

# Direct observation of the influence of cardiolipin and antibiotics on lipid II binding to MurJ

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## 1. Supplementary Methods

### Plasmid construction and expression of *E. coli* MurJ and FtsW

The plasmids used for over-expression of the proteins in this study are described in **Supplementary Table 1**. *murJ* and *ftsW* genes were amplified from *E. coli* BL21(DE3) genomic DNA by polymerase chain reaction (PCR) using the primers (Integrated DNA Technologies Inc., Leuven, Belgium) listed in **Supplementary Table 1** and cloned into a modified pET15b (1) and pET28a vectors using an In-Fusion kit (Clontech). The restriction sites highlighted in bold (only for the plasmids that express wildtype proteins) were used to construct the plasmids indicated. The DNA sequences were verified by sequencing (Source Bioscience). The MurJ protein containing a 6×His tag at the C-terminus was overexpressed in *E. coli* C43(DE3) (Lucigen) cells, grown in LB medium containing ampicillin (100 µg ml<sup>-1</sup>). When the culture reached an absorbance at 600nm of ~0.5, expression was induced with 0.5 mM isopropyl β-thiogalactopyranoside (IPTG) for 3h at 37 °C. N-terminally His-tagged FtsW was overexpressed in *E. coli* C43 (DE3) cells, grown in LB medium containing kanamycin (50 µg ml<sup>-1</sup>). When the culture reached an absorbance at 600 nm of ~0.5, the cells were induced with 1 mM IPTG for 4h at 37 °C.

### Site-directed mutagenesis

Site-directed point mutations on residues A29, N49, S263, D269, and E273, which were expected to be critical for the function of the MurJ (2), were performed to generate the single point mutants A29C, A29P, N49A, S263A, D269A and E273A. These MurJ mutants were prepared by a PCR-based method, using the plasmid that was used for expressing wild-type protein as the template. The primers (Integrated DNA Technologies Inc., Leuven, Belgium) used for these mutations are listed in **Supplementary Table 1**. Expression and purification of mutant proteins was performed in the same manner as for the MurJ wild-type protein.

### Lipids, Lipid II, antibiotics and MTSES

Unless stated otherwise, all lipids [*E. coli* cardiolipins and 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine PE 16:0/18:1(9z)] used in this study were obtained from Avanti Polar Lipids powders and stock solutions were prepared following a previously established method (3). The L-lysine form of lipid II was purchased from BACWAN at University of Warwick. Stock solution was dried under direct nitrogen gas flow and dissolved in a buffer containing 200 mM ammonium acetate and 0.05% (w/v) LDAO to the required concentration. Vancomycin, ramoplanin and MTSES were purchased from Sigma, Insight Biotechnology Ltd. and Cambridge Bioscience Ltd. Respectively. These compounds were dissolved and diluted in 200mM ammonium acetate supplemented with 0.05% (w/v) LDAO.

### Native mass spectrometry

#### Lipid II binding experiments

Lipid II binding experiments were performed with the protein in 200 mM ammonium acetate supplemented with 0.05% (w/v) LDAO. In order to obtain the binding constant for the interaction between lipid II and MurJ, lipid II was added in increasing amounts (**Supplementary Table 2**) while keeping the protein concentration constant. Peak intensities were extracted and the ratios of the

intensity of the lipid II bound peak versus the total intensity of all observed species (in one charge state) were calculated (4). Average and standard deviation of these ratios from at least 5 charge states from three independent experiments were plotted against lipid II concentration. The data were fitted globally using GraphPad Prism 5.0 with the equation  $Y=B_{max} * X / (X + K_d)$  where  $B_{max}$  is the maximum specific binding in the same units as  $Y$ . Using our methods, we could not deduce the low-affinity  $K_d$  for the second lipid II binding as higher concentrations of lipid II binding to the protein were impractical to measure using our mass spectrometry method due to associated decreases in spectral quality, and the potential for favouring the capturing of non-specific binding events through the electrospray process.

### **Antibiotic binding**

Antibiotics (vancomycin and ramoplanin) were diluted into buffer containing 200 mM ammonium acetate and 0.05% (w/v) LDAO and were added in different ratios either to solutions of MurJ in the same buffer or to the MurJ-lipid II complex in 200 mM ammonium acetate and 0.05% (w/v) LDAO. All experiments were repeated three times.

