctly less numerous and larger patches of MreB-RFP<sup>SW</sup> that accumulate at the periphery of ΔrodZ cells (arrows) in either minimal (B) or rich (C) medium. Strains used were FB90/pTB63 [mreB-<i>rfp</i>ΔmrdAB/ftsQAZ] (A), and FB85/pTB63 [mreB-<i>rfp</i>ΔrodZ/ftsQAZ] (B and C). Overnight cultures in M9-mal were diluted to OD<sub>600</sub>=0.1 in the same (A and B) or in LB (C) and growth was continued at 30ºC to OD<sub>600</sub>=0.4-0.5.

Panels D-F show that depletion of RodZ leads to the formation of such MreB patches well before cells become grossly misshapen. The RodZ-depletion strain FB81 [mreB-<i>rfp</i>ΔP<sub>BAD</sub>::rodZ] was grown overnight in M9-mal with 0.5% arabinose, diluted to OD<sub>600</sub>=0.05 (D and E) and OD<sub>600</sub>=0.01 (F) in LB with 0.5% (D) or no (E and F) arabinose, and growth was continued at 30ºC to OD<sub>600</sub>=0.4-0.5.

G-L, Co-localization of MreB and Van-FL in the presence and absence of RodZ. G,I,K,L Shown are corresponding DIC (panels 1), RFP (panels 2) and Van-FL (panels 3) fluorescence, and merged fluorescence (panels 4) images of live cells. Strain FB109 [mreB-<i>rfp</i>swΔyhdE<>ftr imp4213] harboring either pBAD33 vector control (G and H) or pJE80 [P<sub>BAD</sub>::<i>sflA</i>] (I and J) were grown overnight in M9-mal supplemented with Cm and 0.1% glucose at 30ºC and diluted 1:1000 into LB supplemented with Cm and 0.1% arabinose. Growth was continued at 30ºC until OD<sub>600</sub>=0.3-0.4. Cells were then stained with Van-FL (see methods). H and J show a magnified view of the boxed regions in G and I, respectively. Arrow-heads indicate overlapping zones of both RFP and Van-FL fluorescence. K and L, Strain FB110/pFB310 [imp4213 rodZ<>aphl P<sub>λR</sub>:: mreB-<i>rfp</i>sw] was grown overnight in M9-mal supplemented with Spec at 30ºC and diluted to an OD<sub>600</sub>=0.01. Growth was continued at 30ºC to an OD<sub>600</sub>=0.1 and cells were stained with Van-FL (see methods). Arrow-heads indicate overlapping zones of both RFP and Van-FL fluorescence. Large arrow indicates Van-FL staining that does not have corresponding MreB-RFP<sup>SW</sup> fluorescence and most likely overlaps with the assembly of FtsZ.
Figure 3-5. Effects on cell shape by over-expression of RodZ and/or MreB.

Panels A-C show over-expression of RodZ (B) and MreB (C) lead to spherical shape when grown in M9 medium at 30°C. Overnight cultures of FB83 [mreB-rfp<sup>sw</sup> yhdE<>frt] harboring vector pJF188EH (A), pFB291 [P<sub>lac</sub>::rodZ] (B), or pFB214 [P<sub>lac</sub>::mreB] (C), grown in LB 0.1% glucose and appropriate antibiotics at 30°C were diluted to OD<sub>600</sub>=0.005 in M9-mal supplemented with the appropriate antibiotics and either 250 µM IPTG (A and C) or 100 µM IPTG (B). Cells were grown at 30°C to OD<sub>600</sub>=0.5, chemically fixed, and imaged. Shown are DIC (1), and RFP (2) fluorescence images of representative cells. Note the clustering of MreB-RFP<sup>SW</sup> in cell over-expressing RodZ (arrows, B2). Bar equals 2 µ.

