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Chemical–Biological Studies of Subcellular Organization in Bacteria

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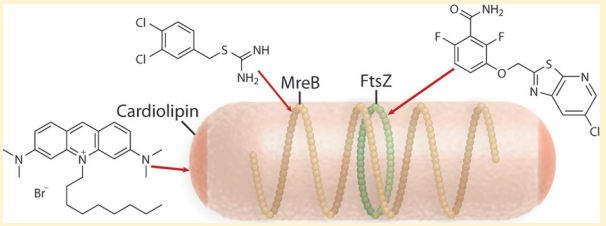
ABSTRACT: The subcellular organization of biological molecules is a critical determinant of many bacterial processes, including growth, replication of the genome, and division, yet the details of many mechanisms that control intracellular organization remain unknown. Decoding this information will impact the field of bacterial physiology and can provide insight into eukaryotic biology, including related processes in mitochondria and chloroplasts. Small molecule probes provide unique advantages in studying these mechanisms and manipulating the organization of biomolecules in live bacterial cells. In this review, we describe small molecules that are available for investigating subcellular organization in bacteria, specifically targeting FtsZ, MreB, peptidoglycan, and lipid bilayers. We discuss how these probes have been used to study microbiological questions and conclude by providing suggestions about important areas in which chemical–biological approaches will have a revolutionary impact on the study of bacterial physiology.



The subcellular organization of proteins in bacteria has striking similarities to that of eukaryotic cells.[1−6] For example, three major families of eukaryotic cytoskeletal proteins (tubulin, actin, and intermediate filaments) are homologous to bacterial proteins, including FtsZ, MreB, and crescentin, respectively.[7] Despite their structural similarities, eukaryotic and bacterial cytoskeletal proteins evolved different physiological functions, and the in vivo roles of the bacterial proteins are still not entirely understood.[8,9] Chemical–biological approaches to the study of intracellular organization in bacteria are gaining momentum and are poised to have an important impact on this field, just as they have in eukaryotic cell biology.

The discovery of small molecules that target specific proteins and modulate their structure and activity in vivo transformed the field of eukaryotic cell biology. The characterization of many of these compounds initially captured the attention of biologists and chemists because of the cytotoxic properties of the compounds and later enabled cell biologists to inhibit the function of proteins and query their role in the cell.[10] Colchicine is an example of this class of small molecules. Colchicine binds tubulin and inhibits its polymerization, and studies of this process provided insight into the function of this cytoskeletal protein.[11] The discovery of paclitaxel as a stabilizing factor that prevents microtubule depolymerization yielded an inhibitor with a biological function that was complementary to colchicine; together, these compounds enabled biologists to control the state of tubulin in cells and to study its structure and function. The development of a toolbox of small molecule inhibitors for cytoskeletal proteins in eukaryotic cells made it possible for biologists to regulate the structure, function, and localization of proteins in ways that were difficult to achieve solely by genetics. In summary, chemical–biological tools revolutionized modern cell biology.

In this review, we describe small molecules for studying the subcellular organization of biomolecules in bacteria. The application of chemical tools to study these areas has several useful characteristics. (1) They act quickly. (2) They do not require manipulation of the chromosome. (3) They may be reversible or nonreversible. (4) Their activity may be easily modulated as a function of dose. (5) Inhibitors targeting conserved cellular processes may be applicable across a broad range of bacterial genera or species. These properties enable the study of cellular organization in live bacterial cells. We discuss specific areas in which chemical approaches have elucidated biological functions centered on cell growth, division, and physiology. Specifically, we review chemical studies of the tubulin homologue FtsZ, the actin homologue MreB, the peptidoglycan (PG), and membranes. We discuss inhibitors of FtsZ and MreB and highlight potential challenges or advantages of these probes for studying bacterial organization. We conclude each section with a short discussion of unanswered questions that could benefit from the use of small molecule probes.

FTSZ: A BACTERIAL TUBULIN HOMOLOGUE

The discovery of the protein FtsZ in bacteria and its structural similarity to tubulin provided the earliest evidence of the cytoskeleton as an ancient mechanism for controlling structure and organization in cells. FtsZ protofilaments form the scaffold upon which the Z-ring, a protein complex that defines the division plane, is assembled and mediates the process of septation during cell division.[2,3,12] The localization of FtsZ to the midcell during division is illustrated in Figure 1A.

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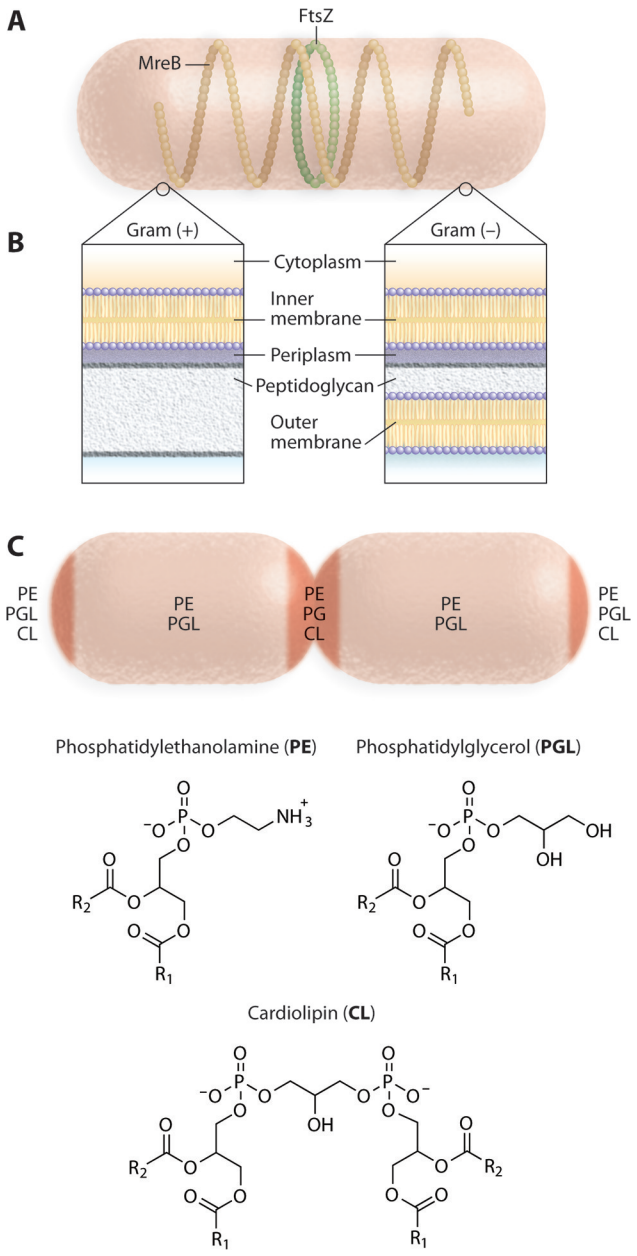


Figure 1. Subcellular localization of FtsZ, MreB, peptidoglycan, and lipids in bacterial cells. (A) Cartoon depicting the localization of the tubulin homologue FtsZ and actin homologue MreB. (B) Structure of the cell wall of Gram-positive and Gram-negative bacteria. The dark lines separating the peptidoglycan from adjacent layers were added to indicate the boundaries. (C) Cartoon depicting the distribution of the membrane lipids phosphatidylethanolamine (PE), phosphatidylglycerol (PGL), and cardiolipin (CL).

A hallmark of loss of FtsZ function is the stalling of septation, which causes cells to filament, makes them mechanically unstable, and eventually causes lysis. The Z-ring recruits numerous proteins to the division site, including enzymes that degrade and build the cell wall. There are several unknown properties of FtsZ and the Z-ring, including the structure of FtsZ protofilaments in vivo and the mechanism by which the Z-ring generates a contractile force that is required for cell division.[13] FtsZ and tubulin share a similar structure and polymerization properties; however, these proteins are not sensitive to the same small molecule inhibitors. Several putative FtsZ inhibitors are described below and are summarized in Table 1.

