Cardiolipin Alters *Rhodobacter sphaeroides* Cell Shape by Affecting Peptidoglycan Precursor Biosynthesis

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**ABSTRACT** Cardiolipin (CL) is an anionic phospholipid that plays an important role in regulating protein biochemistry in bacteria and mitochondria. Deleting the CL synthase gene (∆cls) in *Rhodobacter sphaeroides* depletes CL and decreases cell length by 20%. Using a chemical biology approach, we found that a CL deficiency does not impair the function of the cell wall elongasome in *R. sphaeroides*; instead, biosynthesis of the peptidoglycan (PG) precursor lipid II is decreased. Treating *R. sphaeroides* cells with fosfomycin and D-cycloserine inhibits lipid II biosynthesis and creates phenotypes in cell shape, PG composition, and spatial PG assembly that are strikingly similar to those seen with *R. sphaeroides* ∆cls cells, suggesting that CL deficiency alters the elongation of *R. sphaeroides* cells by reducing lipid II biosynthesis. We found that MurG—a glycosyltransferase that performs the last step of lipid II biosynthesis—interacts with anionic phospholipids in native (i.e., *R. sphaeroides*) and artificial membranes. Lipid II production decreases 25% in *R. sphaeroides* ∆cls cells compared to wild-type cells, and overexpression of MurG in *R. sphaeroides* ∆cls cells restores their rod shape, indicating that CL deficiency decreases MurG activity and alters cell shape. The *R. sphaeroides* ∆cls mutant is more sensitive than the wild-type strain to antibiotics targeting PG synthesis, including fosfomycin, D-cycloserine, S-(3,4-dichlorobenzyl)isothiourea (A22), mecillinam, and ampicillin, suggesting that CL biosynthesis may be a potential target for combination chemotherapies that block the bacterial cell wall.

**IMPORTANCE** The phospholipid composition of the cell membrane influences the spatial and temporal biochemistry of cells. We studied molecular mechanisms connecting membrane composition to cell morphology in the model bacterium *Rhodobacter sphaeroides*. The peptidoglycan (PG) layer of the cell wall is a dominant component of cell mechanical properties; consequently, it has been an important antibiotic target. We found that the anionic phospholipid cardiolipin (CL) plays a role in determination of the shape of *R. sphaeroides* cells by affecting lipid II biosynthesis. Removing CL in *R. sphaeroides* alters cell morphology and increases its sensitivity to antibiotics targeting proteins synthesizing PG. These studies provide a connection to spatial biochemical control in mitochondria, which contain an inner membrane with topological features in common with *R. sphaeroides*.

**KEYWORDS** MreB, MurG, cardiolipin, cell shape, peptidoglycan
attention to the underlying machinery controlling PG assembly and the catastrophic effect of inhibiting its biosynthesis (5, 6). PG is synthesized in three distinct steps in Gram-negative bacteria (Fig. 1). (i) Monosaccharide-peptide precursors are synthesized in the cytoplasm by seven enzymes: MurABCDEF and DdlA. (ii) A lipid attached by MraY tethers this precursor to the inner leaflet of the cytoplasmic membrane (referred to as lipid I), a second sugar is attached by MurG to create a disaccharide-peptide precursor (referred to as lipid II), and the molecule is flipped to the outer leaflet of the bilayer by MurJ. (iii) Lipid II is polymerized in the periplasmic space by a glycosyltransferase (GTase) and the nascent glycan strands are incorporated into the existing PG by a transpeptidase (TPase) that cross-links peptides on adjacent lipid II molecules.

In rod-shaped bacteria, PG synthesis occurs at different sites in the cell at different stages of the cell cycle. During cell division, FtsZ organizes the divisome, which synthesizes PG at the division site. During cell elongation, the elongasome is associated with MreB, which directs PG synthesis at the sidewalls. See text for details. GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; Ala, alanine; Glu, glutamic acid; Dap, meso-diaminopimelic acid.

**FIG 1** PG synthesis in Gram-negative bacteria. PG synthesis in Gram-negative bacteria is conserved and proceeds in three distinct steps. The process starts with the biosynthesis of monosaccharide-peptide precursors in the cytoplasm followed by the formation of lipid I and lipid II at the cell membrane. Lipid II is then oriented from the cytoplasm to the periplasm, where PG assembly occurs. In rod-shaped bacteria, cytoskeletal proteins guide the location of PG synthesis at different stages of the cell cycle. During cell division, FtsZ organizes the divisome, which synthesizes PG at the division site. For clarity, we include only a subset of cell division proteins in the diagram. During cell elongation, the elongasome is associated with MreB, which directs PG synthesis at the sidewalls.
Cardiolipin Deficiency Alters Bacterial Cell Shape

Previous studies demonstrating a connection between membrane composition and bacterial biochemistry (7) motivated us to explore the role of the phospholipid bilayer in synthesis of PG and determination of bacterial cell shape. We previously observed that a mutant (strain Δcls) of the Gram-negative, rod-shaped bacterium *Rhodobacter sphaeroides* lacking the anionic, intrinsically curved phospholipid cardiolipin (CL) creates cells that are shorter in length than those created by the wild-type (wt) strain (8). A similar change in cell morphology has not been observed in other rod-shaped bacterial cells lacking CL, e.g., *Escherichia coli* and *Bacillus subtilis* (9, 10), suggesting that this phenotype may be unique to alphaproteobacteria. In this paper, we describe *R. sphaeroides* using a strategy similar to that used by other bacteria—i.e., MreB-directed cell elongation—to determine the rod shape of cells. Despite a reduction in growth along its cylindrical cell body, the CL-deficient mutant of *R. sphaeroides* has an elongosome that functions normally. We found that the influence of the depletion of CL on *R. sphaeroides* cell shape arises from effects on the biosynthesis of the PG precursor, lipid II. We describe experiments supporting the hypothesis that a CL deficiency in *R. sphaeroides* reduces the supply of lipid II to the elongasome for PG assembly at the sidewalls of the cell, which alters cell shape and makes cells susceptible to PG-targeting antibiotics.

