Exploring the Conformations of Nonfunctional Variants of the Lipid II Flippase MurJ in *Escherichia coli.*

Research Thesis

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with research distinction in the undergraduate colleges of The Ohio State University

by

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Abstract

Biosynthesis of the bacterial peptidoglycan cell wall is essential for determining bacterial shape and protection from differences in osmolaric pressure. One necessary step for the construction of the bacterial cell wall is the translocation of the lipid-linked peptidoglycan precursor, Lipid II, across the cytoplasmic membrane. MurJ is the flippase that translocates lipid II in *Escherichia coli* anda target of recently discovered antibacterials. Little is known about the mechanism of flippase proteins such as MurJ. However, crystal structures recently have shown MurJ in multiple inward- and outward-open conformations. These crystal structures, along with structure-guided *in vivo* cysteine cross-linking data, have suggested an alternating-access mechanism of function for MurJ. Since this cross-linking method allows probing of the different conformations of MurJ *in vivo,* here, we utilized this method to examine how changes to residues critical to MurJ function affect the conformation of the flippase *in vivo*. Our data suggest that MurJ mutant proteins containing R18E stall or slow down function so they are mostly found in an inward-open conformation, while those containing R24E may slightly favor the outward-open conformation.

Introduction

In gram-negative bacteria like *Escherichia coli*, the cellular envelope contains four main components: the inner membrane (IM), the outer membrane (OM), the aqueous compartment between them known as the periplasm, and the peptidoglycan layer (PG) (**Figure 1**) (Silhavy *et al*. 2010). The peptidoglycan layer is an essential layer that is also known as the cell wall in bacteria. It is a polymeric lattice made up of glycan chains of two alternating sugars, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) crosslinked by a β-(1,4)-glycosidic bond. NAM molecules are monosaccharides with five amino acids attached to them. These peptides allow adjacent glycan strands to be crosslinked together to form the mesh-like peptidoglycan structure. The peptidoglycan layer gives bacteria structural support and their shape. It also protects their cytoplasmic or inner membrane from hypotonic environments that would cause lysis. Given these essential functions of the peptidoglycan cell wall and the fact that it is unique to bacterial cells, as human cells do not synthesize peptidoglycan, peptidoglycan biogenesis is a target for many antibiotics like the penicillins (Typas *et al.* 2011, Lovering *et al.* 2012).

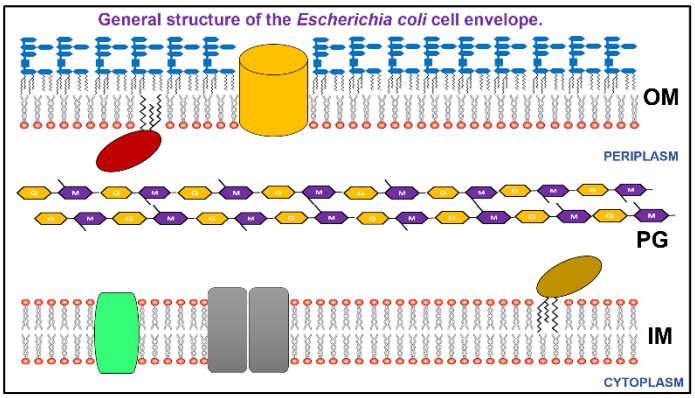

Figure 1: **Bacterial Envelope Overview**. OM is the outer membrane, PG is the peptidoglycan layer, IM is the inner membrane (Figure adapted from Sujeet Kumar).

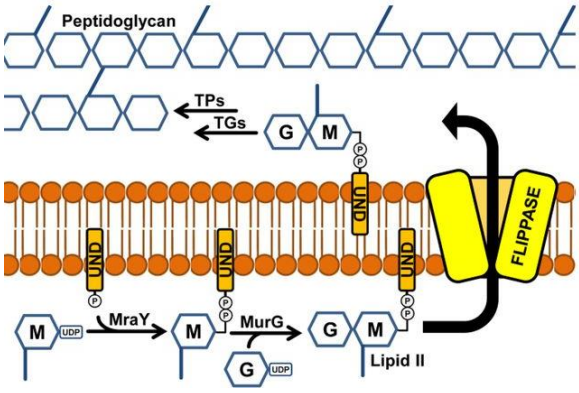

Figure 2: Synthesis of Lipid II and Peptidoglycan (Figure from Butler *et al*. 2013).

