Published in final edited form as:

J Am Chem Soc. 2018 April 04; 140(13): 4481–4484. doi:10.1021/jacs.8b00942.

Membrane potential is required for MurJ function

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Abstract

MurJ, the flippase that exports the bacterial cell wall monomer Lipid II to the periplasm, is a target for new antibiotics, which are desperately needed to treat Gram-negative infections. Quantitative methods to monitor MurJ activity are required to characterize inhibitors but are challenging to develop because the lipid-linked substrate is not chemically altered in a flippase reaction. Here we show that MurJ inhibition can be quantified by measuring the accumulation of intracellular Lipid II using a biotin-tagging strategy. We have exploited this assay to show that MurJ is inhibited in the presence of a compound that dissipates the membrane potential. By probing cysteine accessibility we have found that under this condition MurJ relaxes into an inactive, outward-facing conformation reminiscent of that targeted by the peptide antibiotic Lys^M. We conclude that membrane potential is required for MurJ function in *E. coli*, and we anticipate that the ability to accumulate this inactive conformation will lead to structures useful for inhibitor design.

Graphical abstract

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Notes

The authors declare no competing financial interest.

Supporting Information. Experimental procedures, LC/MS analysis, Western blot analysis, and strain information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Antibiotic-resistant infections caused by Gram-negative pathogens pose a major threat to human health. New antibiotics to treat these infections are desperately needed. The bacterial cell wall, also known as peptidoglycan, is required for survival, and its biosynthesis has proven to be an excellent target for antibiotics.^{1–3} Lipid II, the building block of peptidoglycan, is a lipid-linked disaccharide-pentapeptide that is synthesized on the inner leaflet of the cytoplasmic membrane and then flipped to the periplasmic leaflet where it is polymerized and crosslinked (Figure 1a, b).^{4–10} Although most peptidoglycan biosynthetic enzymes were discovered decades ago, the essential membrane protein MurJ was only recently identified as the Lipid II flippase.^{7,8,11} Exploiting MurJ as a target for new antibiotics requires quantitative assays to monitor inhibition and a better understanding of its transport mechanism.

We previously developed methods to detect changes in Lipid II pools in *S. aureus* upon antibiotic treatment and showed that these changes can provide useful information about antibiotic mechanisms.¹² Lipid II detection was accomplished by labeling the extracted precursor with biotin-D-Lys and then western blotting (Figure 1c).^{12–14} We found that treating *S. aureus* with moenomycin, an inhibitor of the penicillin binding proteins (called aPBPs) that polymerize Lipid II, resulted in a large increase in cellular pools of this precursor (Figure 1a). Applying the same inhibitor to *E. coli* resulted in only marginal accumulation of Lipid II; however, we found that Lipid II pools increased substantially in the presence of vancomycin, a glycopeptide antibiotic that binds the terminal D-Ala-D-Ala dipeptide of Lipid II, preventing substrate consumption (Figure 1a, d, **S1**). We have concluded that it is possible to accumulate Lipid II if all enzymatic processing of the precursor is fully blocked. Because inhibiting the aPBPs, which are susceptible to moenomycin, does not result in Lipid II accumulation, it follows that the substrate must be consumed by another cellular pathway.

Recently, RodA, a member of the shape, elongation, division, sporulation (SEDS) family of proteins was shown to polymerize Lipid II in *E. coli* (Figure 1a).^{15,16} RodA is not sensitive to moenomycin, explaining why Lipid II did not accumulate in the presence of moenomycin alone. We reasoned that inhibiting both classes of polymerases in *E. coli* would result in Lipid II buildup in the *E. coli* periplasm. There are no known inhibitors of RodA, but its cellular function depends on the presence of MreB filaments at the cytoplasmic membrane. MreB filament assembly can be inhibited by the small molecule A22 (Figure S1), resulting in inactivation of RodA.^{15,17} A second SEDS protein, FtsW, is proposed to function at the *E. coli* divisome,[Hongbaek 2016] though it has not been demonstrated to possess polymerase activity. Formation of the divisome can be inhibited by the overexpression of *sulA*. [Bi & Lutkenhaus, 1993] Therefore, we co-treated *E. coli* cultures with moenomycin and A22 under conditions of *sulA* overexpression and assessed Lipid II levels after 10 min (Figure 1e, **S4**). Whereas moenomycin alone caused no Lipid II buildup, and A22 alone caused only slight Lipid II buildup, co-treatment caused substantial buildup. Notably, the induction of *sulA* did not influence Lipid II levels (data not shown). Therefore, both RodA and aPBPs contribute to Lipid II consumption in the *E. coli* periplasm.