RESULTS

**CL plays a role in determining *R. sphaeroides* cell shape.** The *R. sphaeroides* cell membrane contains four major classes of phospholipids, namely, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PGL), and CL, in a ratio of approximately 5:2:2:4.0:6 (PE/PC/PGL/CL) by weight (11). A CL-deficient mutant of *R. sphaeroides* lacking the CL synthase gene (strain Δcls) produces a reduced amount of CL (0.5% of total lipids, representing an ~90% decrease in CL) and displays a cell shape phenotype with a characteristic decrease (20%) in cell length compared to the wt strain (Fig. 2A and B) (8, 11). To investigate whether the change in the cell shape of the *R. sphaeroides* Δcls mutant is due to an increased cell division rate (relative to cell growth), we performed single-cell time-lapse imaging of both *R. sphaeroides* wild-type (wt) and Δcls cells (Fig. S1 in the supplemental material). We imaged cells, tracked their growth directly between cell division events, and found that the wt and Δcls strains had comparable doubling times of ~150 min. The Δcls mutant, however, displayed a 40% decrease in the rate of cell elongation compared to the wt strain, suggesting that the morphological change in the *R. sphaeroides* Δcls mutant may arise from a defect in cell elongation. Complementation of the Δcls mutant by ectopically expressing CL synthase in cells from the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression vector pIND (12) increased the concentration of CL to 2.3% of total lipids (~50% of the level in wt cells) and restored the cell elongation rate by 20% (Fig. S1), suggesting that CL contributes to sculpting the rod shape of *R. sphaeroides* cells.

**CL deficiency does not impair *R. sphaeroides* elongasome function.** MreB is a bacterial cytoskeletal protein that is homologous to eukaryotic actin (13). In rod-shaped bacteria, MreB polymerizes into filamentous structures positioned on the inner leaflet of the cytoplasmic membrane that is associated with elongasome components, including the cell elongation-specific TPase PBP2. MreB filaments move circumferentially around the cell with a velocity that is correlated to the rate of PG assembly and in an orientation that may guide elongation of the PG layer of the cell wall (14–16). In *R. sphaeroides*, MreB, MreC, MreD, PBP2, and RodA are hypothetically encoded at the same locus (Fig. S2A) (17). MreB requires MreC and MreD for its cellular localization, and PBP2 requires RodA for its TPase activity (3). RodA has recently been reported to also possess GTase activity (18, 19). We used a chemical biology approach to investigate whether *R. sphaeroides* uses a elongasome strategy for shape-determination similar to that summarized above. We treated *R. sphaeroides* cells with the small-molecule inhibitors S-(3,4-dichlorobenzyl)isothiourea (A22; binds MreB and disassembles MreB filaments) and mecillinam (MEC; binds PBP2 and inhibits its TPase activity required for cell elongation). The MIC of A22 was 60 μg/ml for *R. sphaeroides*, and the MIC of MEC was
FIG 2  CL participates in cell shape determination by affecting lipid II biosynthesis. (A to F) Probability density histogram of the cell length distribution of *R. sphaeroides* wt cells (A), Δcls cells (B), wt cells treated with A22 (C), wt cells treated with mecillinam (MEC) (D), wt cells (E) treated with FOS (E), and wt cells treated with DCS (F).

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3 μg/ml. Treatment using sub-MICs of both drugs (A22, 10 μg/ml; MEC, 0.5 μg/ml) did not significantly impair *R. sphaeroides* cell growth and yet decreased cell length (A22, 40%; MEC, 30%), which matches the phenotype of these compounds in other rod-shaped bacteria (3) and suggests a conserved cell elongation machinery in *R. sphaeroides* (Fig. 2C and D). We observed a similar phenotype (i.e., shorter cell length) in comparisons between *R. sphaeroides* cells with reduced CL concentrations and cells treated with A22 and MEC; this result led us to hypothesize that an altered elongosome function in the *R. sphaeroides Δcls* mutant may reduce PG assembly at the sidewalls of the cell.

We used real-time quantitative PCR (qPCR) to test if transcription of genes coding for elongosome components is altered in *R. sphaeroides Δcls* cells. We found no significant difference in the levels of transcription of elongosome genes in wt and Δcls cells (Fig. S2B). We also treated *R. sphaeroides* cells with the PBP1a inhibitor cefsulodin to inhibit its TPase activity. The MIC of cefsulodin for *R. sphaeroides* cells is 20 μg/ml; a sub-MIC (5 μg/ml) of the drug did not cause significant growth defects or changes in cell shape (Fig. S3), suggesting that PBP1a does not participate in cell elongation (19).

MreB filaments are involved in the process of PG assembly at the sidewalls of cells during cell elongation. We examined whether the structure and function of MreB are disrupted in *R. sphaeroides Δcls* cells by comparing the PG growth patterns of *R. sphaeroides* wt and Δcls strains. We probed PG assembly in cells at different stages of the cell cycle with HADA, a fluorescent analog of d-alanine containing a coumarin moiety that can be covalently incorporated into newly synthesized PG (20, 21). PG assembly in *R. sphaeroides* cells occurred along the sidewalls and accompanied cell elongation (Fig. 2G). At early and late stages of cell division (i.e., cell septation), PG assembly is focused at the division plane. After the mother and daughter cells are separated, PG assembly returns to the sidewalls as cells elongate. This cycle of PG assembly was not observed in *R. sphaeroides* wt cells treated with A22 in which the structure of MreB was disrupted (Fig. 2H); A22-treated *R. sphaeroides* cells exhibited three aberrant phenotypes: round shapes, irregular shapes, and asymmetric cell division. Round *R. sphaeroides* cells displayed peripheral PG assembly and divided at the mid-cell. PG was assembled at apparently random positions in cells with irregular shapes. Cells undergoing asymmetrical division displayed asymmetrical labeling of septal PG. *R. sphaeroides* wt cells treated with MEC had phenotypes similar to those of A22-treated cells, suggesting that inhibiting PBP2 activity may disrupt the structure of MreB or retard the movement of MreB filaments (Fig. 2H). The pattern of PG assembly in *R. sphaeroides Δcls* cells was similar to that in wt cells (Fig. 2G), suggesting that a change in shape is unlikely to arise from disrupting the MreB structure or inhibiting PBP2 activity.

**Inhibition of lipid II biosynthesis results in a phenotype that resembles that of *R. sphaeroides Δcls* cells.** In rod-shaped bacteria, lipid II biosynthesis colocalizes with PG assembly at different stages of the cell cycle (22). The change in cell shape that we observed in the *R. sphaeroides Δcls* mutant may have arisen from a reduction in lipid II biosynthesis. To test this hypothesis, we reduced lipid II biosynthesis by treating *R. sphaeroides* cells with the MurA inhibitor fosfomycin (FOS) and the DdlA inhibitor d-cycloserine (DCS). MurA converts UDP-N-acetylglucosamine (UDP-GlcNAc) into UDP-
N-acetylmuramic acid (UDP-MurNAc) in the first concerted step in lipid II biosynthesis. DdlA is a D-alanine (D-Ala) ligase that produces D-Ala-D-Ala for incorporation into lipid II (Fig. 1). The MICs of FOS and DCS for *R. sphaeroides* were 1.5 mg/ml and 1 μg/ml, respectively. *R. sphaeroides* cells treated with sub-MICs of either drug (FOS, 250 μg/ml; DCS, 0.05 μg/ml) had a decrease in cell length (FOS, 15%; DCS, 25%) and lacked significant growth defects (Fig. 2E and F). HADA labeling experiments indicated that these two drugs did not affect the pattern of PG assembly in *R. sphaeroides* cells (Fig. 2G), suggesting that a change in the shape of *R. sphaeroides* Δcls cells may arise from a decrease in lipid II biosynthesis arising from a CL deficiency.