In *E. coli*, it has been previously shown that a protein called MurJ, located in the inner membrane, is essential for peptidoglycan synthesis (Ruiz 2008, Inoue *et al.* 2008). MurJ transports lipid II, the lipid-linked building block for the peptidoglycan layer, across the inner membrane (**Figure 2**) (Ruiz 2008, Inoue *et al.* 2008, Sham *et al*. 2014). This transport is a necessary step of peptidoglycan synthesis because lipid II is synthesized in the inner leaflet on the inner membrane but can only be utilized on the outer leaflet of the inner membrane. However, the mechanism for how MurJ transports this molecule is unknown. MurJ is known to be a flippase, but in general, very little is understood about the mechanism of flippase proteins (Butler *et al.* 2014).

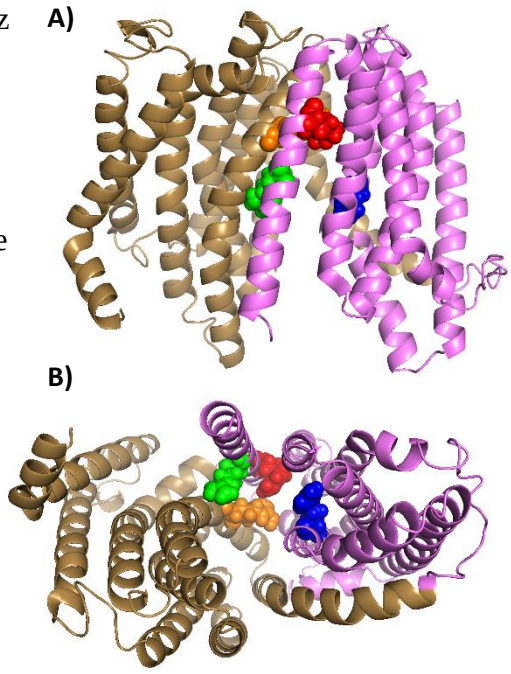

Figure 3: **MurJ Structure.** (**A**) Residue R18 is represented as the green spheres, R24 as the red spheres, R52 as the blue spheres and R270 as orange spheres. (**B**) Cytoplasmic up view of MurJ with highlight residues.

MurJ is conserved in bacteria that produce peptidoglycan, so understanding important components to its function could be useful in developing new antibiotics that target this protein in many pathogens such as (Mohamed *et. al* 2014, Huber *et. al* 2009). This is especially of interest because antibiotics that would target peptidoglycan synthesis will lessen the potential for negatively impacting animal cells, since the peptidoglycan layer is unique to the bacterial envelope.

MurJ is a V-shaped protein with 14 transmembrane domains (Butler *et. al* 2013). These transmembrane domains can be split into two groups: The amino-terminal (N) lobe, that consists of transmembrane domains 1-6, and the carboxyl-terminal (C) lobe, that consists of transmembrane domains 7-14. Inside the V-shape these two lobes form, there is a large cationic cavity. The cavity is formed by transmembrane domains 1, 2, 7 and 8 (Kuk *et al.* 2017, Zheng *et al.* 2018, Butler *et. al* 2013, Butler *et. al* 2014). Within MurJ's central cavity, there are eight charged residues critical for function. When these residues are changed to other amino acids, they cause either a total or a partial loss of MurJ function (Butler *et al.* 2014). Four notable residues that specifically render MurJ nonfunctional when changed to non-positively charged residues are R18, R24, R52 and R270 (**Figure 3**). However, we do not understand the specific role these residues play on MurJ function, and this study explores the relationship between these four arginine amino acid residues that are essential for function and their effect on the conformational state of MurJ.

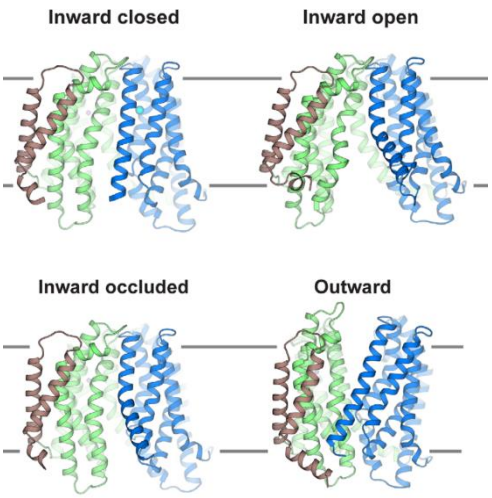

Figure 4: Conformational Structures of MurJ (Figure adapted from Kuk *et al*. 2019).