Panel D shows co-overexpression of both MreB and RodZ can restore rod growth but also lead to a division defect. Overnight culture of FB83/pFB216 /pFB291 [mreB-rfp<sup>sw</sup> yhdE<>frt / P<sub>lac</sub>::mreB / P<sub>lac</sub>::rodZ] grown in LB 0.1% glucose and appropriate antibiotics at 30°C were diluted to OD<sub>600</sub>=0.005 in M9-mal supplemented with the appropriate antibiotics and 250 µM IPTG. Cells were grown at 30°C to OD<sub>600</sub>=0.5, chemically fixed, and incubated with 0.25 µg/ml DAPI prior to imaging. Shown are DIC (1), DAPI (2) and RFP (3) fluorescence. Arrow-head indicates zones of MreB-RFP<sup>SW</sup> enrichment and absence of DNA. Large arrow indicates a bulge centered around accumulation of both MreB-RFP<sup>SW</sup> and DNA.

Panel E shows that shortly after co-overexpression of MreB and RodZ, MreB-RFP<sup>SW</sup> and GFP-RodZ accumulate at the cell tips which are also DNA-free. Overnight culture of strain FB83/pFB299/pFB309 [mreB-rfp<sup>sw</sup> yhdE<>frt/P<sub>lac</sub>::mreB rodZ/ P<sub>syn35</sub>::gfp-t-rodZ] grown in LB 0.1% glucose was diluted 1:600 into M9-mal. Cells were grown at 30°C to OD<sub>600</sub>=0.5, chemically fixed, and incubated with 0.25 µg/ml DAPI prior to imaging. Shown are DIC (1), DAPI and RFP merged (2), RFP (3), and GFP (4) fluorescence images of representative cells.

Panel F shows over-expression levels of MreB and RodZ relative to wt. Cultures of strain TB28 with plasmid pairs pJF188EH and pBAD33 (wt, 1), pFB291 and pBAD33 (RodZ+++ 2), pFB216 and pJF118EH (MreB+++ 3) and pFB291 and pFB216 (RodZ+++ MreB+++, 4) grown as described from panels A-C and prepared for quantitative Western analysis. Fold induction is shown below each blot.
Figure 3-6. DNA segregation and positioning in RodZ- cells.

Panels A-F show RodZ- (D and E), Mrd- (F) and Mre- (C) cells do not show nucleoid segregation defects. Overnight cultures of strains TB28 (A), TB28/pTB63 (B), FB30/pTB63 (C), FB60 (D), FB60/pTB63 (E) and FB38/pTB63 (F) grown in M9-mal (Tet was included for strains carrying pTB63) at 30°C were diluted to OD_{600}=0.1 in the same media. Cells were grown at 30°C to OD_{600}=0.3 and chemically fixed. Prior to imaging DAPI was added to 0.25 µg/ml. Shown are DIC (1) and DAPI fluorescence (2). 200 cells
from each strain were analyzed for the absence of DAPI staining indicating the presence of DNA-less cells. This revealed a total of 1, 1, 4, and 0 DNA-less cells for TB28/pTB63, FB30/pTB63, FB38/pTB63 and FB60/pTB63, respectively.

Panels G-N show loss of RodZ does not greatly affect segregation of origins and termini of replication. Overnight cultures of strains TB99(iFB274) [ori-parS (Plac::gfp-Δ30parB)] (G and I), FB88(iFB274) [ori-parS ParBAD::rodZ (Plac::gfp-Δ30parB)] (H and J), FB86(iFB274) [dif-parS (Plac::gfp-Δ30parB)] (K and M) and FB89(iFB274) [dif-parS ParBAD::rodZ (Plac::gfp-Δ30parB)] (L and N) grown in M9-mal with 0.5% arabinose at 30°C were diluted to OD$_{600}$=0.01 into M9-mal lacking arabinose but supplemented with 100 µM IPTG and growth was continued at 30°C. At OD$_{600}$=0.1 (T1) and 0.4 (T2) aliquots were taken for preparation of whole cell extracts and for chemical fixation. Prior to imaging, DAPI was added to 0.25 µg/ml. Shown are DIC (1), DAPI (2) and GFP (3) fluorescence images for cells from T1 (G,H,K and L) and T2 (I,J,M and N).

Panel O shows Western blot confirming depletion of RodZ. Equal amounts of whole cell extracts (10 µg) made from cultures G-N were probed with αRodZ antibody.