To test this hypothesis, we determined the PG composition of *R. sphaeroides* cells using ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) (Table 1; see also Fig. S4). We treated *R. sphaeroides* wt cells with A22 and MEC, isolated PG, digested it with mutanolysin (an N-acetylmuramidase), analyzed the material by UPLC-MS (23), and found that both compounds changed the PG composition of cells (Fig. 3A). Compared to wt cells, treatment with either A22 or MEC decreased the concentration of disaccharide-peptide (muropeptide) monomer by 33% and increased the concentration of crossed-linked muropeptides (dimer and trimer) by 20%, resulting in a 32% increase in the degree of cross-linking (Fig. 3B). Treatment with both A22 and MEC led to a 60% increase in the concentration of anhydromuramyl muropeptides present at the terminating end of the glycan strand, reflecting a 43% decrease in the average glycan strand length (Fig. 3C) (24). Similar results were found in *E. coli* cells treated with A22 (25). Surprisingly, *R. sphaeroides* Δcls cells have a PG composition indistinguishable from that seen in wt cells or in wt cells treated with FOS or DCS (Fig. 3), suggesting that CL deficiency does not impair the elongasome function and that a change in cell shape of the *R. sphaeroides* Δcls mutant may arise from a reduction in lipid II biosynthesis.

**CL affects lipid II biosynthesis.** We found no significant differences in the levels of transcription of genes coding for enzymes that synthesize lipid II in *R. sphaeroides* wt and Δcls cells (Fig. S5). We then investigated the impact of CL on the activity of specific enzymes involved in lipid II biosynthesis. MurG is a GTase and a hypothetical peripheral membrane protein that catalyzes the rate-limiting step of lipid II formation by transferring the GlcNAc from UDP-GlcNAc to lipid I (Fig. 1) (26, 27). We fused *gfp* to the genomic *murG* in both *R. sphaeroides* wt and Δcls strains (resulting in TyL1 and TyL2 strains, respectively; Fig. S6) and imaged the cells. During elongation, MurG localized to the membrane and concentrated at the cell poles. During division, MurG localized at the division plane (Fig. 4A). This subcellular MurG localization pattern was unchanged in *R. sphaeroides* Δcls cells. Cell fractionation experiments indicated no significant difference between the amounts of membrane-localized MurG (~75%) in *R. sphaeroides* wt cells and Δcls cells (Fig. 4B). Apparently, membrane localization of MurG does not

### Table 1: Muropeptides analyzed by UPLC-MS in positive-ion mode

| Peak | Retention time (min) | Calculated mass | Observed m/z | Length of the stem peptides |
|------|----------------------|-----------------|--------------|----------------------------|
| 1    | 7.6                  | 870.37          | 871.37 (+1)  | Tri                        |
| 2    | 9                    | 698.29          | 699.29 (+1)  | Di                         |
| 3    | 12                   | 941.41          | 942.41 (+1)  | Tetra                      |
| 4a   | 13.7                 | 1,722.71        | 575.23 (+3)  | Tri-tri                    |
| 4b   | 20.3                 | 1,722.71        | 862.35 (+2)  | Tri-tri                    |
| 5a   | 21.7                 | 1,793.77        | 897.88 (+2)  | Tri-tetra                  |
| 5b   | 22.8                 | 1,793.77        | 897.88 (+2)  | Tri-tetra                  |
| 6    | 24.1                 | 1,864.81        | 933.40 (+2)  | Tetra-tetra                |
| 7a   | 28                   | 2,717.15        | 906.71 (+3)  | Tetra-tetra-tetra          |
| 7b   | 29.2                 | 2,717.15        | 906.71 (+3)  | Tri-tetra-tetra            |
| 8    | 30.1                 | 2,788.21        | 930.40 (+3)  | Tetra-tetra-tetra          |
| 9a   | 30.7                 | 1,773.74        | 887.87 (+2)  | Anhydro tri-tetra          |
| 9b   | 31.9                 | 1,773.74        | 887.87 (+2)  | Anhydro tri-tetra          |
| 10   | 33.2                 | 1,844.77        | 923.38 (+2)  | Anhydro tetra-tetra-tetra  |

*Refer to chromatograms in Fig. S4.*
require CL and changes in R. sphaeroides Δcls cell shape are not due to MurG mislocalization.

Anionic phospholipids, such as CL and PGL, have been shown to accumulate at the polar regions of rod-shaped bacterial cells, where they may reduce the surface energy of curvature-mediated membrane strain (9, 28). We labeled R. sphaeroides membranes with the fluorescent probe 10-N-nonyl acridine orange (NAO), which has a preference for interacting with anionic phospholipids, and observed an enrichment of the fluorophore at the polar regions of both wt and Δcls cells (Fig. 4C). As PGL is the precursor of CL, R. sphaeroides Δcls cells contained a higher level of PGL than wt cells (11). Enrichment of MurG at the R. sphaeroides wt and Δcls cell poles suggests that its localization may occur through interacting with CL and PGL. We purified recombinant R. sphaeroides MurG and quantified the interaction between MurG and membranes with different phospholipid compositions using in vitro liposome-pelleting assays. In these assays, 67% of the total MurG present interacted with liposomes containing 100 mol% PC and segregated to the pellet fraction. When 10 mol% CL or PGL was incorporated into PC-containing liposomes, the fraction of MurG interacting with membranes increased to 92% for both anionic phospholipids (Fig. 4D), suggesting equal preferences for MurG binding of CL and PGL. Other CL-binding peripheral membrane proteins show a similar relationship between these two lipids (29).