PC190723 as a Potent Inhibitor of FtsZ. 3-Methoxybenzamide underwent a medicinal chemistry "face lift" to increase its potency as an FtsZ inhibitor.[14] PC190723 is one of the compounds that emerged from this effort; the compound stabilizes FtsZ filaments and is a potent antimicrobial agent against *Staphylococcus* species.[15] *Bacillus subtilis* and *Staphylococcus aureus* mutants that are resistant to PC190723 and other 3-methoxybenzamide derivatives have mutations that cluster to a region of FtsZ associated with the paclitaxel-binding site in eukaryotic tubulin.[14,15] Certain *S. aureus* mutants require PC190723 for growth as the compound stabilizes specific FtsZ mutations. This phenotype is indicative of a specific interaction between the inhibitor and its target protein. PC190723 has a K_d of 10 μM to recombinant FtsZ from *B. subtilis*.[16] In vitro, PC190723 increases the stability of FtsZ protofilaments from recombinant FtsZ purified from *B. subtilis* and *S. aureus* and reduces the dynamics and GTPase activity of the protein.[15,16] The formation of dynamic FtsZ polymers is crucial for functional Z-ring formation in vivo. The observation that PC190723 causes rod-shaped *B. subtilis* cells to filament and spherical *S. aureus* cells to increase in diameter is consistent with its influence on FtsZ function.[14,15] This compound is hence a promising in vivo probe for studying FtsZ function in Gram-positive bacteria; its activity, however, is limited in Gram-negative organisms.

Chemical–Biological Studies of FtsZ Using PC190723 Analogue 8j. FtsZ has recently been studied in vivo using PC190723 analog 8j.[17] 8j has an MIC (0.25 μg/mL) that makes it 4 times more potent than PC190723 (1 μg/mL) against *S. aureus*.[18] The treatment of recombinant FtsZ with 8j stabilizes polymers and decreases GTPase activity.[17] In vivo experiments with *B. subtilis* strain 168 demonstrated that FtsZ was mislocalized and the protein had reduced turnover rates in cells treated with 8j.[17]

Localization experiments demonstrated that FtsZ no longer formed Z-rings in cells treated with 8j; however, components of the Z-ring remained localized within FtsZ foci that were interspersed throughout cells, including ZapA, EzrA, SepF, FtsL, DivIC, penicillin binding protein 2B (PBP2B), and FtsW.[17] Treatment of cells with 8j did not change the localization pattern of the Min system proteins DivIVA, MinC, and MinD or the nucleoid occlusion protein Noc.[17] Small clusters of mislocalized FtsZ did not respond to the Min and nucleoid occlusion systems.[17] The Min proteins prevent localization of FtsZ near the poles, and the nucleoid occlusion system prevents localization of FtsZ in regions occupied by chromosomal DNA. The binding of 8j to FtsZ polymers may reduce access of regulatory domains on FtsZ to the Min and nucleoid occlusion systems.

Inhibitor 8j causes FtsZ to form highly curved and single-stranded polymers in vitro, even in the presence of ions that encourage FtsZ bundling (e.g., Ca^{2+}).[17] The effect of this small molecule on polymer formation may explain the weakening of FtsZ function and atypical Z-ring formation in cells treated with inhibitor. At sub-MIC concentrations of 8j, abnormal cell division occurred in *B. subtilis* and *S. aureus* and was accompanied by helical or twisted division septa.[17] Clusters of FtsZ remained associated with PG synthesis machinery; however, the function of these proteins was altered.[17] Compound 8j appears to abolish the remodeling of PG at

Table 1. FtsZ Inhibitors[a]

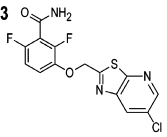
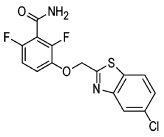
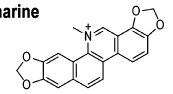
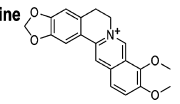
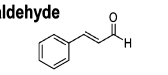
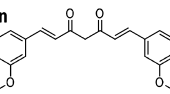
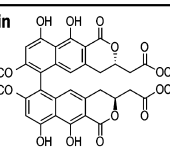
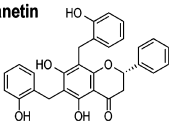
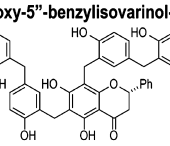
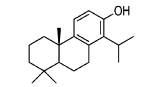
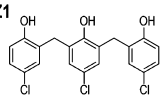
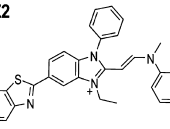
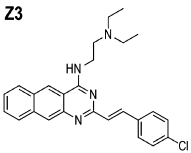
Compound Structure	MIC values	FtsZ binding data	Refs
PC190723 	2.8 μM (*B. subtilis*, *S. aureus* MDRSA), other strains tested	Binding site at H7 of FtsZ (Taxol-tubulin binding site); *B. subtilis* FtsZ, Kd = 10μM	(14-16)
8j 	0.7 μM (*S. aureus* ATCC 29213), other strains tested	Binding site at H7 of FtsZ	(17,18)
Sanguinarine 	10 μM (*B. subtilis* 168) 75 μM (*E. coli* BL21)	*E. coli* FtsZ, Kd = 30 μM	(26)
Berberine 	1.5 mM (*E. coli* MG1655)	*E. coli* FtsZ, Kd = 20 nM	(27, 28)
Cinnamaldehyde 	7.6 mM (*E. coli*) 3.8 mM (*B. subtilis*) 1.9 mM (*S. aureus* MRSA)	Binding site at H7 of FtsZ (SulA-FtsZ binding site); *E. coli* FtsZ, Kd = 1.0 μM	(32)
Curcumin 	100 μM (*B. subtilis* 168) >100 μM (*E. coli* MG1655)	*E. coli* FtsZ, Kd = 7.3 μM	(33)
Viriditoxin 	12 μM (*S. aureus* MRSA) 38 μM (*E. coli* MB 5431 tetR)	ND	(36)
Dichamanetin 	1.7 μM (*S. aureus*) 1.7 μM (*B. subtilis*) 3.4 μM (*M. smegmatis*)	ND	(37)
2'''-hydroxy-5''-benzylisovarinol-B 	10.7 μM (*S. aureus*) 2.6 μM (*B. subtilis*) 3.8 μM (*M. smegmatis*) 2.3 μM (*E. coli*) 15.4 μM (*P. aeruginosa*)	ND	(37)
Totarol 	2 μM (*B. subtilis* 168) 56 μM (*S. aureus*, *M. tuberculosis*)	*M. tuberculosis* FtsZ, Kd = 11 μM	(41)
Zantrin Z1 	20 μM (*E. coli* MC1000) 40 μM (*P. aeruginosa* K) 1.25 μM (*B. subtilis* JH 642) 2.5 μM (*S. aureus* H, MRSA) various others tested	ND	(38)
Zantrin Z2 	40 μM (*E. coli* MC1000) >80 μM (*P. aeruginosa* K) 2.5 μM (*B. subtilis* JH 642) 1.25-2.5 μM (*S. aureus* H, MRSA) various others tested	ND	(38)
Zantrin Z3 	20 μM (*E. coli* MC1000) 40 μM (*P. aeruginosa* K) 1.25 μM (*B. subtilis* JH 642) 2.5 μM (*S. aureus* H, MRSA) various others tested	ND	(38)

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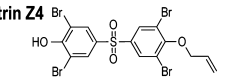
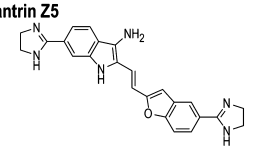
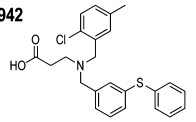
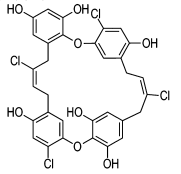
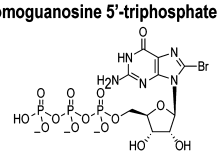
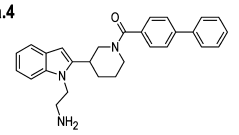
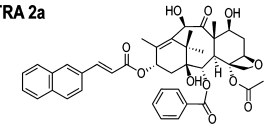
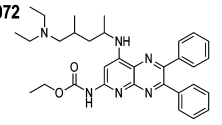
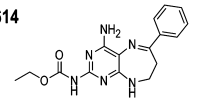
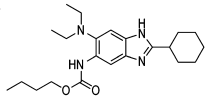
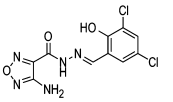
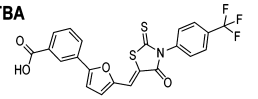
Table 1. Continued