Based on recent crystal structure data, it is now known that MurJ exists in several structural confirmations, including inward closed, inward open, inward occluded and outward open (**Figure 4**). These structures suggest an alternating-access mechanism for function of MurJ (Kuk *et al*. 2019).

Further evidence to support the alternating-access mechanism for MurJ function was obtained in vivo. The proton motive force (PMF) was previously found to be necessary for MurJ flippase activity. Specifically, membrane potential is required

for function, and disruption of membrane potential led to a conformational change of MurJ to favor an outward-open state, suggesting that it exists *in vivo* (Rubino *et al.* 2018). Then, by structure-guided *in vivo* cysteine cross-linking and proteolysis-coupled gel analysis, both an inward-open and an outward-open conformation could be probed for and visualized, illustrating conformational structures that exist *in vivo* (Kumar *et al.* 2019). Using this method, cysteines were introduced and crosslinked either the periplasmic or cytoplasmic side of MurJ. In one conformation, these cysteines would not come in a cross-linking distance by the crosslinkers used. In the other conformation, the cysteines would then come close enough together to be crosslinked, thus demonstrating the existence of that conformation. Since results showed that MurJ can be in both inward-open and outward-open conformations *in vivo*, MurJ is thought to function by an alternate-access mechanism (Kumar *et al.* 2019, Alvin *et al*. 2019, Rubino *et al.* 2018). Therefore, the current model is that Lipid II enters MurJ from the inner leaflet of the inner membrane while MurJ is in an inward-open facing state, and then lipid II is flipped across the membrane by MurJ, which opens to an outward-facing conformation. Afterwards, by some unknown mechanism requiring membrane potential, MurJ flips back to an inward-facing conformation (**Figure 5**). In this work, we test whether substitutions in the MurJ cavity that render the flippase non-functional lock MurJ in one of these conformations.

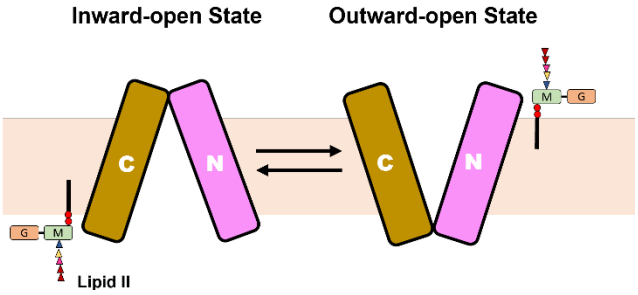

Figure 5: Current model of MurJ flippase function. Two conformations are shown in this model, an inward-facing conformation facing toward the cytoplasm and an outward-facing model opening to the periplasm. (Adapted from Kumar *et al.* 2019)

Methods

Bacterial strains and growth conditions.

Strains used are listed in Table 1. Cells grew at 37°C with aeration in lysogeny broth (LB) with ampicillin (125 μg/ml), and growth was monitored by optical density at 600 nm (OD600).

Table 1: Strains used in the study

Strain	Genotype	Source
DH5α	F^- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE*44 λ^- *thi*-1	Life Technologies
MC4100	F^- *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	(Inoue *et al*. 2008)
NR754	MC4100 *ara*$^+$	(Silhavy *et al*. 2010)
NR3267	NR754 Δ*murJ::FRT* (pRC7KanMurJ)	(Kumar *et al*. 2019)
NR 5795	NR754 (pET23/42FLAGMurJΔCys-222Thrombin) WT	This study
NR5755	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/V43C/T251C)	(Kumar *et al*. 2019)
NR5757	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/S73C/A296C)	(Kumar *et al*. 2019)
NR5796	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/R18E/V43C/T251C)	This study
NR5826	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/R18E/S73C/A296C)	This study
NR5797	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/R24E/V43C/T251C)	This study
NR5846	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/R24E/S73C/A296C)	This study
NR5798	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/R52E/V43C/T251C)	This study
NR 5827	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/R52E/S73C/A296C)	This study
NR 5809	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/R270E/V43C/T251C)	This study
NR 5828	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/R270E/S73C/A296C)	This study
NR 5829	NR754 (pET23/42FLAGMurJΔCys-222Thrombin/A29W/V43C/T251C)	This study

Plasmids construction and site-directed mutagenesis (SDM).

