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MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis

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Abstract

Peptidoglycan (PG) is a polysaccharide matrix that protects bacteria from osmotic lysis. Inhibition of its biogenesis is a proven strategy for killing bacteria with antibiotics. The assembly of PG requires disaccharide-pentapeptide building blocks attached to a polyisoprene lipid carrier called lipid II. Although the stages of lipid II synthesis are known, the identity of the essential flippase that translocates it across the cytoplasmic membrane for PG polymerization is unclear. We developed an assay for lipid II flippase activity and used a chemical genetic strategy to rapidly and specifically block flippase function. We combined these approaches to demonstrate that MurJ is the lipid II flippase in *Escherichia coli*.

Bacteria use polyisoprenoid-linked oligosaccharides to assemble the essential peptidoglycan (PG) matrix that surrounds their cytoplasmic membrane and fortifies their cell envelope against high internal osmotic pressure (1). The building block of PG (cell wall) is a disaccharide-pentapeptide that is synthesized at the cytoplasmic leaflet of the inner membrane (IM) as a precursor known as lipid II (Fig. 1A) (1, 2). This precursor must be flipped across the membrane for cell wall synthesis.

The identity of the lipid II flippase has been controversial with the debate centered on two candidates: MurJ-like and FtsW/RodA-like proteins (3–6). MurJ is a polytopic IM protein and member of the MOP (multidrug/oligo-saccharidyl-lipid/polysaccharide) exporter

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For additional data, see Supporting Online Material.

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superfamily (7). It is essential in *Escherichia coli*. Cells depleted of MurJ fail to complete PG biogenesis, accumulate PG precursors, and lyse (4, 6). A 3-D structural model and corresponding transmembrane topology of MurJ is similar to that of MOP exporters of amphipathic drugs and undecaprenyl-PP-linked oligosaccharides (8). Furthermore, a hydrophilic central cavity in MurJ is essential for function. FtsW and its paralog RodA are polytopic IM proteins that belong to the SEDS (shape, elongation, division, and sporulation) superfamily and are required for PG synthesis during division or elongation, respectively (3, 5, 9). Support for SEDS proteins functioning as flippases is based on in vitro studies where lipid II flippase activity was detected for purified FtsW incorporated into liposomes (10). The identity of the lipid II flippase has been sought after for decades (2). Determining which proteins flip lipid II in vivo requires a sensitive method to detect lipid II flippase activity and a method to connect this activity to a specific protein within the cell. When added to *E. coli*, the protein toxin colicin M (ColM) is translocated into the periplasm where it cleaves lipid II (Fig. 1A) (11, 12). We therefore reasoned that ColM could be used in an assay to detect freshly flipped lipid II. To evaluate this possibility, cells were metabolically labeled with [^{3}H]-mDAP, an amino acid unique to the PG peptide, and either left untreated or incubated with purified ColM. Cells were then extracted with hot water followed by butanol to separate soluble PG intermediates and ColM-derived products from lipid-linked PG precursors, respectively. HPLC analysis of the water-soluble extract revealed a new peak in the ColM-treated samples (Figs. 1B and S1) and its appearance correlated with the loss of radiolabel in the butanol extract (Fig. 1C). Moreover, increasing the cellular lipid II concentration by overproducing the lipid-carrier synthase UppS (2) enhanced the production of the ColM-specific peak (Fig. S2). The ColM-specific product was identified as PP-M_{pep4}-G (Figs. S3–S4), which presumably results from the processing of the ColM product, PP-M_{pep5}-G, by a carboxypeptidase (Fig. 1A). Because carboxypeptidases function only in the periplasm (1), this result confirms that ColM acts on flipped lipid II.

To test whether MurJ flips lipid II, a method to rapidly and specifically inactivate it was needed. A collection of 39 functional single-Cys MurJ variants modifiable by the Cys-reacting molecule MTSES were previously used to determine the membrane topology of MurJ (8). We asked whether any of these mutant proteins were rendered non-functional by derivitization with MTSES. Treatment of Cys-free MurJ ($MurJ^{WT}$) cells with MTSES had no effect on growth, but addition of MTSES to cells producing derivatives with Cys substitutions at positions 29, 49, 263, and 269 rapidly induced lysis, suggesting that MurJ function, and thus PG synthesis, was inhibited (Fig. 2 and S5). In contrast, treatment of $MurJ^{E273C}$ cells with MTSES caused cell shape defects and limited lysis indicative of an incomplete PG synthesis block due to partial MurJ inhibition. The toxicity of MTSES labeling was suppressed in all five strains by the presence of the wild-type *murJ* allele (Fig. 2 and S5). Thus, MTSES specifically and rapidly inhibits these single-Cys MurJ variants. We chose $MurJ^{A29C}$ (Figs. 2, S6 and S7) to assess the effect of MurJ inactivation on lipid II flipping.