### **MTSES and MurJ variants data**

MTSES was diluted in 200 mM ammonium acetate and 0.05% (w/v) LDAO. Both MurJ<sub>WT</sub> and MurJ<sub>A29C</sub> were treated with MTSES before adding lipid II. Lipid II binding for MurJ variants was analysed using analogous methods to the wild-type protein. The relative ratios of the intensities of lipid II bound and unbound were plotted. Standard deviations are calculated from four charge states in three independent repeats.

### **Lipid II binding to MurJ in presence of other lipids**

Effect of cardiolipins and PE on lipid II binding to delipidated MurJ was monitored by acquiring the mass spectra in presence of increasing amounts lipids to the pre-formed MurJ-lipid II complex. The relative ratios of MurJ-bound lipid II versus the total intensity of unbound and cardiolipin-bound MurJ from three independent datasets were plotted as a function of cardiolipin concentration. All experiments were performed three times and that the standard deviations are calculated from four charge states in these 3 datasets.

### **Absolute quantification of cellular cardiolipins**

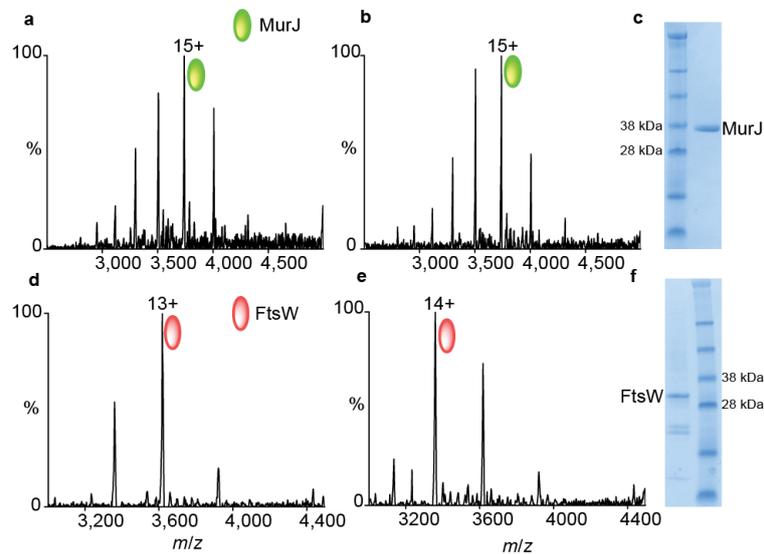
Cellular levels of cardiolipins were quantified by following (5). Briefly, cells were pelleted by centrifugation (1000g for 10 min at 4 °C). The pellet was washed with 20 mM Tris (pH 8.0), 150 mM NaCl twice, re-suspended in 20 mM Tris (pH 8.0), 150 mM NaCl buffer to a final volume of 1 ml and transferred to a glass vial. The absorbance of cells at 600 nm was measured to count the cell numbers. Then, the cells in 20 mM Tris (pH 8.0), 150 mM NaCl buffer were mixed with 2 ml methanol and 0.8 ml chloroform, sonicated for 10 min and blended with 1 ml chloroform and 1 ml 20 mM Tris (pH 8.0), 150 mM NaCl. After centrifugation (5000g for 10 min), the chloroform phase was transferred to a new glass vial by a glass Pasteur pipette and dried under a gentle stream of nitrogen. The extracted lipids were mixed with cardiolipin standard (14:1(3)-15:1 CA, LM-1802, Avanti) and dissolved in lipidomics buffer A for lipidomics quantification by following the same protocol above. Data was analysed by extracting ion chromatogram (XIC) and its area under the curve (AUC) of each cardiolipin was processed and integrated in Xcalibur 2.2 (Thermo) with 50 ppm

mass tolerance and 7 point Gaussian smoothing. The absolute quantity of cardiolipins in sample was calculated based on the ratio of their AUCs to the AUC of cardiolipin standard. Experiments were repeated three times.