Compound Structure	MIC values	FtsZ binding data	Refs
Zantrin Z4 	2 μM (*B. subtilis* 168)	ND	(38)
Zantrin Z5 	0.28 μM (*M. tuberculosis* H37Rv)	ND	(38)
PC170942 	>600 μM (*E. coli* ATCC 25922) >600 μM (P. aeruginosa 101021) 38 μM (B. subtilis 168) 150 μM (S. aureus ATCC 601055) various others tested	FtsZ binding site at the edge of the GTP binding pocket	(45)
Chrysophaentin A 	MIC_{50} values 2.7 ± 0.9 μM (*S. aureus* ATCC 25923) 2.2 ± 1.0 μM (*S. aureus* ATCC BAA-44) 5.6 ± 2.8 μM (*E. faecium* ATCC 49032) 4.3 ± 1.2 μM (*E. faecium* ATCC 70022)	FtsZ binding site adjacent to and partially occludes the GTP binding pocket	(46)
8-bromoguanosine 5'-triphosphate 	ND	*M. jannaschii* FtsZ, Kd, app = 126 ± 38 nM	(19, 20)
16.a.4 	50 μM (*E. coli*) 100 μM (*H. influenzae*) 12.5 μM (*M. catarrhalis*) 25 μM (*B. subtilis*) 25 μM (*S. aureus*) 12.5 (*S. pneumoniae*)	*M. jannaschii* FtsZ, Kd = 105 μM	(21)
TRA 2a 	5 μM (*M. tuberculosis* H37Rv) 2.5 μM (*M. tuberculosis* IMCJ946.K2)	ND	(22)
SRI-3072 	0.28 μM (*M. tuberculosis* H37Rv)	ND	(23)
SRI-7614 	19.2 μM (*M. tuberculosis* H37Rv)	ND	(23)
1a-G7 	2.0 μM (*M. tuberculosis* H37Rv) various other *M. tuberculosis* strains tested	ND	(24)
A189 	200-400 μM (*E. coli*) 50 μM (*S. aureus*)	ND	(25)
OTBA 	2 μM (*B. subtilis* 168)	*E. coli* FtsZ, Kd = 15 μM	(26)

[a]These compounds have been described in the literature as FtsZ inhibitors. This table summarizes the structures of inhibitors, their antimicrobial activities, and FtsZ binding characteristics. MIC values represent MIC_{99} unless otherwise specified. Values that have not been reported are specified as ND.

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the septum by synthases associated with FtsZ but does not affect the insertion of cell wall precursors along the cylindrical region of cells.[17]

Evaluation of the Potency and Specificity of Putative FtsZ Inhibitors in Vivo. Several other small molecules have been identified as FtsZ inhibitors; below we introduce many of these compounds and discuss their activity and specificity for FtsZ. Additional compounds not discussed in the text are listed in Table 1.[19−25] Small molecule inhibitors require several characteristics to be useful as probes for biological studies, including their binding a target specifically. It is thus important that the inhibitor has minimal off-target effects.

There are several off-target effects that may be misinterpreted as direct FtsZ inhibition in vivo, including direct DNA damage, stalling DNA replication, and perturbing the transmembrane potential. Sanguinarine and berberine have been described as FtsZ inhibitors; they have also been characterized as intercalating agents that perturb DNA topology and may cause DNA damage.[26,27] The SOS response pathway is responsible for blocking cell division when the cell is stressed, for example, by DNA damage, and stimulates the expression of the FtsZ inhibitor protein SulA. The study of bacterial filamentation caused by berberine concluded that SulA and the SOS response pathway were not responsible for this phenotype and suggested it was initiated by FtsZ inhibition.[28] However, bacteria can filament via SulA-independent mechanisms that arise from DNA damage or stalled replication, and a variety of mechanisms can trigger these events.[29,30] Mitomycin C, a DNA-damaging agent with no known FtsZ binding properties, causes cell filamentation in an *Escherichia coli* Δ*sulA* mutant.[31] Sanguinarine and berberine may have no direct effect on FtsZ, and their DNA binding properties may explain their toxicity.

Other potential DNA-damaging agents include the small molecules cinnamaldehyde and curcumin, which have been described as FtsZ inhibitors.[32,33] Both molecules contain a reactive aromatic α,β-unsaturated carbonyl group that may react with nucleophilic amino acid side chains of proteins via Michael addition reactions. Several studies have demonstrated that cinnamaldehyde and curcumin participate in the formation of reactive oxygen species in cells, which may initiate an SOS response and cause filamentation in bacteria.[34,35] Ruling out the general reactivity of these compounds in vivo and characterizing their off-target effects would be beneficial for their application to the study of FtsZ in vivo.

Viriditoxin, dichamanetin, and 2‴-hydroxy-5″-benzylisovarinol-B are polyphenolic natural products that have been described as FtsZ inhibitors;[36,37] in the following section, we describe a structurally related polyphenol, Zantrin Z1.[38] The binding affinity and putative FtsZ binding site have not yet been determined for viriditoxin, dichamanetin, or 2‴-hydroxy-5″-benzylisovarinol-B. In vitro studies with these compounds demonstrated that they inhibit the GTPase activity of FtsZ and destabilize polymer filaments. The overexpression of FtsZ rescued cells treated with viriditoxin. The authors assessed the DNA damage and replication effects of viriditoxin in cells lacking the SOS response protein SulA. However, cell elongation due to DNA damage or the perturbation of DNA replication still occurs in the absence of SulA and makes it difficult to build direct connections between cell filamentation and FtsZ inhibition. For example, the antimicrobial compound 534F6 (now termed gyramide A) was originally found in a screen for FtsZ inhibitors and causes Δ*sulA E. coli* to filament.[39] Gyramide A and structurally related analogues were recently characterized as a new class of DNA gyrase inhibitors.[40] The inhibition of DNA topology regulation is also a trigger for cell filamentation.

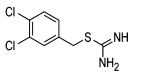
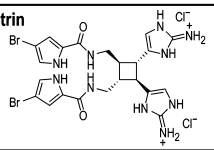
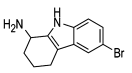
Another class of putative FtsZ inhibitors that may have significant off-target effects based on their in vivo activity is the Zantrins. The Zantrins consist of a family of small molecules discovered in a screen for modulators of the GTPase activity of recombinant FtsZ.[38] Five GTPase inhibitors were identified in the screen: Z1 and Z4 were inhibitors of FtsZ polymerization, and Z2, Z3, and Z5 stabilized FtsZ filaments. The compounds disrupted Z-ring formation in vivo; however, only Z5 produced filamentous cells. As cell filamentation is a hallmark of inhibiting FtsZ function, the absence of this phenotype with Z1−Z4 suggests off-target effects on growth or metabolism. No data are available for the binding site or affinity for any of the compounds in this family.

Membrane perturbations represent another category of off-target effects that may be relevant to the response of cells to the Zantrins as well as another inhibitor, totarol. Totarol is a natural product isolated from the tree *Podocarpus totara* and has been described as an inhibitor of FtsZ.[41] *B. subtilis* cells become elongated after being treated with totarol, which suggests a potential defect in cell division. The K_d for the association between totarol and *Mycobacterium tuberculosis* FtsZ was determined to be 11 μM; however, the region of FtsZ to which the compound binds is unknown. Several different mechanisms of antibacterial action have been proposed for totarol against Gram-positive strains, including the disruption of bacterial respiration.[42] The localization of cytoskeletal and cell division structures in bacteria was recently demonstrated to be sensitive to membrane depolarization.[43] The effect of totarol is reminiscent of oxygen-deprived *B. subtilis* in which the cells become elongated and wider. A useful strategy for assessing whether the effects of totarol are due to changes in respiration may be to evaluate the localization of other important division-related proteins, including MinD in cells treated with this small molecule. The localization of MinD in cells requires the transmembrane electric potential and may thus be a useful indicator of its depletion.