Primers are listed in Table 2. Plasmids were constructed using Phusion polymerase. Construction of pRC7KanMurJ and pET23/42FLAGMurJΔCysthrombin was previously described (Kumar *et al*. 2019).

The pET23/42FLAGMurJΔCysthrombin derivatives with glutamate or tryptophan substitutions were generated using SDM PCR. Plasmids with double Cys-codon substitutions in Flag-murJΔCys-thrombin were used as templates to introduce the third mutation, the glutamate to arginine mutation or alanine to tryptophan mutation.

All the substitutions were made by using SDM PCR with Phusion polymerase (95ºC for 2 min, followed by 19 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 14 min and a final extension of 72ºC for 12 min) according to the manufacturer's instructions. The variants made in pET23/42FLAGMurJΔCys-thrombin were electroporated into DH5α and transformants were selected on LB agar containing 125 μg/mL ampicillin.

Table 2: Primers used in the study

PRIMER NAME	SEQUENCE 5'-3'	PURPOSE
5murJR18E	CATGTTTTCGGAGGTGCTTGGCTTCGCACGAG	SDM
3murJR18E	CCAAGCACCTCCGAAAACATGGTCATCGAGC	SDM
5murJR24E	GGCTTCGCAGAGGACGCAATTGTCGCCAGAATCTTTG	SDM
3murJR24E	CAATTGCGTCCTCTGCGAAGCCAAGCACACGCG	SDM
5murJR52E	CTTGTTAGAGCGTATCTTTGCCGAAG	SDM
3murJR52E	CAAAGATACGCTCTAACAAGTTAGGAAGTTTAAAAGCG	SDM
5murJR270E	CGCCGACGAGTTAATGGAGTTTCCGTCCGG	SDM
3murJR270E	CTCCATTAACTCGTCGGCGTAATACATCCAAG	SDM
5murJA29W	CAATTGTCTGGAGAATCTTTGGCGCAGGGATGGC	SDM

3murJA29W	CAAAGATTCTCCAGACAATTGCGTCTCGTGCG	SDM

Functionality testing for pET23/42FLAGMurJΔCys-thrombin derivatives.

The ability of pET23/42FLAGMurJΔCysthrombin-derived plasmids to functionally complement the loss of murJ was evaluated as described previously (Butler *et. al* 2013, Butler *et. al* 2014, Chamakura *et. al* 2017) using strain NR3267 (NR754 Δ*murJ::frt* pRC7KanMurJ) (Elhenawy *et. al* 2016).

In vivo cysteine cross-linking using a maleimide homobifunctional cross-linker.

The *in vivo* cysteine cross-linking procedure used was previous described (Kumar *et. al* 2019). Strains were grown to OD600 1 in LB medium containing ampicillin and 10 ml of culture was used. Each suspension was treated with either dimethyl sulfoxide (DMSO; solvent used for dissolving crosslinkers) or with the homobifunctional maleimide cross-linker N,N′-(o-phenylene)dimaleimide (oPDM; Sigma) at a final concentration of 1 mM. Samples were incubated on a rotator at room temperature for 5 min in the dark during crosslinking treatment, then quenched with L-cysteine (Sigma; 10 mM final concentration) for 5 min at room temperature on a rotator. For spheroplast formation, the samples were resuspended in spheroplast buffer (50 mM Tris-HCl, pH 8.0, 1 M sucrose, 2 mM EDTA) with 0.125 mg/ml lysozyme. After incubating at room temperature for 15 min, 40 µl of 1M $MgCl_2$ was added. Spheroplast formation was confirmed through microscopy, then collected by centrifugation and resuspended in 500 µl of 50 mM Tris-HCl, pH 8.0 containing 1 µl Benzonase (Novagen). Spheroplast membranes were pelleted using ultracentrifugation at 100,000 rpm for 1hr at 4ºC in a Optima-MAX-TL ultracentrifuge (Beckman Coulter) using TLA120.2 rotor. The membrane pellet was resuspended in thrombin reaction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, 1% DDM) (Hennon *et. al* 2014), and samples were centrifuged to remove