This chemical genetic method for MurJ inactivation was compatible with the in vivo flippase assay. MTSES treatment of $MurJ^{WT}$ cells did not affect lipid II processing by ColM (Figs. 1B–C and S1). Additionally, in the absence of MTSES, $MurJ^{A29C}$ cells behaved like

MurJ^{WT} cells (Figs. 1B–C and S1). However, simultaneous addition of MTSES and ColM to MurJ^{A29C} cells failed to produce significant quantities of the ColM-dependent product PP-M$_{pep4}$-G. In fact, radiolabel in the lipid fraction increased in these samples (Figs. 1B–C and S1). Thus, when MurJ^{A29C} was inactivated with MTSES, lipid II was protected from ColM cleavage and label accumulated in the lipid fraction as observed previously for MurJ-depletion strains (4, 6).

The protection of lipid II from ColM cleavage upon MurJ^{A29C} inactivation suggests that either lipid II is not flipped or that inhibiting MurJ^{A29C} somehow interferes with ColM import or activity. To investigate this, we performed our assay using spheroplasting to remove the OM barrier (13) and provide ColM with direct access to flipped lipid II. In the absence of MTSES, ColM treatment of MurJ^{WT} or MurJ^{A29C} spheroplasts reduced the amount of label in the lipid fraction (Fig. 3), indicating that lipid II was actively flipped and thus cleaved by ColM. Although MTSES did not affect ColM activity on MurJ^{WT} spheroplasts, it completely abolished lipid II processing by ColM in MurJ^{A29C} spheroplasts (Fig. 3). Moreover, lysis of MTSES-treated MurJ^{A29C} spheroplasts restored lipid II processing, indicating that the intact IM impeded access of ColM to lipid II. Thus, MurJ appears to act as a lipid II flippase.

When MurJ^{A29C} was inactivated with MTSES, flippase activity was reduced to a level that was barely detectable and incompatible with life. This observation indicates that the essential function of MurJ is to translocate lipid II and that other factors catalyzing lipid II flipping are unlikely to exist in *E. coli*. Nevertheless, we investigated the requirement of SEDS proteins for flippase activity by depleting FtsW in a Δ*rodA* strain. We found that lipid II flipping remained robust in this background (Figs. S8–S9). Although it is possible that residual FtsW in these cells was sufficient for the observed activity, this result suggests that SEDS proteins are not responsible for lipid II flippase activity in vivo. Alternatively, the decrease in levels of PG lipid intermediates upon FtsW depletion (Fig. S9) suggests that either synthesis of PG precursors or recycling of undecaprenyl-P might be affected by the loss of SEDS activity. From these data and the fact that MurJ contains a central, solvent-exposed cavity that is essential for function (8), we conclude that MurJ is the lipid II flippase in *E. coli*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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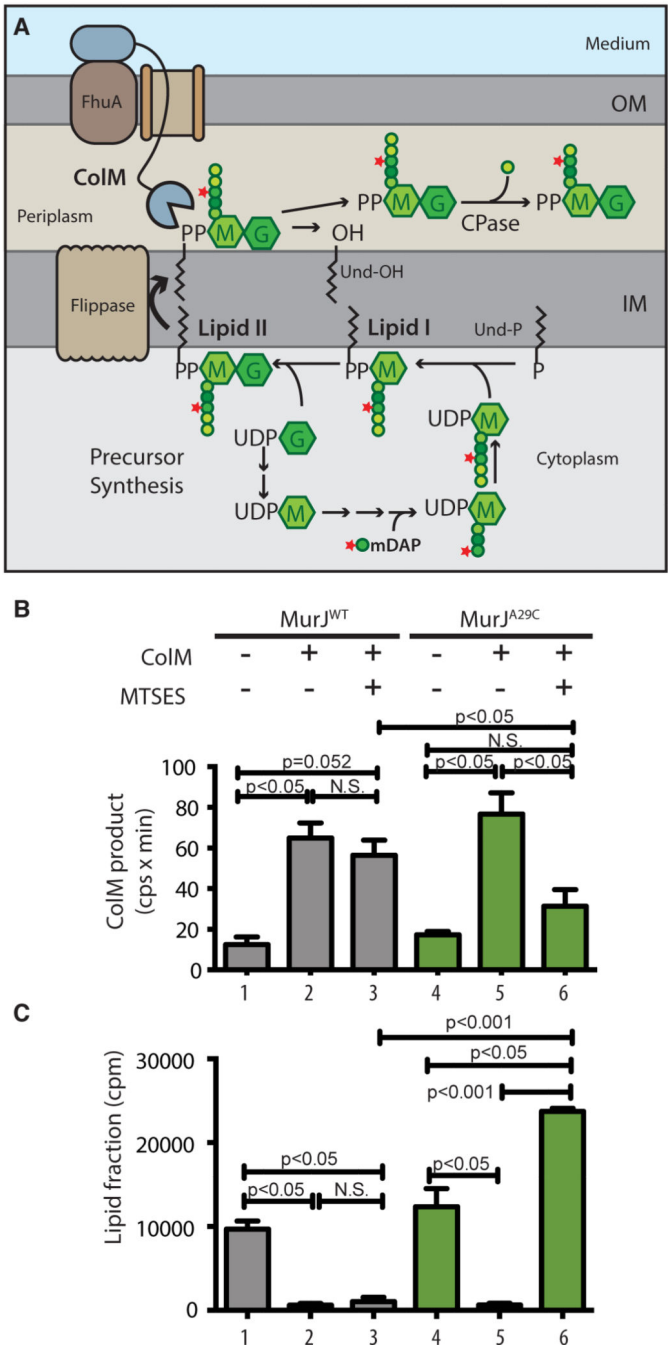