A hydrophobic patch in E. coli MurG was proposed to bind the cell membrane (30). Sequence alignment revealed that this membrane-binding interface is conserved in R. sphaeroides MurG (Fig. S7). The hydrophobic patch of amino acids in E. coli MurG is surrounded by several basic amino acid residues that may contribute to its preferential interaction with CL (30, 31). With the exception of R77 (corresponding to R79 in E. coli MurG), these residues are not conserved in R. sphaeroides MurG (Fig. S7). We purified R. sphaeroides MurG<sup>R77E</sup> protein and found that the R77E mutation decreased the amount of MurG interacting with liposomes containing 100 mol% PC by 17% and abolished its preference for anionic phospholipids (Fig. 4D).
R. sphaeroides MurG interacts with CL, and the interaction is important for its enzymatic activity and cell elongation. (A) Representative micrographs of TyL1 and TyL2 cells, which are R. sphaeroides wt and Δcls cells in which the genomic murG was replaced with gfp-murG, respectively. Cells were grown to log phase (absorbance of 0.6, λ = 600 nm) and imaged using phase-contrast bright-field and fluorescence microscopy. Arrows indicate the pole-localized MurG. (B) Biochemical fractionation of TyL1 and TyL2 cells. We fractionated TyL1 and TyL2 cell lysates that had the same amount of proteins into cytosolic and membrane fractions and performed Western blot (WB) analysis using a monoclonal antibody against GFP. The percentage of membrane-localized MurG was determined by quantifying the optical densitometry signal using ImageJ. Data represent mean values ± standard deviations obtained from three independent experiments. (C) Representative micrographs of R. sphaeroides wt and Δcls cells stained with NAO. Cells were grown to early log phase (absorbance of 0.3, λ = 600 nm), stained with NAO, and imaged using fluorescence microscopy. Arrows indicate the polar enrichment of NAO. (D) Liposome-pelleting assays of MurG and MurGR77E with liposomes containing the indicated phospholipids. The percentage of pelleted MurG was determined by quantifying the optical densitometry signal using ImageJ. Data represent mean values ± standard deviations obtained from three independent experiments. S, supernatant; P, pellet. (E) A diagram depicting the incorporation of HADA into the lipid II molecule by MurG and its assembly into the existing PG by PBP proteins. We hypothesize that CL interacts with MurG and is important for its activity. As CL does not affect PG assembly, we assessed the effect of CL on lipid II production (Continued on next page)
CL and PGL are both important for the function of *E. coli* MurG; however, CL is more effective at stimulating the GTase activity of MurG (31). Although the MurG-membrane interaction is unlikely to be affected in *R. sphaeroides Δcls* cells (due to the presence of PGL), the depletion of CL in the cell membrane should in principle decrease MurG activity and reduce the biosynthesis of lipid II available for cell elongation. To test this hypothesis, we treated *R. sphaeroides* cells with the MurG inhibitor murgocil (32). Regrettably, murgocil is specific to staphylococcal species and was inactive against *R. sphaeroides* due to amino acid differences between the two strains in the MurG-murgocil binding site (Fig. S7). Alternatively, we indirectly compared the amounts of lipid II produced by *R. sphaeroides* wt and Δcls cells by quantifying the incorporation of HADA into the cell wall (Fig. 4E). Removing CL decreased the production of lipid II in *R. sphaeroides* by 25% (Fig. 4F). Treating *R. sphaeroides* cells with FOS and DCS reduced HADA incorporation by 21% and 24%, respectively (Fig. S8A). Complementation of CL synthase in *R. sphaeroides Δcls* cells (which restores ~50% of the concentration in wt cells and the rate of cell elongation by 20%; Fig. S1) restored lipid II production by 14% as measured in this assay (Fig. 4F), suggesting that CL is important for the enzymatic activity of MurG and cell elongation. Although murgocil is ineffective against Gram-negative bacteria, we performed a control experiment in which we treated *Staphylococcus aureus* cells with murgocil and found an 81% decrease in HADA incorporation (Fig. S8B), thereby correlating MurG activity to PG assembly.

**Overexpression of MurG in *R. sphaeroides Δcls* cells restores the rod shape of cells.** To further explore the correlation between changes in MurG activity and cell length, we increased MurG activity in *R. sphaeroides* cells by overexpressing MurG (an N-terminal green fluorescent protein [GFP] fusion protein) from pIND. Leaky expression of GFP-MurG from the plasmid did not affect the subcellular localization of MurG and cell growth (as did 1 and 10 μM IPTG); consequently, we used leaky expression to increase the intracellular concentration of MurG. This approach increased *R. sphaeroides* cell length by 21% compared to control cells expressing cytoplasmic GFP. Expressing GFP-MurGL77E (which should have reduced binding to the membrane) in *R. sphaeroides* cells abolished polar localization of GFP and increased the cell length by 16% compared to cells expressing GFP (Fig. 5A to C). These results correlate MurG activity with the length of *R. sphaeroides* cells.

We created *R. sphaeroides* MurG mutant L76E (corresponding to residue L78 in the proposed hydrophobic patch of *E. coli* MurG) and expressed GFP-MurG^{L76E} in *R. sphaeroides* cells by the leaky-expression approach. GFP-MurG^{L76E} was localized to the division plane, failed to bind the *R. sphaeroides* cytoplasmic membrane, and increased the length of *R. sphaeroides* cells by only 5% compared to cells expressing GFP, demonstrating the importance of MurG-membrane interaction for cell elongation (Fig. 5D).

To compensate for the decreased MurG activity in *R. sphaeroides Δcls* cells, we also increased MurG expression in cells using a leaky promoter. MurG expression increased the length of *R. sphaeroides Δcls* cells by 13% compared to Δcls cells expressing GFP (Fig. 5E and F), suggesting that the morphological abnormality of the *R. sphaeroides Δcls* mutant is a result of a decrease in MurG activity that reduces the supply of lipid II for cell elongation.

**CL deficiency increases the sensitivity of *R. sphaeroides* to antibiotics targeting PG synthesis.** As CL alters the activity of MurG, which catalyzes the rate-limiting step in the biosynthesis of lipid II, we hypothesized that the *R. sphaeroides Δcls* mutant may have increased susceptibility to antibiotics that inhibit lipid II biosynthesis and its assembly. To test this hypothesis, we performed spot-titer assays and found that *R.
sphaeroides Δcls cells—compared to wt cells—had increased sensitivity to drugs interfering with lipid II biosynthesis, including FOS and DCS, and drugs interrupting PG assembly, including A22, MEC, and ampicillin (AMP; binds penicillin-binding proteins [PBPs] and inhibits their TPase activities) (Fig. 6). These results further
support our hypothesis that a CL deficiency impedes the production of lipid II in \textit{R. sphaeroides}.

**DISCUSSION**

This study demonstrated that CL contributes to bacterial cell shape by altering cell wall synthesis. Our data led us to hypothesize that CL may regulate the activity of enzymes synthesizing lipid II during cell elongation. Of the nine lipid II synthases shown in Fig. 1, MurG catalyzes the rate-limiting step of lipid II biosynthesis and preferentially interacts with CL (versus PE and PGL) in \textit{E. coli} (31). We demonstrated that the change in \textit{R. sphaeroides} Δ\textit{cls} cell shape arises from a decrease in MurG activity. A CL deficiency may also decrease the activities of other lipid II synthases in \textit{R. sphaeroides}, such as the integral membrane protein MraY and the lipid II flippase MurJ. In addition to decreased cell length, \textit{R. sphaeroides} cells that were Δ\textit{cls} or treated with inhibitors FOS or DCS also had an increased cell width (Fig. 2B, E, and F), which suggests that inhibiting lipid II production may alter the orientation of MreB filaments and their relationship to cell diameter (33).