insolubilized or aggregated proteins. After centrifugation, 0.4 units of thrombin protease (Sigma) were added to 50 µl of the solubilized membrane fraction only for treated samples. The reaction was next incubated overnight at room temperature. Then, equal volume of 2X AB buffer (6.84 mM Na_2HPO_4, 3.16 mM NaH_2PO_4, 50 mM Tris-HCl, pH 6.8, 6 M urea, 1% β-mercaptoethanol, 3% SDS, 10% glycerol, 0.1% bromophenol blue) (Butler *et. al* 2013, Butler *et. al* 2014) was added to the samples and loaded onto 10% SDS-polyacrylamide gels for electrophoresis and detection by immunoblotting.

Immunoblotting for FLAG-MurJ detection.

Samples were prepared as explained above for the cysteine cross-linking experiments or as follows for biogenesis of FLAGMurJ by immunoblotting since the selected arginine and alanine reside mutations are total loss of function mutations of MurJ.

Cells were grown overnight and normalized by dividing 400 by OD600 values. Then, they were pelleted by centrifugation and lysed with 50 µl of BugBuster protein extraction reagent (Novagen) and 1 µl of Benzonase (Novagen). After incubating the samples from the biogenesis or cross-linking procedures at room temperature, 50 µl of 2XAB buffer was added.

Samples were loaded onto a 10% SDS-polyacrylamide gel for electrophoresis. Proteins were then transferred from the gel to a polyvinylidene difluoride (PVDF) membrane at 10V for 2 h using semi-dry transfer apparatus (BioRad). PVDF membranes were probed with anti-FLAG M2 (1:10,000; SigmaAldrich) and anti-mouse horseradish peroxidase (HRP; 1:10,000; GE Amersham) antibodies. Signals were developed using the Clarity Western ECL substrate as per the manufacturer's instructions (Bio-Rad) and detected using a ChemiDoc XRS+ system (Bio-Rad).

Results

Experimental rationale.

We wanted to use site-directed cysteine *in vivo* cross-linking to test if non-functional mutations that change specific essential arginine or alanine residues stalled MurJ in one conformation, either a conformation that was open to the cytoplasm (i.e. inward open) or one open to the periplasm (i.e. outward open) (Butler *et. al* 2013, Butler *et. al* 2014). Several charged residues in MurJ's central cavity are critical for its function, including residues R18, R24, R52, and R270 (Butler *et. al* 2014). We hypothesized that substitutions at these sites cause MurJ to be stalled in one conformation, thus halting MurJ's function. Another residue examined was A29. Residue A29 is at the interface of the N-lobe and C-lobe in the periplasmic side of MurJ. We know that the $MurJ^{A29C}$ variant is functional. However, when $MurJ^{A29C}$ was treated with sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES), which covalently binds to cysteines, it became nonfunctional. We hypothesized that the addition of MTSES to position A29C adds bulkiness that prevents MurJ from being able to adopt an inward-open conformation, causing MurJ to be stuck in the outward-open state and making it nonfunctional.

For this strategy, we introduced paired cysteine residues into each of the lobes of MurJ and used a homobifunctional cross-linking reagent that could cross-link specific cysteine pairs when the two lobes adopted only one of the two conformations, as described previously (Kumar *et. al* 2019). All variants were encoded in pET23/42FLAGMurJΔCys-thrombin plasmids.

Construction of loss-of-function variants.

In order to test the function of the *murJ* alleles encoding the desired MurJ variants, we used strain NR3267, which carries the pCR7KanMurJ plasmid (Elhenawy 2017). The pRC7KanMurJ plasmid has two important features that allow us to screen for functionality of

murJ alleles on different plasmids. First, pRC7KanMurJ has a partitioning defect, so it does not equally segregate into daughter cells during cellular division. Because of this defect, the plasmid is lost from a population of cells in the absence of selection. This selection can either be the absence of a functional *murJ* allele in the cell from another introduced plasmid, as NR3267 is chromosomally Δ*murJ*, or from selection by kanamycin present in the media. Secondly, pRC7KanMurJ encodes β-galactosidase. This allows us to use blue/white screening of colonies in the presence of X-gal and IPTG to determine the loss of pRC7KanMurJ from the population.