Fig. 1. In vivo assay for lipid II flippase activity

(**A**) PG precursor synthesis starts with the conversion of UDP-N-acetylglucosamine (UDP-G) to UDP-N-acetylmuramic acid (UDP-M), followed by the addition of amino acids (represented by colored spheres) to UDP-M to form the pentapeptide (pep5) stem (L-Ala-γ-D-Glu-m-DAP-D-Ala-D-Ala). [^{3}H]-mDAP label is indicated by the star. The UDP-sugars are transferred to undecaprenol-P (Und-P) in the IM to form lipid II, which is flipped across the IM to expose the disaccharide-pep5 (M_{pep5}-G) for polymerization and crosslinking into PG (not illustrated). Exogenous ColM binds to FhuA and is translocated across the OM

presumably through a porin. In the periplasm, ColM cleaves lipid II into undecaprenol (Und-OH) and soluble PP-M_{pep5}-G, which is further processed by carboxypeptidases (CPase) to produce PP-M_{pep4}-G. (**B-C**) Cells of Δ*murJ* Δ*lysA* strains producing FLAGMurJ lacking endogenous Cys residues referred to as $MurJ^{WT}$ (NR2592) or its derivative $MurJ^{A29C}$ (NR2593) were labeled with [^{3}H]-mDAP. After 15 min, ColM and/or MTSES were added as indicated and growth was continued for 10 min. Samples were then withdrawn and either extracted with hot water alone or sequentially with water then butanol. Hot water extracts were subjected to high-performance liquid chromatography (HPLC) and radiodetection to quantify the labeled ColM product (**B**); scintillation counting was used to quantify label in the lipid (butanol) fraction (**C**). See figs. S1–S4 for experimental details and peak identification. Shown are the mean ± SD; $N = 3$. P value determined with Student's t test. N.S., not significant.