The observation that Lipid II cannot accumulate outside the cytoplasm unless all peptidoglycan polymerase activity is blocked led us to speculate that it might be possible to

develop a quantitative assay to monitor flippase activity by measuring changes in intracellular Lipid II pools. We have previously shown that it is possible to block flippase activity of a cysteine variant of MurJ, MurJ^{Cys/A29C}, by treating it with the thiol-labeling reagent MTSES (2-sulfonatoethyl methanethiosulfonate).⁸ A recently-reported MurJ crystal structure shows that Ala-29 is located at the interface between the two lobes of the transporter at the periplasmic face of the membrane and suggests that MTSES functions by inhibiting the interchange between outward-facing and inward-facing conformations required for transport (Figure 2a).¹⁸ To evaluate prospects for a quantitative inhibition assay, we treated exponential phase cultures expressing MurJ^{Cys/A29C} and MurJ^{Cys} with increasing concentrations of MTSES as we monitored growth. MurJ^{Cys} continued to grow in the presence of 400 μ M MTSES, but MurJ^{Cys/A29C} showed dose-dependent killing, with lysis beginning 10 min after treatment (Figure 2b). We extracted Lipid II from treated and untreated cultures at a time point just before the onset of lysis and found that the MurJ^{Cys} cultures showed no difference in Lipid II levels even at 400 μ M MTSES; in contrast, the MurJ^{Cys/A29C} cultures showed a dose-dependent increase in Lipid II pools (Figure 2c). Because dose-dependent Lipid II accumulation was only observed upon treatment of the MurJ^{Cys/A29C} mutant, and coincided with dose-dependent killing, we concluded that the rise in Lipid II levels was due to inhibition of this essential flippase. These studies establish that inhibition of MurJ can be detected in cells by monitoring the increase in intracellular Lipid II pools.

We have also shown that this assay can be adapted to spheroplasts, which lack both a peptidoglycan layer and an outer membrane. When treated with MTSES for 10 min, *E. coli* spheroplasts harboring only the MurJ^{Cys/A29C} variant showed a marked increase in Lipid II levels (Figure 3a, compare lanes 1 and 4). Subsequent treatment with dithiothreitol (DTT), which reduces the disulfide linkage between Cys-29 and MTSES (Figure 2a), resulted in a return to baseline Lipid II levels within 10 min (Figure 3a, lanes 4 – 6). The return to baseline is consistent with restoration of transport to the periplasmic face of the membrane, with consumption of Lipid II by polymerases. By treating spheroplasts with a gradient of MTSES concentrations for 10 min and measuring relative Lipid II abundance, we were able to measure an IC₅₀ value of $\sim$ 60 μ M for MTSES (Figure 3b, Figure 3c). While MTSES itself is a covalent inactivator with no potential as an inhibitor, the ability to measure IC₅₀ values in a spheroplast assay allows MurJ inhibitors to be compared quantitatively without factoring in their permeability or efflux.

We wondered whether we could use our ability to monitor MurJ activity to address whether Lipid II transport requires a membrane potential. MurJ is a member of the MOP exporter superfamily of transporters,^{7,19} and some of them are driven by sodium or proton electrochemical gradients.^{20–24} To assess whether MurJ flipping activity is similarly powered, we treated cells expressing the MurJ^{Cys/A29C} variant with the protonophore TCS (3,3',4',5-tetrachlorosalicylanilide), which dissipates both the membrane potential and proton chemical gradient (combined, the proton-motive force). We found that Lipid II levels were similarly elevated when cells were treated with either TCS or MTSES (Figure 4a), or with the structurally distinct protonophore CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone, Figure S6a, b). Lipid II accumulation with protonophore treatment can be specifically attributed to dissipation of the membrane potential rather than the proton

chemical gradient because valinomycin, but not nigericin, also causes accumulation (Figure S7). As peptidoglycan polymerases do not require energy to polymerize Lipid II, we have concluded that Lipid II consumption ceases in the absence of membrane potential because transport to the periplasm stops. Protonophore washout restores cell growth, indicating repolarization of the membrane, with concomitant return of Lipid II pools to baseline levels (Figure S6c). Therefore, the membrane potential is required for Lipid II export.