Taking advantage of these features of pRC7KanMurJ, we transformed strain NR3267 with mutagenized pET23/42FLAGMurJΔCys-thrombin plasmids. Since all of the mutations we generated are known to cause a total loss-of-function phenotype, there was no loss of pRC7KanMurJ in cells. Therefore, cells encoding non-functional pET23/42FLAGMurJΔCys-thrombin glutamate or tryptophan derivatives yielded blue colonies, while cells encoding functional or non-mutated pET23/42FLAGMurJΔCys-thrombin plasmids yielded white colonies. Therefore, the alleles we generated behaved as expected.

Detection of pET23/42FLAGMurJΔCys-thrombin variants.

To generate pET23/42FLAGMurJΔCys-thrombin variants with glutamate or tryptophan substitutions, we used a plasmid containing paired cysteine mutants that had been previously described for probing for conformational changes in MurJ (Kumar *et. al* 2019). The two paired cysteine combinations we used were V43C/T251C, the periplasmic pair that would only crosslink in the inward-facing conformation, and S73C/A296C, the cytoplasmic pair that would only crosslink in the outward-facing conformation. Using SDM, residues R18, R24, R52 and

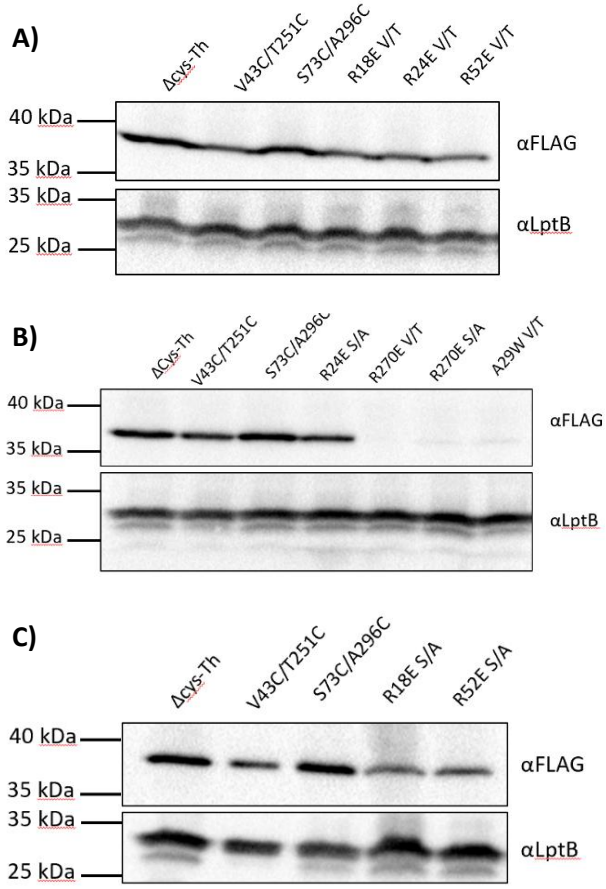

Figure 6. Proteolytic analysis of the FLAG-MurJΔCys-thrombin variants used to probe conformational states of MurJ. FLAG immunoblotting of samples from merodiploid *murJ* strains producing FLAGMurJΔCys-thrombin (ΔCys-Th) and its variants to determine biogenesis of FLAG-MurJ. LptB immunoblotting (bottom panel) was used as a loading control. **(A)** Expression of R18E, R24E and R52E in the V43C/T251C background. **(B)** Expression of R18E and R52E in the S73C/T251C background. **(C)** Expression of R24E S73C/A296C, R270E V43C/T251C and S73C/A296C and A29W V43C/T251C.

R270 individually were changed to a glutamate residue in both plasmids. Residue A29 was changed to a tryptophan residue only in the pET23/42FLAGMurJΔCys-thrombin V43C/T251C background.

Once mutated, we transformed these plasmids into the wild-type strain NR754 to examine the biogenesis of the FLAGMurJ variants. Since the plasmids were introduced into NR754, which contains chromosomal *murJ*, the resulting strains were viable on LB with ampicillin, since the pET23/42FLAGMurJΔCys-thrombin plasmid contains the ampicillin resistance gene. Once transformed, the cells were lysed, and cellular proteins extracted for immunoblotting using anti-FLAG antibodies.