Panel P has a histogram indicating appearance of nucleoids from RodZ+ (blue) and RodZ depleted (red) cells from time points T1 (left panel) and T2 (right panel). Cells analyzed include those from origin and termini experiments (n=200).

Panels Q and R has a histogram showing the number of foci of the GFP-Δ30ParB protein bound to parS sequences near the origin (Q) and terminus (R) in RodZ+ (blue) and RodZ- (red) cells from time points T1 (left panel) and T2 (right panel) (n=100).
Figure 3-7. Defining RodZ domains necessary for localization and rod-shape.

Shown is the ability of various GFP-tagged constructs expressed from integrated plasmids to correct the spherical shape of the ∆rodZ knockout strain FB60 (1 upper) and to localize in FB60 (1 lower) and wt strain TB28 (2 lower). Integrated plasmids used were iFB273 (A), iYT22 (B), iFB285 (C), iFB289 (D), iFB319 (E), iYT27 (F), iFB293 (F), iFB312 (H) and iFB321 (I). Above each set of images is a cartoon depicting the domain architecture of the tested construct. Domains are HTH, Helix-turn-Helix (aa 10-85); +++, RodZ basic domain (86-110); Tm, RodZ transmembrane domain (111-133); P, RodZ periplasmic domain (134-337); +, MalF cytoplasmic domain (2-14); M, MalF transmembrane helix 1 (15-34); RFP, mCherry RFP. All constructs have GFP appended to the N terminus via the T7 linker peptide. Strains were grown overnight in M9-mal with 250 µM IPTG at 30°C and diluted to OD600=0.05 in the same media. Cells were grown at 30°C to OD600=0.3-0.5 and imaged live. Shown are DIC and GFP fluorescent images of representative cells. The arrows in panels F3 and F4 show faint foci formed by GFP-RodZ83-337 (iYT27). Shown are DIC (upper) and optical slices of GFP fluorescence from the middle (middle) and top of the cell (lower).
Figure 3-8. Model for RodZ function in *E. coli*. The juxta-membrane domain of RodZ (red +) prevents MreB patch formation when localized to MreB via either the HTH or periplasmic domain. In the absence of RodZ altogether, MreB accumulates into patches, or clusters, which are still capable of directing murein synthesis. Without the HTH domain, the periplasmic domain properly localizes the juxta-membrane domain through a putative interaction with one of the other shape proteins, such as MreC. This interaction is predicted to be relatively weak as only a fraction of HTH- RodZ molecules localizes in spirals. Without the periplasmic domain, the HTH domain, which we predict strongly and directly interacts with MreB, properly localizes the juxta-membrane domain to prevent MreB patch formation.
Chapter 4

Future Studies
A role for Mrd and Mre proteins in regulating phospholipid synthesis?

One feature common to all six shape mutants (*mrd*, *mre*, and *rodZ*) is the accumulation of extensive phospholipid membrane systems in their interior. We propose that these membrane systems contribute significantly to the conditional division defect by recruiting essential division protein into non-productive assemblies. It is still unclear why shape mutants accumulate extra membrane. Why does the cell not adjust the levels of phospholipid to meet its actual surface requirement? We considered the possibility that the rate of phospholipid synthesis is simply hard wired to the amounts for a rod and coupled to the increase in cell mass. Another possibility is that the rate of phospholipid synthesis is regulated by the putative shape complex. Perhaps the shape complex "senses" the surface requirements and communicates with the phospholipid synthesis machinery to fine tune the proper levels.

Mechanisms that regulate the rate of phospholipids synthesis are not well understood (38). It is attractive to think that one reason why virtually nothing is known about such mechanisms is because they are intimately connected to the mechanisms that determine cell shape. It would be difficult to interpret aberrations in phospholipid synthesis rates if they were also accompanied by shape defects. Moreover, it is not intrinsically obvious what the phenotype would be of cells that made either half or twice the amounts of phospholipids that are required. If a cell made half the required phospholipids would they turn spherical or simply reduce their growth rate? If a cell made twice as much, would the extra membrane simply involve into the interior of the cell similar to what happens with
the shape mutants? What effect would this have on shape or division? Perhaps it would be illuminating to attempt to establish protein-protein interactions between phospholipid synthases and the shape proteins. Also, it is possible that the synthases co-localize with MreB or one of the other shape proteins, and this should be worth investigating.