CL has four acyl tails and a relatively small head group that creates a large intrinsic curvature of $\sim 1.3 \text{ nm}^{-1}$ (34). In rod-shaped bacteria, CL has a preference for localizing in the inner leaflet of the cell membrane and concentrates at regions of high membrane curvature (e.g., cell poles), where it reduces the local elastic strain on this liquid crystalline material (28); this phenomenon is particularly relevant to liquid crystals in the field of materials science (35). PGL has a significantly smaller curvature ($\sim 0.11 \text{ nm}^{-1}$) than CL and also concentrates at the polar regions of cells through
unknown mechanisms, which may entail electrostatic interactions with proteins (9). The heterogeneous distribution of anionic phospholipids in membranes has been hypothesized to function as a mechanism for localizing membrane-associated proteins into different regions within bacterial cells (7). We found that MurG interacts with anionic phospholipids—equally preferring CL and PGL—and is enriched at the poles of *R. sphaeroides* cells, where PG is relatively inert. Polar enrichment of MurG has been observed in *E. coli* cells and has been hypothesized to provide a mechanism for the storage of excess protein (36).

Although we were unable to test it directly, our data support a model in which binding to CL influences the structure of MurG and stimulates its GTase activity. Within membranes that are energy transducing and have parallels to those found in *R. sphaeroides*, CL binds tightly to respiratory complexes in the electron transport chain, stabilizes protein complexes, and promotes ATP synthesis (37, 38). In *E. coli*, CL binds tightly to the SecYEG protein complex, stabilizes its dimeric form, and regulates protein translocation (39, 40). In *Vibrio cholerae*, CL stabilizes the oligomerization state of EpsE and stimulates its Eps system, which exports cholera toxin across the outer membrane (41). Purified, recombinant MurG and other GTases have been reported to be dimers or oligomers (42) that may be stabilized and activated by CL.

Although MurG also binds PGL and its subcellular localization in *R. sphaeroides Δcls* cells is unchanged, PGL is unable to restore the observed reduction in MurG activity. A recent study demonstrated that *E. coli* RecA displays different tryptophan fluorescence emission spectra upon binding to CL- and PGL-containing liposomes and suggested that RecA adopts different conformations when interacting with different families of anionic phospholipids (43). To explore whether the structure of MurG may be altered upon membrane binding, we measured the tryptophan fluorescence of *R. sphaeroides* MurG (containing two tryptophan residues; see Fig. S7 in the supplemental material) and found difference in emission spectra in the presence of liposomes containing different anionic phospholipids (e.g., CL and PGL; Fig. S9). The structural mechanisms differentiating the effects of the two anionic phospholipids on MurG activity require further studies.

*E. coli* mutants lacking CL do not have an altered morphology because the MurG activity is increased in cells due to the upregulation of MurG expression (31). In contrast, the expression of MurG in *R. sphaeroides Δcls* cells remained unchanged compared to *R. sphaeroides* wt cells (Fig. S10). Overexpression of MurG in *R. sphaeroides* wt cells increases the length of cells, correlating MurG activity to the growth of cells at the sidewalls. We overexpressed MurG in *R. sphaeroides Δcls* cells to restore its activity and found that the cells partially recovered their rod-shaped morphology.

*R. sphaeroides* MurG mutant R77E interacts with the membrane, loses its preference for anionic phospholipids, and does not localize to cell poles. Expression of the MurG<sup>R77E</sup> mutation increased the length of cells, suggesting that the mutation did not abolish MurG enzyme activity. However, the cells expressing MurG<sup>R77E</sup> were shorter than the cells overexpressing wild-type MurG, which suggests that anionic phospholipids optimize the interaction of MurG with membranes and its activity. The molecular mechanisms underlying the preferential interaction between MurG and anionic phospholipids remain to be determined. Interestingly, the L76E MurG membrane-binding mutant was still able to localize at the division plane, suggesting that MurG interacts with the divisome using a motif that is different from the hydrophobic patch and does not interact with membranes during cell division. This observation may explain why a CL deficiency did not affect MurG activity at the septum and cause a defect in cell division in *R. sphaeroides*.

A growing body of evidence indicates that CL plays a fundamental role in a variety of bacterial processes, including ATP synthesis, DNA replication and repair, cell division, protein translocation, osmoadaptation, biofilm formation, and pathogenicity (7, 8, 29, 44–48). Our research provides the first example of the role of CL in determining bacterial cell shape. The PG layer of the cell wall is the canonical target for antibiotics; many of the antibiotics in this family have lost activities due to resistance. Although the
TABLE 2 Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Source or reference |
|-------------------|-------------------------|---------------------|
| **E. coli strains** |                         |                     |
| DH5α              | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR Δ(lacZYA-argF)U169 λ (φ80d lacZΔM15) | Laboratory collection (CGSC#12384) |
| S17-1             | recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 | Laboratory collection |
| BL21(DE3)         | Host strain for recombinant protein expression |                     |
| **R. sphaeroides strains** |                         |                     |
| Δcls              | Wild type | ATCC 17023 |
| Δcls::pIND5       | 2.4.1 containing a kanamycin resistance cassette in place of the genomic cls | This study |
| TyL1              | 2.4.1 murG::gfp-murG | This study |
| TyL2              | Δcls murG::gfp-murG | This study |

Plasmids

| plIND5p           | A derivative of plND4 in which the Ncol site is replaced with an NdeI site; plND4 is an IPTG-inducible expression vector for R. sphaeroides; kanamycin resistance | 8 |
| plIND5TetR        | A variant of plND5 with a tetracycline resistance cassette in place of the kanamycin resistance cassette | This study |
| gfp-plND5         | plND5 containing gfp | This study |
| gfp-murG-plND5    | plND5 containing gfp-murG | This study |
| gfp-plND5TetR     | plND5TetR containing gfp | This study |
| gfp-murG::plND5TetR | plND5TetR containing gfp-murG | This study |
| gfp-murG=pIND5    | plND5 containing gfp-murG | This study |
| gfp-murG=pIND5TetR| plND5TetR containing gfp-murG | This study |
| pk18mobasB        | Suicide plasmid for allele exchange; kanamycin resistance | Laboratory collection |
| pk18mobasBtetR    | A variant of pk18mobasB with a tetracycline resistance cassette in place of the kanamycin resistance cassette | This study |
| 1k-gfp-murG-pK18  | pk18mobasBtetR containing murG and its 5’ upstream DNA sequence, with a gfp inserted between the two | This study |
| mobsasBtetR       | A vector for expression of proteins with an N-terminal His tag/thrombin/T7 tag in the E. coli pET system | Novagen |
| murG::pET28b(+)   | A vector for expression containing murG | This study |
| clsp::pIND5sp     | pIND5sp containing cls; pIND5sp is a variant of plND5 with a spectinomycin resistance cassette in place of the kanamycin resistance cassette | 8 |