3-{5-[4-Oxo-2-thioxo-3-(3-trifluoromethylphenyl)-thiazolidin-5-ylidenemethyl]furan-2-yl}benzoic acid (OTBA) is a small molecule discovered in a screen that induced filamentation in *B. subtilis* cells. OTBA decreased GTPase activity of recombinant FtsZ and increased the degree of bundling of FtsZ filaments.[44] Treatment of cells with OTBA disrupts the localization of FtsZ, prevents Z-rings from forming, causes cells to elongate, and does not affect DNA replication or segregation. The site of binding of OTBA to FtsZ has not been identified; the K_d for binding of OTBA to *E. coli* FtsZ was reported to be 15 μM. Identification and characterization of a binding site would be useful in validating this potential FtsZ inhibitor.

PC170942 and chrysophaentin A are FtsZ inhibitors for which binding sites have been proposed. PC170942 is an analogue of PC58538, which was identified in a sporulation cell-based screen.[45] PC58538 causes *B. subtilis* 168 cells to filament, and the phenotype can be reverted by overexpressing FtsZ. *B. subtilis* mutants that are resistant to PC58538 contain mutations to amino acids near the GTP binding site of FtsZ and produce cells that have a minimal or reduced level of

Table 2. MreB Inhibitors[a]

Compound Structure	MIC values	MreB binding data	Refs
S-(3,4-dichlorobenzyl)isothiourea (A22) 	13 μM (*E. coli*) 53 μM (*S. aureus*) >425 μM (*B. subtilis*) 43 μM (*C. crescentus* CB15N)	*T. maritima* MreB, Kd = 1.32 μM	(53, 60-68)
Sceptrin 	22 μM (*S. aureus*)	Monomeric G-actin, Kd = 19.2 μM	(68-71)
CBR-4830 	128 μM (*P. aeruginosa* PAO1) 1 μM (*P. aeruginosa* CB398, strain lacks 5 separate RND efflux pumps)	ND	(72)

[a]These compounds have been described in the literature as MreB inhibitors. The structure, antimicrobial activity, and MreB binding characteristics of this class of compounds are given.

filamentation compared to wild-type cells. Analogue PC170942 has improved potency and inhibits both the formation of FtsZ filaments and GTPase activity with IC_{50} values of 44 and 1100 μM against *B. subtilis* and *E. coli* FtsZ, respectively.

The recently identified natural product chyrsophaentin A has antimicrobial activity against Gram-positive strains, including drug-resistant *S. aureus* and *Enterococcus faecium*.[46] Chyrsophaentin A reduced the level of polymerization of *E. coli* FtsZ and inhibited its GTPase activity with an IC_{50} of 9 μM. Chyrsophaentin A competes with GTP for binding FtsZ, as it occludes the triphosphate region of the GTP binding pocket and partially blocks the guanine binding site. It will be important to determine if this compound and its analogues have in vivo activity against Gram-negative and Gram-positive strains. The characterization of the effect of chyrsophaentin on cells in vivo will also be useful in gauging its utility as a cellular probe.

In summary, although numerous putative FtsZ inhibitors have been described, many of these compounds lack the level of characterization in vivo that is required to confirm their specificity for targeting FtsZ. The two best characterized FtsZ inhibitors are PC190723 and its derivative 8j; however, they are limited to Gram-positive bacterial studies. For further reading, additional reviews describe small molecule inhibitors of FtsZ.[47,48]

Future Studies: Dissecting FtsZ Function Using Small Molecule Probes. *FtsZ Control of PG Remodeling.* Inhibitor 8j provides a glimpse into the structure and function of FtsZ polymers in vivo. FtsZ appears to localize the cell division PG remodeling machinery and to regulate its activity. The inactivation and localization of PBP2B to FtsZ foci distributed throughout cells treated with 8j suggest that the enzymes that synthesize PG may be active only when the Z-ring is intact. Identifying and characterizing this mechanism may be useful in developing new antibiotics that inhibit bacterial cell division and would be particularly beneficial if the control of the active state of PG remodeling within intact Z-rings is conserved across various species of bacteria.

Structure of FtsZ Polymers in Vivo. Experimental data support a hypothesis for the importance of lateral contacts between FtsZ protofilaments in Z-ring formation. On the basis of the reported effects of 8j, we suggest that the curvature of FtsZ filaments induced by 8j may perturb the formation of lateral contacts. Characterization of the curved filaments may contribute to understanding how FtsZ protofilaments bundle and self-associate, and the identification of contacts that are required for associations. The structure of PC190723 docked in silico against helix 7 of FtsZ and its similarity to the paclitaxel binding site suggest this region of the protein may be involved in regulating polymer structure and/or the ability to form lateral contacts.[15] Despite the availability of crystallographic data for FtsZ, the structure of FtsZ polymers in vivo is still not understood. Further characterization is required to understand the biological relevance of the 8j binding site and its effects on FtsZ polymer structure and dynamics.

MREB: A BACTERIAL ACTIN HOMOLOGUE

Actin homologues participate in several key physiological processes in bacteria, including regulating cell shape, maintaining cell polarity, and participating in chromosome segregation.[2,3,12,49,50] A hallmark of these processes is the requirement for the spatial and temporal control over the positioning of biomolecules within the cell. For example, the biochemical regulation of bacterial cell shape occurs via positioning cell wall-modifying enzymes on actin-like filaments.[51] Members of this family of bacterial proteins include MreB, Mbl, MreBH, FtsA, ParM, AlfA, and MamK. MreB is required for maintaining the polar localization of several proteins and participates in chromosome segregation.[52−54] The localization of MreB in the cell is shown in Figure 1A, but it should be noted that the current understanding of MreB localization has recently been challenged.[55−58] MreB has been demonstrated to form patches, rather than a helical structure across the cell.[55,56,58] Another recent study demonstrated that MreB controls the location of cell wall synthesis during growth and provides a mechanical role in defining the stiffness of cells.[59] Several small molecule inhibitors have been identified and characterized for MreB; one compound in particular (A22, shown in Table 2) has been utilized for studying MreB function in vivo.

***S*-(3,4-Dichlorobenzyl)isothiourea (A22) as a Potent MreB Inhibitor.** *S*-(3,4-Dichlorobenzyl)isothiourea (A22) was discovered in a screen for compounds that caused chromosome partitioning defects in *E. coli*.[60] The synthesis and evaluation of structural analogues indicated that the *S*-benzylisothiourea motif was a key component of the activity of this compound in vivo.[61] Replacement of the benzyl group or isothiourea moiety abolished biological activity. The incorporation of chlorine at the 3- and/or 4-position on the benzyl

group enhanced biological activity.[62] A22 binds MreB and perturbs the ATP-binding region of the protein, as the locations of the alleles in A22-resistant *Caulobacter crescentus* mutants map to or near the ATP-binding pocket of MreB.[53] The K_d for binding of A22 to *Thermotoga maritima* MreB in the absence of ATP is 1.3 μM.[63] ATP and A22 likely compete for binding to MreB because of the overlap in their binding sites, and thus, A22 must be used at concentrations much higher than the K_d to observe saturating effects. Below, we discuss MreB studies that have taken advantage of A22.