**Do the Mrd and Mre shape proteins co-localize?**

An important component of this work is the construction of a functional MreB-RFP sandwich fusion protein. It represents the first functional fluorescent fusion protein to MreB in *E. coli* or related organisms. The MreB-RFP^{SW} fusion was found to localize to spiral-like structures similar to those seen in *B. subtilis*. Furthermore, it proved to be ideal for establishing co-localization of MreB with RodZ, which was tagged with GFP. In this same way the sandwich fusion can be used to ask the question of whether the additional shape proteins join MreB and RodZ in the same sub-cellular spiral. Protein-protein interactions suggest this will be the case, however it is important to place physiological relevance to these interactions. Co-localization between all of the shape proteins would further support the model the proteins function as a complex.

The MreB-scaffold model for rod-shape determination predicts that the other shape proteins will depend on MreB for proper localization. It would be interesting to establish a dependence of localization of shape proteins on each other. This may serve to establish a hierarchical series of interactions that could be useful to predict function of the components.
How do RodA, MreC and MreD contribute to cell shape determination?

As discussed in chapter 1, the polytopic membrane protein RodA has been suggested to be a lipid II “flipase” that delivers the murein precursor to the synthases. This, however, has not been established. It may be useful to test this possibility by utilizing the fluorescence-based assay originally developed for studying phospholipid membrane asymmetry (102, 121) and recently adapted for studying transport of lipid II (171). This method relies on the ability to label lipid II with a fluorescent compound that can subsequently be incorporated into vesicles derived from synthetic phospholipids or cell membrane. The fluorophore from one side of the vesicles can be rendered non-fluorescent either by adding a reducing agent or with specific antibody. From here, the transport of lipid II from one side to the other can be measured over time by additional treatment and measuring loss of fluorescence (171). Performing these lipid II flipping assays with purified RodA, or alternatively, with cell membranes from shape mutants, may prove to be a useful approach to identify and characterize the lipid II flipase.

The extensive interactions between \textit{B. subtilis} MreC and a number of high and low weight PBPs suggests this protein may be important to stabilize a complex of murein synthases and hydrolases. An additional function of MreC cannot be ruled out at this time however. Perhaps the periplasmic domain, which is structurally similar to the chymotrypsin family of peptidases, has murein hydrolase activity. The protein responsible for L,D carboxypeptidase mediated maturation of murein is not known (chapter 1). It may be worthwhile exploring the
possibility this activity is carried out by MreC, or possibly the periplasmic domain of RodZ. Both MreC and RodZ are found in cocci species of bacteria that lack MreB (not shown), possibly suggesting a general role in murein synthesis.

MreD is a relatively small (162 aa) polytopic integral membrane protein with five predicted transmembrane helices. Interestingly, these appear to show higher degrees of conservation that the short membrane domain-connecting loops (not shown) suggesting a function in the membrane interior. Again, the function of MreD could be to serve as a shape complex-stabilizer, similar to MreC.

**How large is the putative shape complex?**

Here we provide direct evidence supporting the MreB-scaffold model of rod-shape determination. We imagine a scenario where MurG, after catalyzing the formation of lipid II, hands the murein precursor off to the putative shape complex, which in turn transports it across the inner membrane and incorporates it into the existing sacculus. As discussed above, incorporating new material likely requires breakage of the existing sacculus. To prevent compromising the integrity of the sacculus, these events, namely breaking and making the murein bonds, are probably highly regulated and coordinated. It is reasonable to imagine that the shape complex could include numerous hydrolases as well, perhaps coordinated by MreC (173). It would therefore be worthwhile to determine the sub-cellular localization of additional murein synthases and hydrolases. It is also conceivable that, in addition to MraY and MurG, many of the enzymes involved in generation of lipid II precursor are also associated with the complex. We can
imagine a scenario where, similar to the possibility discussed above that phospholipid synthases could by regulated by the shape proteins, the shape proteins could likewise associate with and regulate murein precursor synthases. To expand this even further, perhaps the synthesis of the entire cell envelope is carried out by several large complexes, or cell envelope synthesizing organelles, that are spatially coordinated by the cytoskeletal systems of MreB and FtsZ.