R. sphaeroides Δcls mutant has no significant defect in growth, it is more sensitive to antibiotics that target PG than the R. sphaeroides wt strain. It has been shown that a Pseudomonas putida Δcls mutant is susceptible to several antibiotics, as its drug efflux system is impaired (49). These data suggest that in addition to the interesting biochemistry regulated by CL, its biosynthesis may be a potential target to explore for antibiotics or their adjuvants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. R. sphaeroides strains were grown aerobically in Sistrom’s succinate medium at 30°C with shaking at 200 rpm. When required, kanamycin (25 μg/ml), spectinomycin (5 μg/ml), or tetracycline (0.025 μg/ml) was added to the medium. E. coli strains were grown in LB broth at 37°C with shaking at 200 rpm. When required, kanamycin (25 μg/ml) or tetracycline (10 μg/ml) was added to the medium. The bacterial strains and plasmids used in this study are described in Table 2.

Analysis of cell morphology. An aliquot of cell cultures in log phase (absorbance of 0.6, λ = 600 nm) was transferred to the surface of a 2% (wt/vol) agarose pad prepared in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4), covered with a glass coverslip, and imaged with an inverted Nikon Eclipse Ti microscope equipped with a Photometrics CoolSNAP HQ2 charge-coupled-device (CCD) camera and a 120-W mercury arc lamp (X-cite Series 120; EXFO). Images were acquired with a 100× objective (Nikon Plan Apo 100×/1.40 oil Ph3 DM) and the Nikon Instruments Software (NIS-Elements Advanced Research (AR) microscope imaging software program (version 4.000.07)). Cell width and length were determined using ImageJ. To track the cell growth at single-cell level, cells were transferred to the surface of a 2% (wt/vol) agarose pad prepared in Sistrom’s succinate medium and imaged every 5 min for 6 h at 30°C.

Plasmid constructions. The primers used in this study are listed in Table 3. Cloning of PCR fragments into vectors were performed by In-Fusion cloning (Clontech) in accordance with the user manual unless otherwise noted. The plND5 and pk18mobasB constructs were transformed into E. coli S17-1 and subsequently mobilized via conjugation into the recipient R. sphaeroides wt or Δcls strain as described previously (8).

(i) gfp-plND5. gfp was amplified by PCR using primers NdeI-gfp_p5 and BglII-gfp_p5 and cloned into plND5 at the Ndel and BglII sites.
TABLE 3 Primers used in this study

| Primer name | Primer sequence |
|-------------|-----------------|
| NdeI-gfp_p5 | GGAGAAATTTACATATGGTGACAGGCGGAGAG |
| BglII-gfp_p5 | GATGGTATGAGATCTCTTGTACAGCTCGTCCAT |
| NdeI-murG_p5 | GGAGAAATTTAACATATGGGCGCGGCTCTCTCTTG |
| BglII-murG_p5 | GATGGTATGAGATCTGTCCTTCTTTGCCCAG |
| NdeI-R77E_S | CGCGCCCGCGAGTCTCGAGGGGGACCAGC |
| murG_L76E_S | GGCCGGATGCCGCTGGGAGCAGCCC |
| murG_L76E_AS | GGCCGGATGCCGCTGGGAGCAGCCC |
| BamHI-murG1k | CGTACCCGGGGATCTCGACAAATGTCG |
| HindIII-murG1k | GGCCCATGCAACGGCTTTCATGTCCTTCTTCTTTCG |
| murG1k+Ndel_S | GGCCGGTGACCATATGGGCGGCC |
| murG1k+Ndel_AS | GGCCGGTGACCATATGGGCGGCC |
| murG1k+BglII-gfp | GGCCGGTGACCATATGGGCGGCC |
| Ndel-murG_p28 | CGCCGGCGAGCATGGAGCCGAGGC |
| Xhol-murG_p28 | GGTTGAGGACGGTCAAGTCG |
| WSG | AGATGTCATATGTCGC |

(ii) gfp-murG-plNDS. murG was amplified by PCR using primers Ndel-murG_p5 and BglII-murG_p5 from R. sphaeroides 2.4.1 genomic DNA and cloned into plNDS at the Ndel and BglII sites to generate murG-plNDS. gfp was then amplified by PCR using primers Ndel-gfp_p5 and murG-Ndel-gfp and cloned into murG-plNDS at the Ndel site.

(iii) plNDS TetR. The tetracycline resistance gene was amplified by PCR using primers RF-TetR-F and RF-TetR-R. The PCR products were used as megaprimer to replace the kanamycin resistance gene in plNDS using the restriction-free method.

(iv) gfp-plNDS TetR. gfp was amplified by PCR using primers Ndel-gfp_p5 and BglII-gfp_p5 and cloned into plNDS TetR at the Ndel and BglII sites.

(v) gfp-murG-plNDS TetR. murG was amplified by PCR using primers Ndel-murG_p5 and BglII-murG_p5 from R. sphaeroides 2.4.1 genomic DNA and cloned into plNDS TetR at the Ndel and BglII sites to generate murG-plNDS TetR. gfp was then amplified by PCR using primers Ndel-gfp_p5 and murG-Ndel-gfp and cloned into murG-plNDS TetR at the Ndel site.

(vi) gfp-murG1k-plNDS. murG1k was created by using a Stratagene QuikChange XL site-directed mutagenesis kit in accordance with the manufacturer’s protocol. The template used was gfp-murG-plNDS, and the primers used were murG_R77E_S and murG_L76E_AS.

(vii) gfp-murG2.4.1-plNDS. murG2.4.1 was created by using a Stratagene QuikChange XL site-directed mutagenesis kit in accordance with the manufacturer’s protocol. The template used was gfp-murG-plNDS, and the primers used were murG1k_L76E_AS and murG1k_L76E_S.

(viii) 1k-gfp-murG-pK18mobsacB TetR. murG and its 5’ upstream DNA sequence (~1 kb) were amplified by PCR using primers BamHI-murG1k and HindIII-murG1k from R. sphaeroides 2.4.1 genomic DNA and cloned into pK18mobsacB TetR at the BamHI and HindIII sites to generate 1k-gfp-murG-pK18mobsacB TetR. An NdeI site was inserted into 1k-gfp-murG-pK18mobsacB TetR between murG and its 5’ upstream DNA sequence by the use of a Stratagene QuikChange XL site-directed mutagenesis kit and primers murG1k+Ndel_S and murG1k+Ndel_AS to create 1k-Ndel-murG1k-pK18mobsacB TetR. gfp was then amplified by PCR using primers murG1k-Ndel-gfp and murG-Ndel-gfp and cloned into 1k-Ndel-murG1k-pK18mobsacB TetR at the Ndel site.