Chemical–Biological Studies of MreB Using A22. The inhibition of MreB by A22 demonstrated that the protein participates in chromosome segregation in *C. crescentus*;[53,64] in contrast, the protein does not appear to be essential to chromosome segregation in *E. coli* and *B. subtilis*.[65,66] Resistant mutants of *C. crescentus* corrected chromosome localization defects in the presence of A22.[53] MreB was also observed to associate with origin-proximal regions of DNA, which suggests that loss of the interaction between MreB and DNA is responsible for the defects observed following A22 treatment of cells.[53]

A22 and the structurally related analogue MP265, a compound that will be described in a later section, were used to study the relationship between MreB and PG biosynthesis. PG synthesis was inhibited in A22-treated cells of rod-shaped bacteria and produced round cells.[67] A22 treatment increased UDP-MurNAc-pentapeptide levels, suggesting that this cell wall precursor is no longer incorporated into the PG. The effect was similar to blocking cell elongation in conditional mutants of PBP2, which had levels of UDP-MurNAc-pentapeptide and PG turnover that were similar to those of cells treated with A22. This observation suggests that MreB function is necessary for PBP2 activity. The addition of A22 did not disrupt degradation of PG by transglycosylases, suggesting that these enzymes are not under direct control of MreB.

It was recently demonstrated that *E. coli* MreB forms patches that move perpendicular to the long axis of the cell and that A22 decreased the number of these patches but not their velocity.[58] This result is consistent with observations of MreB mutants believed to be deficient in ATP hydrolysis, where movement of MreB patches was not blocked, and suggests that MreB polymerization does not contribute to the movement of these protein domains.[55] Photobleaching experiments with fluorescently labeled MreB eliminated MreB treadmilling as a model for MreB patch motility.[56] However, when cells were treated with antibiotics that inhibit PBP2 and PG synthesis, the movement of MreB patches was blocked.[55,56,58] This result indicates that peptidoglycan synthesis is coupled to the movement of MreB patches around the cell.

In agreement with previous studies, A22 treatment of cells decreased the average length of glycan strands.[68] This observation supports the role of MreB in increasing PBP2 activity. The recovery of cells after A22 treatment involves extensive remodeling of the cell wall and has a destabilizing effect on the outer membrane, which is responsible for the loss of outer membrane lipids through vesicle formation. Cells recover from the mislocalization of MreB and return to their normal rod shape.

Chemical–Biological Studies of MreB and Actin Using Sceptrin. Sceptrin is an MreB inhibitor that was identified using a screen for the isolation of natural products with an affinity for *E. coli* proteins.[69] The analysis of sceptrin-resistant *E. coli* K-12 mutants demonstrated that the inhibitor binds to or perturbs the ATP-binding pocket of MreB, like A22, as sceptrin-resistant mutants also confer A22 resistance to *E. coli* cells. Sceptrin has also been described as an inhibitor of eukaryotic actin and decreases the motility of several cancer cell lines.[70] Although a synthetic route to sceptrin has been described,[71] the inhibitor has not yet been used to study MreB directly and may lack the broad-spectrum activity observed for A22. For example, the compound is ineffective at inhibiting MreB in *C. crescentus*.[68]

CBR-4830 and Chemical–Biological Studies of MreB. CBR-4830 is an indole that has been reported to bind MreB.[72] Mutants of *P. aeruginosa* that are resistant to CBR-4830 contain mutations that map to the *mreB* open reading frame. These mutations partially overlap with loci described for A22-resistant mutants in *C. crescentus*.[53] CBR-4830-resistant *P. aeruginosa* strains had improved resistance to A22 compared to wild-type cells, which suggests an overlap in the inhibitory mechanism of these compounds. However, in this study, CBR-4830 inhibited the ATP-dependent polymerization of *P. aeruginosa* MreB, whereas A22 did not. These results suggest a subtle difference in the mechanisms by which the two compounds inhibit MreB. A22 inhibits MreB by competing with ATP, whereas CBR-4830 may not.[63,72]

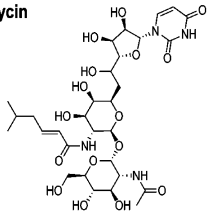
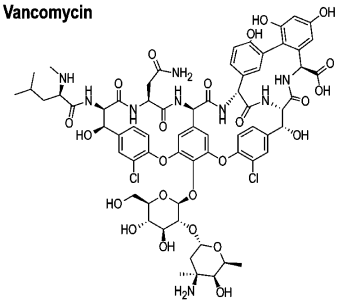
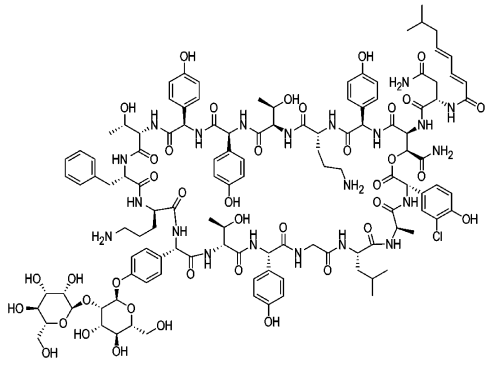
CBR-4830 appears to be less sensitive than A22 to the concentration of ATP during MreB inhibition and is thus an attractive candidate for in vivo studies. It should be noted, however, that CBR-4830 is pumped out of wild-type cells, thus reducing its effectiveness in vivo. This limitation can be transcended by co-application of a drug efflux pump inhibitor or studies in drug efflux pump knockout strains. CBR-4830 has not yet been used to study MreB organization; however, after further validation of off-target binding, it may be a useful tool for in vivo studies.[72]

Future Studies: Dissecting MreB Function Using Small Molecule Probes. *Eliminating the Influence of Off-Target Effects.* The characterization of off-target effects of A22 in the absence of MreB would be particularly helpful for in vivo studies. Removing the 3-chloro group from A22 from the phenyl ring improved the specificity of the analogue, referred to as MP265.[68] For example, treatment of MreB knockout strains with high concentrations of A22 reduced the rate of bacterial growth; treatment with MP265 did not impair growth. These data suggest that MP265 may be more specific for MreB than A22 and, thus, a promising lead compound for further investigations.

Validation of MreB DNA Segregation Function. Studies of the effects of A22 on MreB function in vivo have demonstrated its role in DNA segregation and PG synthesis in *C. crescentus*. It would be helpful to address whether A22 disrupts DNA segregation through the binding of MreB to ParB, or preventing binding of ParB to *parS* sites on the chromosome, as has been previously suggested.[66] An additional possibility is that A22 differentially inhibits other prokaryotic ATPases, such as ParA, in different species of bacteria.

Confirmation of CBR-4830 as an MreB Inhibitor in Vivo. The evaluation of CBR-4830 as an in vivo inhibitor of MreB may provide a complementary probe for studying MreB that transcends some of the limitations of A22. Further studies in vivo are required to evaluate the target and off-target effects of CBR-4830, as this compound has not been vetted for in vivo studies of MreB.

Table 3. Peptidoglycan Labeling Reagents[a]

Compound Structure	Biomolecular target	Fluorescence properties	Refs
Tunicamycin 	Broad inhibitor of glycosylation	NA	(78)
Fosfomycin 	MurA	NA	(79)
Vancomycin 	Nascent PG subunit (a dissacharide pentapeptide)	BODIPY-FL conjugate; λex / λem = 502/510 nm	(81)
Ramoplanin A2 	Lipid II	Fluorescein conjugate; λex / λem = 500/520 nm	(82)

[a]These compounds have been described and utilized as labels for studying PG synthesis. The structure, biomolecular targets, and fluorescence characteristics of this class of compounds are given.