**Are there additional shape determining factors?**

This work identified a novel shape factor, RodZ, necessary for maintaining rod-shape in *E. coli*. It was found in the Rod genetic screen (Requiring an overdose of FtsZ) designed to identify mutants that require overexpression of the division protein FtsZ, a feature common to all six known shape mutants. The screen also identified mutants with a transposon insertion in the regulatory regions of the *mrd* and *mre* operons, but not in the coding regions themselves. These mutants were most likely still able to produce intermediate amounts of their respective shape proteins. This fact may have made them slightly “healthier” than a null mutant and could indicate that the Rod screen may have difficulty identifying the “sicker” null shape mutants. Indeed, the *rodZ* null mutant FB60 was found to have a significant plating defect (Fig.3-2A). Therefore, the screen, although it successfully identified RodZ, may not have been saturated. Additional attempts that aim to screen very large numbers of colonies may identify additional shape factors.
It is also conceivable that additional shape factors are unconditionally essential and that their loss cannot be compensated for by extra FtsZ. These would not be expected to be identified by the Rod screen. As an alternative, it should be possible to screen for additional shape factors by looking for binding partners of the known shape proteins. This could be done using one or more of the shape proteins as bait in a bacterial two-hybrid screen. Additionally, there may be shape factors that only become essential when a portion of RodZ is missing. Cells can tolerate the loss of the HTH or periplasmic domain of RodZ and still grow well while retaining a rod-shape. Perhaps it would be fruitful to perform a synthetic lethal screen asking for mutants that require the RodZ HTH or periplasmic domain. For example, strain FB61 [rodZ155<>aph], which has a deletion of the periplasmic domain, harboring an unstable plasmid encoding the full length RodZ protein, could be mutagenized and screened for mutants that require the unstable plasmid for growth. A similar approach could be tried with a mutant lacking the RodZ HTH domain.

**Does RodZ directly regulate MreB assembly?**

Our results strongly suggest a role for RodZ in ensuring proper assembly of the MreB cytoskeleton. In particular, the juxta-membrane domain of RodZ, when properly localized via either the HTH or periplasmic domain, appears to be required to prevent MreB patch formation (Fig.3-8). This model needs to be tested directly by examining the effects of RodZ on MreB assembly using purified components. This approach may prove to be a challenge. Several attempts to
purify E. coli MreB has not yielded functional protein. The reasons for our inability to do so are not known and will require additional efforts. Nevertheless, if and when functional MreB is purified it can be tested for its ability to hydrolyze ATP, to polymerize as assayed by light scatter and sedimentation, and to form higher order structures visible by electron microscopy. It will be worth testing how RodZ might affect MreB’s ability to perform any one of these expected functions.

**What purpose does the RodZ HTH domain have?**

The dispensable nature of the HTH domain for rod-shape is intriguing. Why does RodZ have this fairly-well conserved domain? Our results suggest it may simply be a localization domain that directly interacts with MreB. If so, it may be coincidental that this domain shows significant homology to the HTH domain of the cl/cro family of transcriptional regulators (Fig.3-1). In the event one is determined, the crystal structure of this domain may shed some light on its precise role in the cell. However, even if the structure of this domain resembles a true HTH it is still conceivable it interacts with MreB and not DNA. It is therefore a high priority to establish if this domain does in fact bind DNA and, if so, why?

Initial attempts to establish RodZ as a DNA-binding protein by electro-mobility shift assays (EMSA) showed promise. However, DNA-binding was found to be independent of the HTH domain and probably carried out by the highly basic juxta-membrane domain (not shown). It may be of greater benefit to examine possible in vivo DNA binding by chromatin immunoprecipitation (ChIP) assays and map potential binding sites by gene chip analysis.
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