(ix) murG-pET28b (+). murG was amplified by PCR using primers Ndel-murG_p28 and Xhol-murG_p28 and cloned into pET28b (+) at the Ndel and Xhol sites. The MurG expressed from the pET28b (+) vector contained an N-terminal His tag followed by a thrombin cleavage sequence.

(x) gfp-murG-pET28b (+). murG was created by using a Stratagene QuikChange XL site-directed mutagenesis kit in accordance with the manufacturer’s protocol. The template used was gfp-pET28b (+), and the primers used were murG_L77E_S and murG_L77E_AS.

qPCR. Cells were grown to log phase (absorbance of 0.6, λ = 600 nm), and RNA was isolated as previously described (8). The RNA samples were then treated with RNase-free DNase (Qiagen, Valencia, CA) and further purified with an RNeasy CleanUp kit (Qiagen, Valencia, CA). cDNA synthesis was performed with a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Five nanograms of cDNA was used for each qPCR reaction. The Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA) with SYBR green chemistry was used to monitor amplification and to quantify the amounts of PCR products. Relative quantitation of gene expression was calculated by the threshold cycle (ΔΔCt) method; the level of rpoZ gene (encodes the Ε subunit of RNA polymerase) expression was used as an internal control. The qPCR primers used in this study are listed in Table 4.
measured with an excitation wavelength of 405 nm and an emission wavelength of 450 nm using a Tecan infinite M200 Pro microplate reader (Tecan, San Jose, CA).

**PG isolation and UPLC-MS analysis.** Cells were grown in plain medium or medium containing the indicated small-molecule inhibitors (A22 [10 μg/ml], MEC [0.5 μg/ml], FOS [250 μg/ml], and DCS [0.05 μg/ml]) to early log phase (absorbance of 0.3, 600 nm) and pelleted by centrifugation at 4,000 g for 10 min at room temperature. Cells were then resuspended in 1 ml 0.25% SDS–0.1 M Tris/HCl (pH 6.8) and boiled for 40 min. The cell suspension was centrifuged at 10,000 g for 5 min at room temperature, and the pellet was washed with double-distilled water (ddH2O). The pellet was then resuspended in 1 ml ddH2O and put in a water bath sonicator for 30 min at room temperature. Nuclease solution (15 μg/ml DNase, 60 μg/ml RNase, 0.1 M Tris/HCl, pH 6.8) (500 μl) was added into the sample, which was then incubated for 40 min at 37°C in a shaker. The sample was augmented with 500 μl trypsin solution (50 μg/ml trypsin–ddH2O), incubated for another 60 min at 37°C in a shaker, and then boiled for 15 min to inactivate the enzymes. The suspension was centrifuged at 10,000 × g for 5 min at room temperature, and the pellet was washed with ddH2O.

### TABLE 4 qPCR primers used in this study

| Gene | Primer type | Primer sequence |
|------|-------------|-----------------|
| rpoZ | Forward     | TGACAAGACCCCTGTCGTAAT |
|      | Reverse     | CGACGCTCTCTGCGATCAGAT |
| mreB | Forward     | CTTCTTGCCTTCCTCAACA |
|      | Reverse     | CGCCAGACCTCGGTGATCAT |
| mreC | Forward     | CTTCTTCAAGCCGCCATC |
|      | Reverse     | TCGAGAATCCGTCCATACAG |
| mreD | Forward     | CACATGCGTCTCCGGAAG |
|      | Reverse     | CATCCTCGTCATCCTGAGAT |
| pgp2 | Forward     | GATGAGCCAGGCTGAATCT |
|      | Reverse     | ATCAACATCCGGCGATCAT |
| rodA | Forward     | GTTCACCGAGATAGAAAGAAG |
|      | Reverse     | CTCGCTCTGTGCTCATAG |
| pgp1A| Forward     | CGTCATGAGCTCGAGAAC |
|      | Reverse     | CGTCCTGTCATGGGATCAC |
| murA | Forward     | CTTCCGAGCGATCTTGTAAT |
|      | Reverse     | CAGATGCGATGCGATCAGAT |
| murB | Forward     | GCATCTCACTCTCAACAG |
|      | Reverse     | CCGATGATTCCTCCATAC |
| murC | Forward     | GCAAGACCGACCGACTAC |
|      | Reverse     | GAGCCATGACGACGATCAC |
| murD | Forward     | CAATGACATCGGCCCTCTTC |
|      | Reverse     | CGACAGGATGATGATGAG |
| murE | Forward     | CGACGCTCGTGCTTTC |
|      | Reverse     | GATGGTGCTTGGTGATCAG |
| murF | Forward     | AAGACCTCGAGCAAGGAGA |
|      | Reverse     | CCCAGTGTTGTTGAGGAG |
| ddaA | Forward     | CTCTTGATGCCAGTCTATT |
|      | Reverse     | GACATAGTCTCGAGCATCAG |
| mraY | Forward     | GCTCGACAAATCTAGTCTC |
|      | Reverse     | CCCTGTTGTCTGCTTTC |
| murG | Forward     | ACCTTCTCCGACAGATTC |
|      | Reverse     | CGGCGATGACGATGATG |
| murJ | Forward     | CTCACTACGCTCCAGGCTT |
|      | Reverse     | ATGTTCCGCGCGCTT |

cardiolipin deficiency alters bacterial cell shape
resuspended in digestion buffer (12.5 mM sodium dihydrogen phosphate, pH 5.5) to reach an level of absorbance of 3.0 (A = 600 nm), and a 1/10 volume of mmutanolysin solution (5.000 U/ml mutanolysin−ddH2O) was added. The sample (~175 μl) was incubated for 16 h at 37°C in a shaker and then boiled for 15 min to inactivate the enzyme. The suspension was centrifuged at 10,000 × g for 5 min at room temperature, and the PG was in the supernatant. A 50-μl volume of reduction solution (20 mg/ml sodium borohydride−0.5 M borax−ddH2O, pH 9.0) was added into the PG solution, which was then incubated for 30 min at room temperature. The sample was adjusted to pH 2.0 to 3.0 with phosphoric acid solution (50%) and analyzed by UPLC-MS.