■ PG AND CHEMICAL BIOLOGY

The PG consists of a network of polysaccharides (i.e., the glycan) that are linked together by peptides. The subunit of this copolymer is a disaccharide tetrapeptide in which *N*-acetylglucosamine and *N*-acetylmuramic acid form the disaccharide monomer; the most common sequence of the mature tetrapeptide consists of L-Ala, D-Glu, *meso*-2,6-diaminopimelic acid, and D-Ala. The subunits are connected through β-1,4-glycosidic bonds to form glycan strands, and amide groups between tetrapeptides link strands together. The composition of the PG subunit and the linkage site between glycan strands vary among different bacterial species. Other factors that influence PG diversity include the three-dimensional structure and organization of the PG network. In Gram-positive bacteria, the meshwork of PG forms the outermost layer of the cell, contains associated teichoic acids, and is thicker than the PG layer found in Gram-negative organisms. In Gram-negative bacteria, the PG is positioned between the inner and outer membranes. A visual comparison of Gram-positive and Gram-negative PG structure is provided in Figure 1B. For detailed information about the chemistry, physical properties, and structure of PG, we suggest several excellent reviews.[73−75]

Antibiotics That Target the PG. A currently accepted model of the spatial organization of PG synthesis entails the cytoskeletal proteins as the shape-determining factors that guide PG synthesis, and PG as the scaffold that physically maintains the cell shape.[76,77] In the past decade of research that led to this model, several different methods were used to probe PG synthesis and correlate its localization with cell division, growth, cytoskeletal proteins, and their interacting partners. These methods include metabolic labeling, antibody labeling, and fluorescent antibiotics that specifically bind to nascent PG substrates. In this section, we focus our discussion on PG-specific antibiotics and their use in studying subcellular organization in bacteria.

The inhibition of cell wall synthesis was among the earliest targets for the development of new antibiotics. Thus, there is a long list of antibiotics that target different stages of PG synthesis, and several of these compounds have been useful in studying subcellular organization (Table 3). Using tunicamycin

as a probe, ManA was determined to play a role in cell shape maintenance.[78] Fosfomycin was used to investigate the hierarchy of protein−protein interactions in cytoskeleton-guided PG synthesis.[79] These and other examples demonstrate how antibiotics can provide insight into the spatial and temporal organization of PG synthesis.[80]

In addition to their use in modulating protein activity in vivo, antibiotics can be covalently modified with fluorescent probes for visualizing cellular organization using epifluorescence microscopy. Three antibiotics, vancomycin, ramoplanin, and phenoxymethylpenicillin (penicillin V, also termed bocillin), have been derivatized with fluorophores for studying PG.[81−83] Vancomycin binds the nascent pentapeptide of a PG subunit and prevents its maturation into a disaccharide tetrapeptide to block PG assembly.[84] Because the maturation step from penta- to tetrapeptide is rapid, labeling of Gram-positive bacteria with fluorescent vancomycin derivatives indicates regions of nascent PG synthesis.[76] Similar to vancomycin, ramoplanin also inhibits the maturation of PG.[82] The antibiotic inhibits trans-glycosylation by binding to lipid II.[85] Ramoplanin is arguably a more specific probe for visualizing nascent PG, as the pentapeptide substrate of vancomycin is also present in mature PG.[82] The third derivatized antibiotic, bocillin, blocks cell wall synthesis by inhibiting transpeptidases.[83] Radiolabeled penicillin derivatives have been used to identify and characterize penicillin-binding proteins (PBPs). Bocillin was introduced as a nonradioactive alternative for visualizing functional PBPs on polyacrylamide gels. It is unclear whether the localization of the probe specifically indicates the sites of PG synthesis; however, its labeling is comparable to that of fluorescent vancomycin in cells of *Corynebacterium glutamicum*.[80]

A significant advantage of using fluorescent derivatives of antibiotics is the high temporal resolution that they provide in contrast to metabolic and antibody labeling methods. Fluorescent antibiotics can be used in conjunction with proteins that are translationally fused to fluorescent proteins, thereby allowing multicolor live cell imaging experiments. This combination of strategies for labeling has been an important tool in studying the dynamics of PG synthesis and the involvement of cytoskeletal proteins in cell shape determination.

Fluorescent antibiotics have several useful characteristics, including their specificity, rapid binding, and compatibility with in vivo imaging; however, these probes are relatively impermeable to the cell wall of Gram-negative bacteria. Bulky glycopeptides cannot penetrate the outer membrane, and the cell wall of Gram-negative bacteria contains multiple sites for vancomycin binding; these characteristics make it difficult to distinguish between old and new PG.[76] However, Gober and co-workers[86] have successfully used fluorescent vancomycin to probe the localization of PG synthesis in *C. crescentus*.

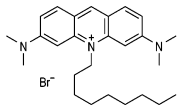
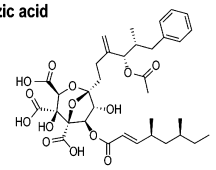
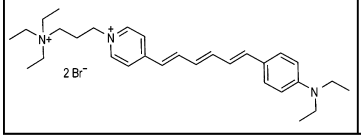
Applications of Fluorescent Vancomycin Probes. The advantages of using fluorescent vancomycin derivatives far outweigh their limitations and have made them widely used probes for studying PG synthesis. This class of small molecules has been particularly useful for studying two general areas of cellular organization in bacteria: (1) the spatial organization of bacterial growth and division and (2) the investigation of the involvement of cytoskeletal proteins and their associated proteins in PG synthesis. In this section, we summarize findings from both of these areas of research.

The first use of fluorescent vancomycin revealed the location of nascent PG in *B. subtilis*, *Streptococcus pneumoniae*, *Streptomyces coelicolor*, and *C. glutamicum* cells.[81] The fluorescent antibiotic has since been used to probe the cell division patterns of several other organisms, including *C. crescentus*,[86] *Mycobacterium* species,[87] *Lactococcus lactis*,[88] and *S. aureus*.[89] This approach for studying PG synthesis transcends the limitations of the spatial resolution of autoradiography and has facilitated the discovery of previously uncharacterized phenomena, including the helical pattern of PG synthesis in *B. subtilis*.[81,82] A recent study of *S. aureus* used the fluorescent probes to investigate how this spherical bacterium can sequentially alternate among three orthogonal planes for division.[90] The authors selectively marked the first division plane with fluorescent vancomycin and tracked the position of the labeled PG during the following two cycles of division. Their approach allowed the correlation between PG labeling and the age of a cell. The results of this study suggest that remodeled PG may serve as a physical marker (e.g., a "landmark") of previous division planes in a spherical bacterium.

The dynamic process of PG remodeling is frequently used as a protein positioning mechanism in the cell. For example, in *Listeria monocytogenes*, the continuous remodeling of PG along the long axis and at the septum of the cell leads to the asymmetric polar localization of ActA.[91] ActA extends from the cytoplasm to the outside of the cell, and its asymmetric distribution is critical for the motility of *L. monocytogenes* cells inside the eukaryotic host. The authors demonstrated that the localization of fluorescent ActA and vancomycin labeling are mutually exclusive, indicating that the degradation of PG and PG-spanning proteins drives the polar localization of ActA. Initially, the protein is randomly inserted into the cell envelope, and repeated cell growth and division spread the patches of ActA into a more uniform distribution across the surface. The distribution of ActA becomes concentrated at one pole as some regions of PG (e.g., the septum) are degraded more rapidly than others (e.g., cell poles). This study suggests that the uneven distribution of PG synthesis and differential PG dynamics can serve as a mechanism for localizing proteins in bacteria.

Fluorescent vancomycin derivatives have also been used to correlate cell wall dynamics and the localization of the cytoskeleton. Several groups have demonstrated that the depletion of one of the three MreB isoforms does not abolish the helical pattern of fluorescent vancomycin in *B. subtilis*, suggesting that these proteins have a redundant function in determining cell shape.[82,92] *E. coli* and *C. crescentus* cells also use MreB for guiding the cylindrical growth of PG, and FtsZ has been shown to modulate the spatial activity of MreB in this process.[86,93] *S. aureus* has a spherical morphology and lacks a copy of *mreB* in its genome. The depletion of *S. aureus* FtsZ resulted in diffuse PG synthesis throughout the entire cell, instead of the typical tight band in the region at which the septum forms.[89] Finally, actinobacteria may have a different mechanism for the localization of PG synthesis, as the organism does not use MreB; instead, DivIVA guides polar PG synthesis in *S. coelicolor*, *C. glutamicum*, and mycobacterial species.[94] Several lines of evidence suggest that DivIVA is required for polar growth and the rod shape of *C. glutamicum*, as fluorescently labeled vancomycin and DivIVA colocalize in these cells.[95] In summary, these studies used vancomycin as a probe for nascent PG and demonstrated the role of cytoskeletal proteins as cell shape determinants.