Variants of FLAGMurJ with R18E, R24E and R52E substitutions in the V43C/T251C background were detected similarly to the V43C/T251C variant (**Figure 6A**). Variants of FLAGMurJ with R18E, R24E, and R52E in the S73C/T251C background showed slightly reduced levels as compared to S73C/A296C (**Figure 6B** and **6C**). The levels of the

FLAGMurJ variant with R270E in V43C/T251C and S73C/A296C were barely at the limit of detection (**Figure 6C**). Since there was nearly no detection, we decided to not move forward with R270E for cross-linking. Introduction of A29W into V43C/T251C also led to no detection; because of this, we did not generate the A29W variant in the S73C/A296C variant and did not move forward with A29W for cross-linking (**Figure 6C**). As a loading control for all biogenesis blots, we detected the LptB protein with an anti-LptB antiserum.

Conformations of pET23/42FLAGMurJΔCys-thrombin variants.

As previous described, the addition of the thrombin protease cut site aids in differentiating between cross-linked and uncross-linked products (Kumar *et. al* 2019). Briefly, the loop connecting the N and C lobes of MurJ contains the thrombin protease cut site. It can be cleaved by the protease thrombin after cells are treated or not with cross-linkers. In the absence of cross-linking, thrombin should cleave this construct into two fragments: one corresponding to the N lobe and the other corresponding to the C lobe. Since the N lobe has the FLAG tag, only the N lobe can be detected by immunoblotting. However, the two fragments should remain covalently linked after protease cleavage if the two lobes were cross-linked prior to proteolysis and be visible by immunoblot as a band between 35 and 40 kDa, where native MurJ runs (Kumar *et. al* 2019). Because we used 10% acrylamide gels for the immunoblots, only full-length or cross-linked variants could be detected in our experiments. The N-lobe fragment

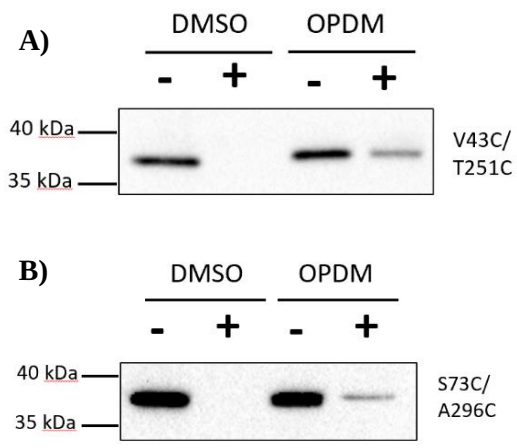

Figure 7. Crosslinking of cysteine variants. A) V43C/T251C, the periplasmic paired mutations, exhibit some crosslinking. **B)** S73C/T251C, the cytoplasmic paired mutations, illustrate some crosslinking.

resulting from cleaving uncross-linked variants with thrombin is too small to be detected when using 10% acrylamide gels.

Two paired cysteine mutations were used for assessing the conformation of the non-functional variants. The V43C and T251C pair is located in the periplasmic side of MurJ. Thus, when these two cysteines were cross-linked by o-PDM, it was indicative of an inward-open conformation (**Figure 7A**). Similarly, the cytoplasmic pair used was S73C and A296C. When this pair was cross-linked by o-PDM, MurJ was in an outward-open conformation (**Figure 7B**).