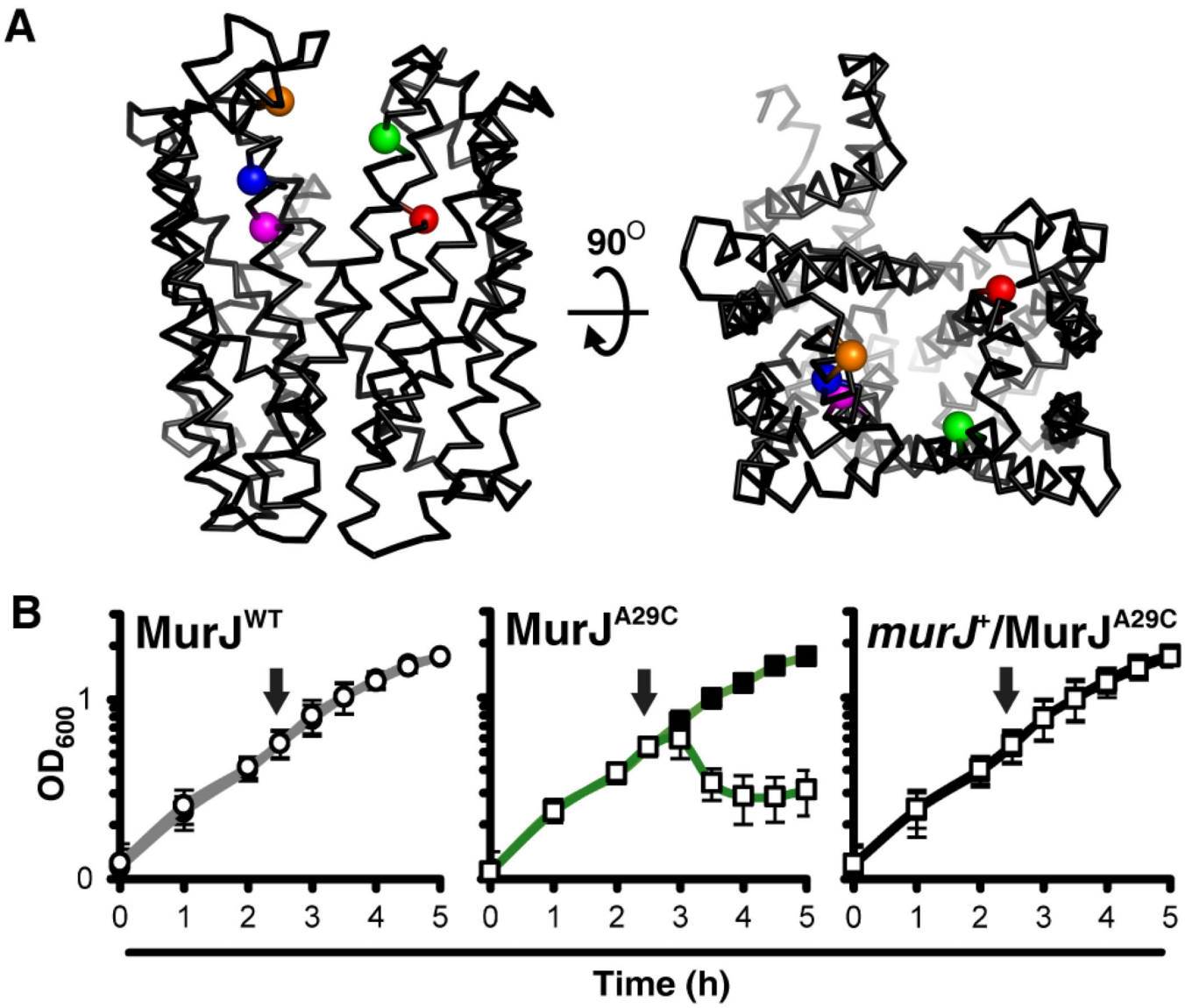

Fig. 2. MTSES specifically inhibits the function of MurJ^{A29C}

(**A**) Structural model of MurJ (8). Sensitivity to MTSES is limited to specific residues within the MurJ cavity: residues 29 (green) in transmembrane domain (TMD) 1, 49 (red) in TMD 2 , and 263 (orange), 269 (blue) and 273 (magenta) in TMD 8. (**B**) Effect of MTSES on the growth of haploid cells producing MurJ^{WT} (left) or MurJ^{A29C} in glucose M63 medium. Lysis of MurJ^{A29C} cells is suppressed by the presence of a wild-type *murJ* allele (right). Arrows indicate time of MTSES addition; filled marker, no MTSES; empty marker, MTSES treated. Data represent mean ± SD; $N = 3$. See fig. S5 for MTSES sensitivity of other variants.