For UPLC-MS, we injected 7.5 μl of purified muropeptides into a Cortecs C18 column (Waters) (2.1 by 100 mm) packed with 1.8-μm-diameter particles and equipped with a Cortecs C18 guard column (Waters). The column temperature was maintained at 52°C using an Acquity standard flow UPLC system equipped with an inline photodiode array (Waters). For muropeptide separation by UPLC, we used solvent A (Optima LC-MS-grade water−0.05% trifluoroacetic acid) and solvent B (30% [vol/vol] Optima LC-MS-grade methanol−Optima LC-MS-grade water−0.05% trifluoroacetic acid) (Fisher Scientific). Muropeptides were eluted from the column with a gradient of increasing solvent B (1 min hold at 1% B to 99% B at 60 min to hold at 99% B 5 min to 1% B at 65.5 min to 4.5 min hold at 1% B) at a flow rate of 0.2 ml/min. We analyzed the eluent from the column using a Bruker MaXis Ultra-High Resolution time-of-flight 4G mass spectrometer (Bruker Daltonik) with an MS method. The capillary voltage was set to 600 V, and the nebulizer pressure was 2 bar, and the drying gas was set to 6.0 liters/m at 220°C. Muropeptides were detected at λ = 205 nm and via MS.

Creation of TyL1 and TyL2. 1k-gfp-murG-pk18mobsac8TetR was introduced into R. sphaeroides wt or Δcls cells by conjugation. The integration of plasmid into the R. sphaeroides genome through homologous recombination was selected by plating cells on plates containing Sistrom’s minimal medium with 1.5% agar. A single colony was picked into plain Sistrom’s minimal medium and grown for 2 days. The excision of the plasmid from the genome through a second homologous recombination step was selected by plating cells on plates containing Sistrom’s minimal medium with 1.5% agar and 10% sucrose. The insertion of gfp between murG and its upstream sequences was confirmed by PCR using primers WSG and HindIII-murG1k.

Cell fractionation. Cells were grown to early log phase (absorbance of 0.3, λ = 600 nm), pelleted by centrifugation at 5,000 × g for 10 min at 4°C, and then resuspended in Buffer T (20 mM Tris-Cl, pH 8.0). Cells were passed through a Constant Systems cell disruptor (Constant Systems Ltd.) at 10,000 lb/in², and the resulting cell lysate was clarified by centrifugation at 5,000 × g for 10 min at 4°C. The clear lysate was quantified, and equal amounts of wt and Δcls lysates were brought to the same volume using Buffer T, centrifuged by centrifugation at 45,000 × g for 30 min at 4°C, and were then divided into fractions of supernatant and pellet (dissolved in Buffer T). The same amounts of proteins in the fractions of supernatant (enriched in cytosolic proteins) and pellet (enriched in membrane proteins) were subjected to Western blot analysis using anti-GFP antibodies.

NAO staining. Cells were grown to early log phase (absorbance of 0.3, λ = 600 nm), and NAO was added at a final concentration of 10 μM into the cultures, which were grown for another 4 h. The cells were then imaged under a fluorescence microscope using 480-nm-excitation and 685-nm-emission filters.

MurG purification. E. coli BL21(DE3) cells transformed with pET28b containing R. sphaeroides murG were grown to an absorbance of 0.5 (A = 600 nm) and induced with 1 mM IPTG for 3 h at 37°C. Cells were pelleted by centrifugation at 4,000 × g for 10 min at 4°C and then resuspended in Buffer T containing 50 mM imidazole and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were passed through a Constant Systems cell disruptor (Constant Systems Ltd.) at 20,000 lb/in², and the resulting cell lysate was mixed with Triton X-100 at a final concentration of 3% for 1 h at 4°C with rotation. The supernatant and insoluble fractions were separated by centrifugation at 25,000 × g for 40 min at 4°C. The supernatant was added to a nickel-nitrioltriacetic acid (Ni-NTA) agarose (Qiagen) column equilibrated with Buffer T. The column was washed stepwise with Buffer T, 25 mM imidazole−Buffer T, and then 50 mM imidazole−Buffer T, and the MurG protein was eluted with 100 mM imidazole−Buffer T. The protein was concentrated into Buffer T (without imidazole) using a 10-kDa-cutoff spin column (Millipore). To remove the His tag from the recombinant protein, the protein concentrate was subjected to thrombin (Millipore) cleavage with a dilution factor for thrombin of 1:200 for 16 h at 22°C. The reaction mixture was mixed with 25 μl of streptavidin agarose beads at 22°C for 30 min with rotation, followed by centrifugation in a spin filter (Millipore). The filtrate, free of biotinylated thrombin, was concentrated into Buffer T using a 10-kDa-cutoff spin column. To remove undigested protein, 25 μl of Ni-NTA agarose was added to the resulting protein concentrate and the sample was incubated at 22°C for 30 min with rotation. The Ni-NTA agarose was removed using a spin filter. The concentration of MurG with a molar extinction coefficient of 21,430 cm⁻¹ M⁻¹ was determined by measuring the absorbance at λ = 280 nm.

Liposome preparation. The following phospholipids used in this study were purchased from Avanti Polar Lipids: 1,2-di-(9Z-octadecenoyl)-sn-gycero-D-phospho-(1′,2′)-sn-glycerol (PG), and 1,1′,2,2′-tetra-(9Z-octadecenoyl) cardiolipin (CL). To prepare liposomes of uniform size, lipid mixtures were dissolved in chloroform, dried in a glass vial, and rehydrated in Buffer T. The resulting liposome solutions were then frozen in liquid nitrogen and thawed in a 37°C water bath 5 times, followed by extrusion (Avanti Polar Lipid Mini Extruder) 15 times through a membrane filter (Whatman) with a pore size of 1 μm.

Liposome-pelleting assay. To block nonspecific binding, phospholipids at 5 mM were preincubated with 0.6 μM of bovine serum albumin (BSA)−Buffer T for 10 min. MurG in Buffer T was then added into the solution, resulting in a final MurG concentration of 6 μM, a phospholipid concentration of 4 mM, and

January/February 2019 Volume 10 Issue 1 e02401-18
a BSA concentration of 0.5 μM. The reaction mixture was incubated for 30 min, centrifuged at 16,300 × g for 30 min, and then divided into fractions of supernatant and pellet (dissolved in Buffer T). All the procedures were performed at room temperature. Proteins in the supernatant and pellet fractions were subjected to SDS-PAGE and visualized by Coomassie blue staining.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02401-18.

FIG S1, TIF file, 0.6 MB.
FIG S2, TIF file, 0.05 MB.
FIG S3, TIF file, 0.1 MB.
FIG S4, TIF file, 0.2 MB.
FIG S5, TIF file, 0.03 MB.
FIG S6, TIF file, 0.3 MB.
FIG S7, TIF file, 0.8 MB.
FIG S8, TIF file, 0.03 MB.
FIG S9, TIF file, 0.04 MB.
FIG S10, TIF file, 0.02 MB.

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