We can address how dissipation of the membrane potential affects the conformation of MurJ using the substituted-cysteine accessibility method (SCAM).^{25–27} In this method, cysteine residues are probed to determine their accessibility to the aqueous environment. Single-cysteine variants of MurJ are first treated with either the membrane-impermeable MTSES or the membrane-permeable *N*-ethylmaleimide (NEM). Proteins are then denatured and treated with maleimide-PEG to label free cysteines, and band shifts for MTSES- and NEM-treated samples are compared. Using this method we assessed accessibility of 12 MurJ residues in untreated cells and in conditions of membrane depolarization (Figure 4b, c). Of eight residues tested that lie in the central cavity, seven positions showed an increase in MTSES accessibility upon membrane depolarization (Figure 4b,c, blue) (Figure 4b,c, red). All four residues located outside of the central cavity showed no change in SCAM pattern between the two conditions (Figure 4b,c, gray, Figure S8). Therefore, membrane depolarization induces a conformational change in MurJ, favoring an outward-open state. Although it is possible the response of MurJ to the loss of membrane potential is indirect, our results are consistent with a mechanism in which MurJ couples substrate transport to the movement of a counterion down its electrochemical gradient, similarly to other MOP exporters.

We note that a recently-reported viral peptide antibiotic, Lys^M, has been found to lock MurJ in an outward-facing state similar to the state reported here.²⁶ This observation shows that the outward-facing state is a viable conformation to target. Although structures of an inward-facing state have been obtained, no structures of an outward-facing conformation are available. The ability to accumulate substantial quantities of the outward-facing state *via* membrane depolarization may lead to structural information which will help guide MurJ inhibitor design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This research was supported by the National Institutes of Health (R01 GM100951 to N.R.; R01 GM066174 to D.K.; U19 AI109764 and R01 GM076710 to D.K. and S.W.).

We thank Tom Bernhardt and Rachelle Gaudet for helpful discussions. We thank the Bernhardt laboratory (Harvard Medical School) for generously sharing plasmid pNP146 and the Schier laboratory (Harvard University) for the use of their microscope. All high-resolution LC/MS data was acquired by Jennifer Wang at the Harvard University Small Molecule Mass Spectrometry Core Facility.

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Figure 2.

Cytoplasmic Lipid II pools accumulate upon MurJ inactivation prior to cell lysis. (a) Crystal structure of MurJ from *T. africanus* in an inward-facing conformation, showing residue Ala-29 located at the interface between the N lobe (light gray) and C lobe (dark gray). Covalent modification of an A29C variant of MurJ with the thiol-labeling probe MTSES inactivates flippase activity. (b) Cultures of NR2131 (pFLAG-MurJ^{Cys}) and NR2186 (pFLAG-MurJ^{Cys/A29C}) expressing N-FLAG-tagged MurJ variants were grown in M63 minimal media to early log phase and treated with the thiol-labeling reagent MTSES. Culture density is monitored by optical density at 600 nm (OD₆₀₀). (c) Cultures from (b) were assessed for Lipid II content after 10 min MTSES treatment (dotted line).

Figure 3.

Efficacy of MurJ inhibitors can be quantified in *E. coli* spheroplasts. (a) NR2186 (pFLAG-MurJ^{Cys/A29C}) cultures were treated with EDTA and lysozyme to generate spheroplasts, which were subsequently treated with MTSES (1 mM) for 10 min. The reducing agent dithiothreitol was added, and Lipid II levels were assessed after time indicated. (b) Spheroplasts as in (a) were treated 10 min with between 3.4 μ M and 2.5 mM MTSES and assessed for Lipid II content. (c) Bands from (b) were quantified and fit to a sigmoidal curve to derive an IC_{50} value of ~60 μ M (see Supporting information). See also Figure S5.

Figure 4. Dissipating the membrane potential alters MurJ conformation, impairing its function. (a) Cultures of NR2186 (pFLAG-MurJ^{Cys/A29C}) were simultaneously treated with MTSES (400 μ M) and the protonophore TCS (100 μ M) and assessed for Lipid II content after 10 min. (b) The substituted-cysteine accessibility method (SCAM) was used to probe conformational changes in MurJ during membrane depolarization. Cultures expressing N-FLAG-tagged single-cysteine variants of MurJ (see Table S1) were pre-treated with the thiol-reactive compounds MTSES (membrane-impermeable) or N-ethylmaleimide (NEM, membrane-permeable) and then subjected to denaturation and maleimide-PEG (Mal-PEG) labeling. Cysteine residues which do not react with MTSES or NEM in the blocking step show a mass shift by Western blot. Membrane depolarization was achieved by 5 min TCS (100 μ M) treatment prior to SCAM. (c) Residues from (b) are mapped onto TM1-12 of inward- and outward-facing homology models of *E. coli* MurJ.^{25,28,29} Positions which show an increased accessibility to the periplasm are shown in blue. Positions which show a decreased accessibility to the aqueous environment are shown in red. Positions with no change in SCAM pattern are shown in gray. See also Figures S6 – S8.