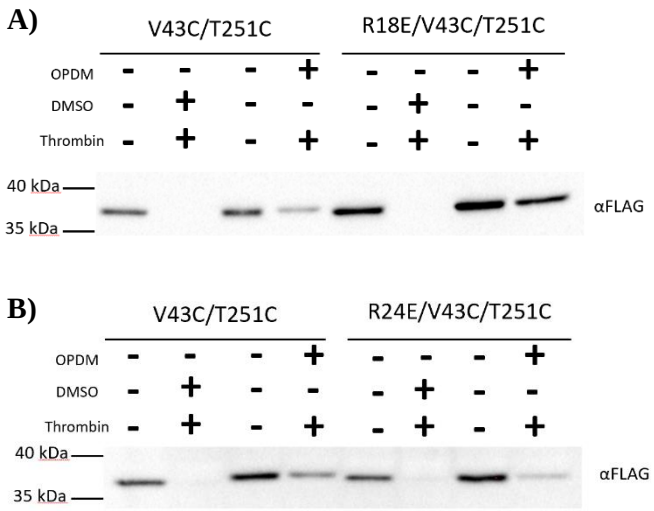

Figure 8. In vivo cysteine cross-linking of arginine mutants in pET23/42FLAGMurJΔCysthrombin V43C/T251C background. (A) Cross-linking of R18E/V43C/T251C. **(B)** Cross-linking of R24E/V43C/T251C.

The first MurJ variant shown in **Figure 8A** carries the R18E/V43C/T251C substitution. While levels of V43C/T251C are somewhat decreased with respect to R18E/V43C/T251C, the R18E/V43C/T251C variant showed an increase in cross-linking. This increase in the amount of MurJ variants in inward-open conformation suggest that the R18E change might somehow interfere with the ability of MurJ to attain the outward-open conformation. In contrast, the second variant, R24E/V43C/T251C exhibited less crosslinking than V43C/T251C (**Figure 8B**), suggesting that the R24E change might lead to a decrease in the ability of MurJ to be the inward-open conformation.

Discussion

MurJ is essential in *E. coli* because of its function in peptidoglycan synthesis, where it flips lipid II from the inner leaflet of the inner membrane to the outer leaflet using an alternating-access mechanism of function (Kumar et. al 2019, Kuk et. al 2019, Sham *et. al* 2014). In this study, we investigated residues that are critical for the function of MurJ to understand how they might participate in inducing conformational structural changes that MurJ must undergo during the transport cycle.

The five residues we looked at were R18, R24, R52, R270 and A29, which are located in the MurJ cavity (Butler *et. al* 2013, Butler *et. al* 2014). As expected, changing these residues caused a total loss of MurJ function. It has been proposed that the arginines might interact with lipid II (Butler *et. al* 2014, Kuk *et. al* 2017, Kuk *et. al* 2019). We also knew that when MTSES, a bulky chemical that reacts with cysteines, was added to a mutant carrying the A29C, MurJ function was inhibited (Sham et. al 2014, Butler *et. al* 2013, Butler *et. al* 2014). Therefore, we hypothesized that the changes we introduced caused a total loss of function because of the charge disruption in the arginine residues or the bulkiness added at residue 29 when substituted by a tryptophan.

Our cross-linking data show that the FLAGMurJΔCys-thrombin R18E/V43C/T251C MurJ variant appears to prefer an inward conformation, while the FLAGMurJΔCys-thrombin R24E/V43C/T251C variant might not. Our hypothesis was that variants with the R18E and R24E substitutions would cross-link in an inward-facing conformation more than the outward-facing conformation because these residues are toward the periplasmic side of MurJ. Residue R18 functioned in line with our hypothesis, as it exhibited more cross-linking in an inward-open conformation than the control. This means that the R18E change might cause MurJ to flip more

slowly to the outward-open conformation, or not flip at all. In contrast, residue R24 did not cross-link more in an inward-open conformation. This means R24E might cause MurJ to stall longer in the outward-open conformation.

Further analysis should be done in Mur variants with the S73C/A296C pair and the R18E and R24E substitutions to confirm the aforementioned preferred conformations. For example, if we expect that the MurJ variant carrying the R18E change prefers the inward-open conformation, then levels of cross-linking reflecting the inward-open conformation should increase, while cross-linking of the S73C/A296C pair should decrease. If MurJ prefers the outward-open confirmation, as seen in residue R24E, then we expect to see less cross-linking in the inward-open cross-linking pair as we did, and more in the outward-open cross-linking pair we plan to test. We also plan to examine the preferred conformation of R52E in both backgrounds to determine if MurJ with this change prefers a specific conformation. To study the impact of residues R270 and A29 on MurJ's conformation, we plan to generate new substitutions at those sites that may compromise function without causing a biogenesis defect. Determining which conformation MurJ prefers with these total loss-of-function mutations can provide us insight to why changes to these residues cause this phenotype. Understanding the importance of these residues might help in generating new antibiotics that might target MurJ.

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