Table 4. Probes for Lipid Studies[a]

Compound Structure	Biomolecular target	Fluorescence properties	Refs
10-N-nonylacridine orange (NAO) 	Cardiolipin	λex / λem = 488/628 nm	(114, 121)
Ro09-0198 Cys-Arg-Gln-Cys-Cys-3-NH2-Ala-Phe-Gly-Pro-Phe-(2S,3S)-2-amino-3-mercaptobutanoyl-Phe-Val-Cys-3-OH-a-Asp-Gly-Asn-(2S,3S)-2-amino-3-mercaptobutanoyl-Lys	Phosphatidyl-ethanolamine	Needs to be tagged for fluorescent antibody binding (e.g. conjugated with biotin for binding with anti-streptavidin antibody)	(119, 120)
Zaragozic acid 	Squalene synthase	NA	(127)
FM 4-64 	Non-specific binding to lipids	λex / λem = 515/640 nm	(133)

[a]These small molecules have been described and utilized in the literature as probes for studying lipid organization. This is a summary of the structure of probes, their biomolecular target or lipid specificity, and fluorescence characteristics.

The bacterial cytoskeleton serves as a blueprint for the structure of the PG; however, it is unclear how these cytoplasmic proteins communicate with PG synthesis machinery located outside of the (inner) membrane. Which molecules link the cytoskeleton to the PG machinery? Many proteins interact with the cytoskeleton in *C. crescentus*. For example, MreC and MreD bridge MreB and the PG machinery.[51] Minor changes in cell shape and minimal vancomycin labeling accompany the depletion of MreC.[86] The small change in cell shape was consistent with intact localization of MreB structures during MreC depletion. These phenotypes indicate that MreC is an integral component in transmitting the organization of MreB to the PG synthesis machinery and influencing cell wall assembly.

Future Studies: PG-Specific Chemical Tools and Their Application to Studying Bacteria. In this section, we discuss unanswered questions regarding the dynamic localization and interactions of proteins in bacterial cells that may be effectively probed and manipulated using small molecules. Small molecules may be advantageous over protein-based probes (e.g., antibodies or translational fusions to fluorescent proteins) when the target is heterogeneous and has a dynamic structure and position in cells (e.g., PG, chromosome, and lipid membranes). Advances in labeling technologies, including the use of Sortase A to attach user-designed molecules to the PG and development of synthetic cell wall precursors that can be incorporated into PG in vivo, will play an important role in imaging the structure and assembly of this biopolymer.[96−100]

Below we summarize several areas in which chemical probes may be particularly useful tools in studying PG-related questions in microbiology.

Cytoskeletal Proteins and PG Synthesis. During cell division, the Z-ring produces a constrictive force that is hypothesized to participate in cytokinesis. How is the force generated by FtsZ transmitted to the PG synthesis machinery? How does the PG machinery establish and maintain the width of the cell?[101] Bacterial genomes contain a variety of genes encoding proteins that may be homologous to tubulin, actin, and intermediate filament. What is the role of these unexplored cytoskeletal elements in the regulation of PG synthesis in bacteria? The discovery of small molecule inhibitors of these proteins will be helpful in studying these questions.

Cell Growth and PG Synthesis. How is the length of a cell determined, and which proteins regulate the process? How is metabolism and environmental sensing coupled to cell growth and PG synthesis? In *M. tuberculosis*, PknA and PknB kinases may participate in the interplay between the extracellular environment and intracellular decisions. Fluorescently labeled vancomycin has been used to demonstrate that the phosphorylation state of the Pkn kinases governs its interactions with PG synthesis machinery.[102] How does a bacterium coordinate cell wall elongation and division? Both of these processes involve PG synthesis. How do the different components interact and communicate? The labeling pattern of *B. subtilis* cells treated with vancomycin and the localization of PBP1 have provided insight into how EzrA and GpsB may coordinate FtsZ- and MreB-based machineries.[103]

Subcellular Localization of PG Synthesis as a Mechanism for Storing Spatial Information and a Force for Moving MreB Filaments. Earlier, we mentioned the hypothesis that spherical bacteria may use remodeled PG from the previous round of cell division as a mechanism for the sequential alternation between division planes. The confirmation of this hypothesis requires an understanding of how the epigenetic information in PG is transmitted to the cytoplasm. Another cellular process that may use the organization of PG as a landmark is the maintenance of cell polarity. Cells maintain polarity before, during, and after division. How does a daughter cell inherit polarity from the mother cell?[104] The localization of PG synthesis may spatially guide the organization of the

bacterial chromosome. The PG synthesis inhibitor tunicamycin caused shape defects in *B. subtilis* cells and simultaneously perturbed the morphology of the nucleoid.[78] The shape of the nucleoid was studied using 4′,6-diamidino-2-phenylindole (DAPI), a small molecule that fluoresces when bound to AT-rich regions of DNA. From these studies, it was proposed that chromosome conformation may follow the organization of PG synthesis.[78] Another role of PG synthesis may be translocation of MreB-associated complexes, as three recent studies demonstrated that inhibition of PG synthesis using antibiotics caused rapid cessation of MreB movements in vivo.[55,56,58]

Peptidoglycan Architecture. Can the optical properties of fluorescent PG-specific probes be tailored for applications in super-resolution microscopy techniques?[105,106] The use of these techniques for studying the organization of PG in live cells below the diffraction limit will provide important details about PG architecture.

SUBCELLULAR ORGANIZATION OF LIPIDS AND CHEMICAL BIOLOGY

Bacterial membranes consist of three majors classes of phospholipids: phosphatidylethanolamine (PE), phosphatidylglycerol (PGL), and cardiolipin (CL). PE has no net charge (i.e., zwitterionic), and PGL and CL are anionic under physiological conditions. All three classes of phospholipids arise from a common precursor via a branched biosynthetic pathway.[107] The phospholipid composition of the membrane varies among species of bacteria and depends on growth conditions.[108] A representation of lipid localization and composition is shown in Figure 1C. In *E. coli*, the cell membrane consists of 75% PE, 20% PGL, and 5% CL.[109]

Probing Lipid Organization Using Small Molecules. Three classes of small molecules for studying the subcellular organization of lipids are introduced and discussed below: photoactivatable cross-linkers, lipid-specific fluorophores, and inhibitors of lipid biosynthesis (Table 4).

A variety of different photoactivated lipid analogues have been synthesized to probe the environment surrounding lipids.[110] For example, phosphatidylcholine can be modified with a diazirine group at the end of the *sn*-2 aliphatic chain. This functional group is converted to a carbene upon UV exposure and reacts rapidly with neighboring molecules to form covalent bonds.[111] Some analogues also contain radioactive elements for quantitative measurements. *Micrococcus luteus* cells can metabolize radiolabeled 9-(2-anthryl)nonanoic acid and transform the lipid analogue into PGL. After the photoreactive lipid is incorporated into the membrane in vivo, the cells are irradiated and lipids are extracted for radioactivity counting.[112] Results from these experiments have provided insight into the lateral distribution and diffusion of lipids in bacterial membranes.