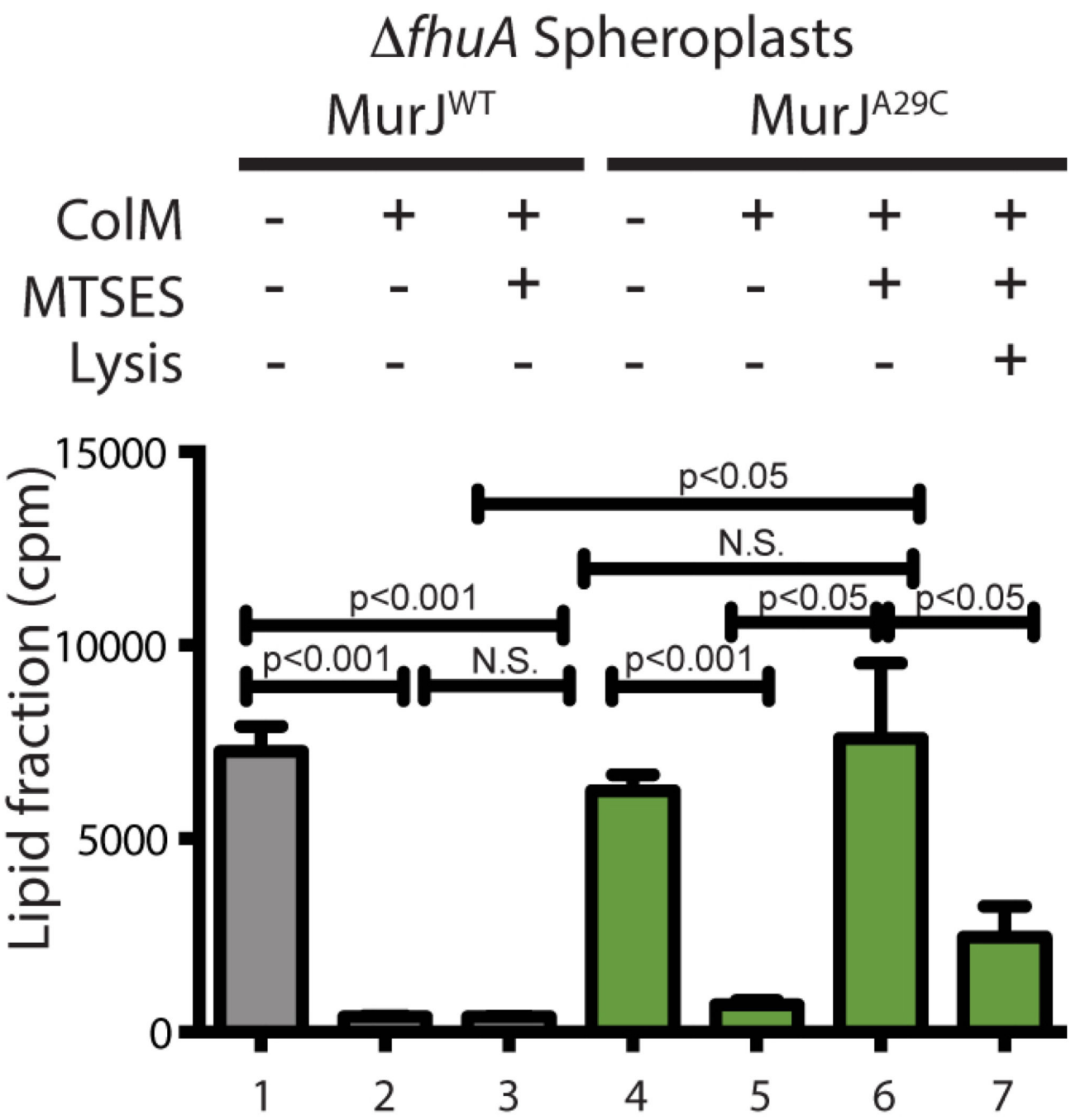

Fig. 3. MurJ activity is required for ColM-dependent cleavage of lipid II in spheroplasts

Cells lacking the ColM receptor FhuA and producing the indicated MurJ variants were grown, labeled, and treated with MTSES as for figure 1. Spheroplasts were then prepared. In all but one case, spheroplasts were pelleted and resuspended in ColM reaction buffer with sucrose and added MTSES (0.8 mM) as indicated. The Lysis + sample was resuspended in buffer lacking sucrose to lyse the spheroplasts. ColM (100 μg) was added to the prepared

spheroplasts as indicated and they were incubated for 15 min at 37°C. Lipid intermediates were detected after butanol extraction by scintillation counting. Statistics are as for figure 1.