Although cross-linking reagents are useful, they must be coupled with biochemical analyses and are not ideal for probing membrane organization in real time. Lipid-specific fluorophores and lipid synthesis inhibitors are more suitable for monitoring the dynamics of lipid domains. Several fluorescent probes have been used to label lipids in vivo.[113−116] Some of these probes have been used for fluorescence microscopy[116] and others for time-resolved fluorescence spectroscopy.[117,118] Most of these probes consist of a lipid with a fluorophore attached to the headgroup. While many fluorophores are not specific to a particular type of lipid, 10-*N*-nonyl acridine orange (NAO) and Ro09-0198 bind to CL and PE, respectively. Ro09-0198 is a hemolysis-inducing peptide, isolated from *Streptoverticillium griseoverticillatum*.[119] Several studies have characterized the binding of Ro09-0198 to PE.[119,120] Binding of NAO to CL arises from intercalation between fluorophores and lipid molecules. When the fluorophores are $\pi-\pi$ stacked, their emission wavelength undergoes a large Stokes shift, which allows NAO bound to CL to be distinguished from NAO bound to PG.[114,121]

Small molecules that inhibit the biosynthesis of particular lipids have also been used to study membrane organization. In eukaryotic cells, cholesterol has been described as a major component of lipid rafts. Compounds that sequester cholesterol or inhibit its biosynthesis (e.g., filipin, nystatin, amphotericin, methyl-β-cyclodextrin, and statins) have been used extensively for studying lipid raft biology.[122,123] Several antibiotics target lipid biosynthesis in bacteria.[124−126] Only recently have these compounds been explored as tools for investigating lipid domains in bacteria. For example, zaragozic acid was used to study lipid domains in *B. subtilis* cells.[127]

Insights Obtained from Using Small Molecules That Bind to Lipids Specifically. Lipid-specific compounds have been useful for identification and characterization of lipid domains in bacterial membranes. Below, we examine how these small molecules provided insight into lipid organization in bacterial membranes.

Initial studies of lipid organization in bacteria detected heterogeneity in bacterial membranes using chemical−biological approaches. For example, *E. coli* cells treated with laurdan, an amphipathic molecule with a fluorescent group, demonstrated the existence of two subpopulations of laurdan in the bacterial membrane.[118] The organization of lipids into two distinct groups was abolished when cells were pretreated with chloramphenicol, a protein synthesis inhibitor. The results indicate that the membrane consists of domains that are either lipid-rich or protein-rich. In a subsequent study,[117] pyrene-labeled phospholipids (pyrene-PE and pyrene-PGL) were used to study *E. coli* and *B. subtilis* cells. A combination of fluorescence spectroscopy and lipid extraction methods demonstrated that pyrene-labeled PE and PGL have different local environments and suggest that presence of lipid domains in bacterial membranes.

Small molecules also allow observation of the lipid organization in vivo. Two early studies reported the non-homogeneous labeling of membranes with lipophilic dyes in *E. coli* and mycobacterial species.[113,116] Several groups later demonstrated that fluorophores that bind to CL and PE (NAO and Ro09-0198, respectively) localized to the septum and poles in *E. coli* and *B. subtilis*.[115,128,129] Interestingly, Ro09-0198 has been observed to localize to the cleavage furrow of Chinese hamster ovary cells;[130] these observations suggest a connection between lipid organization and cell division in both prokaryotes and eukaryotes. Moreover, CL and PE are concentrated in the forespore membrane in *B. subtilis* cells,[128] and their localization contributed to the hypothesis that some lipids may be recruited to regions of membrane curvature.[131,132]

Investigations of the organization of bacterial membranes have provided clues about the biological function of lipid domains. Lipid organization is hypothesized to play a role in many processes, including cell division,[133] DNA replication,[107] sporulation,[128] osmoregulation,[134] protein translocation,[135] PG synthesis,[136] and biofilm formation.[127] Below, we describe two

examples of small molecule-based studies of the biological roles of lipid domains in bacterial membranes.

In *B. subtilis*, the helical pattern of FM4-64 colocalizes with the helical pattern of MinD translationally fused to green fluorescent protein.[133] To quantify the interaction between lipid spirals and MinD, fluorescence resonance energy transfer (FRET) was measured between GFP-MinD (i.e., FRET donor) and FM4-64 (i.e., FRET acceptor). These experiments indicated that the two classes of biomolecules interact within the 10 nm spatial limit of FRET. The close association between anionic lipids and MinD suggests that these lipids may participate in cell division.

The relationship between CL organization and *E. coli* membrane curvature was quantitatively measured using NAO to visualize CL.[137] MinD translationally fused to a fluorescent protein colocalized with CL microdomains at regions of large, negative curvature in *E. coli* membranes. CL localization at regions of large negative curvature may be a landmark for positioning MinD at the poles of *E. coli* cells. This hypothesis is supported by the demonstration that CL modulates the polar localization of the osmosensory transporter ProP in *E. coli* cells.[134] Cells expressing a chimera of a fluorescent protein fused to ProP were labeled with NAO; the polar localization of the protein was observed to be dependent on CL organization in vivo. It was also observed that the polar localization of the mechanosensitive ion channel protein MscS is also CL-dependent.[138]

Future Studies: Lipid-Specific Small Molecules and Their Application to Bacterial Cell Biology. In summary, chemical−biological tools have been useful for probing the subcellular organization of lipids in vitro and in vivo. The discovery and characterization of new classes of compounds that bind specific lipids or substrate analogues that can be incorporated into lipids in vivo will allow this area of microbiology to move forward. Recently, fluorescent analogues of trehalose have been used in *M. tuberculosis* cells to produce fluorescent glycolipids in vivo, and these labeled glycolipids were observed to concentrate at the bacterial poles.[139]

There are many unanswered questions regarding the structure and organization of bacterial membranes in which chemical probes may be particularly helpful. We highlight several areas below.

Mechanism of Controlling Membrane Organization. Several observations suggest that negative membrane curvature stabilizes and localizes CL domains in bacteria.[132] For example, a recent study of *E. coli* spheroplasts visualized CL using NAO while systematically varying membrane curvature by confining the cells in biocompatible microchambers.[137] The characterization of this mechanism in cells would benefit from lipid-specific derivatives of NAO that are more photostable and compatible with super-resolution microscopy techniques.

Biological Function of Lipid Domains. Lipid domains may function as physical landmarks for protein localization and/or may directly modulate the activity of proteins. Several studies suggest that lipid rafts may perform both of these functions in vivo. MinD localization is perturbed in a *B. subtilis* strain that does not synthesize PGL,[133] and CL stimulates the ATPase activity of EpsE, a component of the type II secretion system in *Vibrio cholerae*.[140] If lipid domains directly modulate enzyme activity or participate in the recruitment of proteins, it will be important to address the molecular mechanism of their recognition and interaction.

Relationship between Cytoskeletal Elements and Lipid Domains. In eukaryotic cells, lipid rafts are often associated with actin filaments. Do the lipid domains in bacterial membranes interact with cytoskeletal proteins? Inhibitors of these proteins may play an important role in deciphering this relationship.

Proteins Associated with Bacterial Lipid Domains. Perturbing the organization of squalene causes delocalization of flotilin and KinC and halts the signaling cascade for biofilm formation in *B. subtilis*.[127] The identification of other signaling cascades that are modulated by lipid domains and the characterization of proteins that are associated with lipid domains in bacteria will play an important role in understanding the physiological role of this mechanism in bacteria.

SUMMARY

Chemical−biological approaches provide a unique tool for the study of subcellular organization in bacteria. The biochemical function and localization of FtsZ, MreB, and other proteins regulate several essential processes in bacteria. The structure, dynamics, and function of these proteins in vivo are still largely unknown. Genetic approaches for the study of these proteins have provided important evidence of their function. As many of these proteins function as molecular hubs for controlling the localization and function of other proteins, knockouts may affect associated proteins and produce phenotypes that can be misinterpreted. The success of chemical−biological approaches in studying the function of the eukaryotic cytoskeleton provides a precedent for the identification and characterization of small molecules and their application to studying homologues in bacteria. Furthermore, small molecules that modulate the intracellular organization of bacterial cells can alter essential physiological processes and provide new opportunities for the development of therapeutic agents.

Characterizing the mechanism by which localization occurs is a frontier area of microbiology that may have important implications in prokaryotic and eukaryotic cell biology. Chemical−biological studies using small molecules as probes of cytoskeletal proteins, cell wall structure and assembly, and membrane organization may provide a revolutionary level of detail regarding the structure and organization of biomolecules within bacterial cells.

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■ ABBREVIATIONS

PG peptidoglycan; PBP penicillin binding protein; PE phosphatidylethanolamine; PGL phosphatidylglycerol; CL cardiolipin; NAO 10-*N*-nonyl acridine orange; FRET fluorescence resonance energy transfer.

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