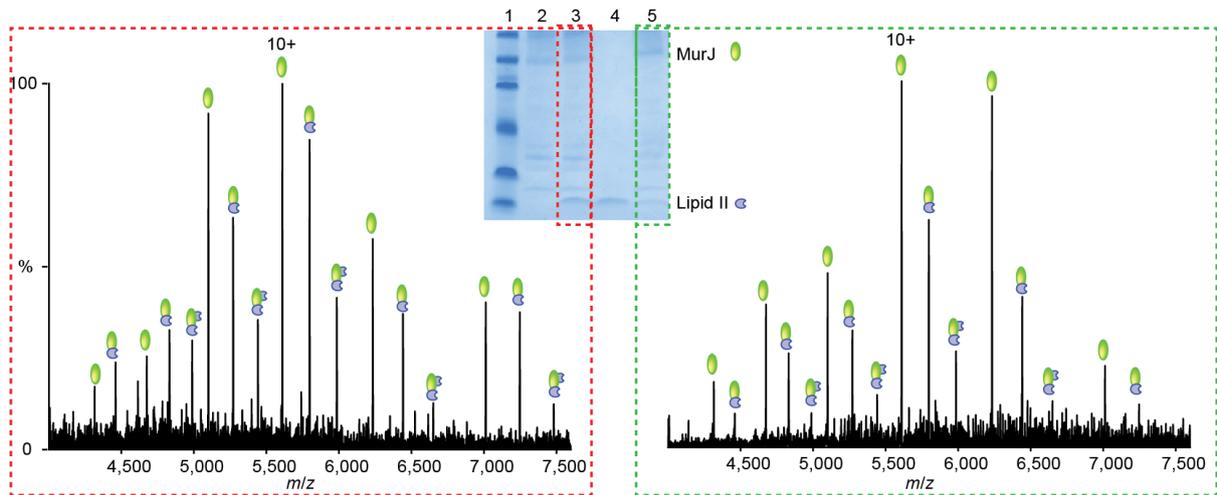
### Estimation of cellular levels of proteins and lipids

We have calculated the cellular levels of proteins using the protein abundance information found on the PaxDb website (<http://pax-db.org>). Combining this information with the estimation of 2000 lipid II molecules per cell (6), yields relative ratios of MurJ:FtsW:lipid II as 1.2:1:45. CDLs compose 5% of *E. coli* total lipids. Considering the total number of lipids per cell ( $2 \times 10^7$ ) (7), we calculate the total number of CDLs per cell as  $1 \times 10^6$ .

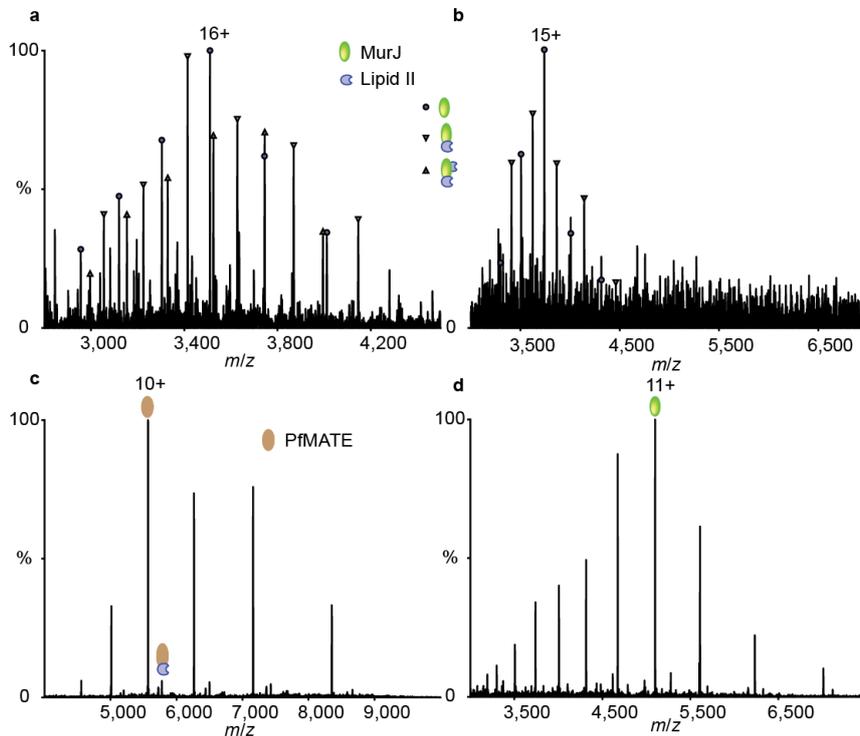
## 2. Supplementary Figures



**Supplementary Fig. 1: Mass spectra of MurJ and FtsW in other detergents.** Mass spectra of MurJ in 0.02% DDM (a) and 0.12% OGNG (b), and mass spectra of FtsW in 0.02% DDM (d), and 0.12% OGNG (e). These spectra indicate that these proteins are monomers in solution and have higher charge states than in LDAO. SDS-PAGE images of purified MurJ (c) and FtsW (f) proteins.

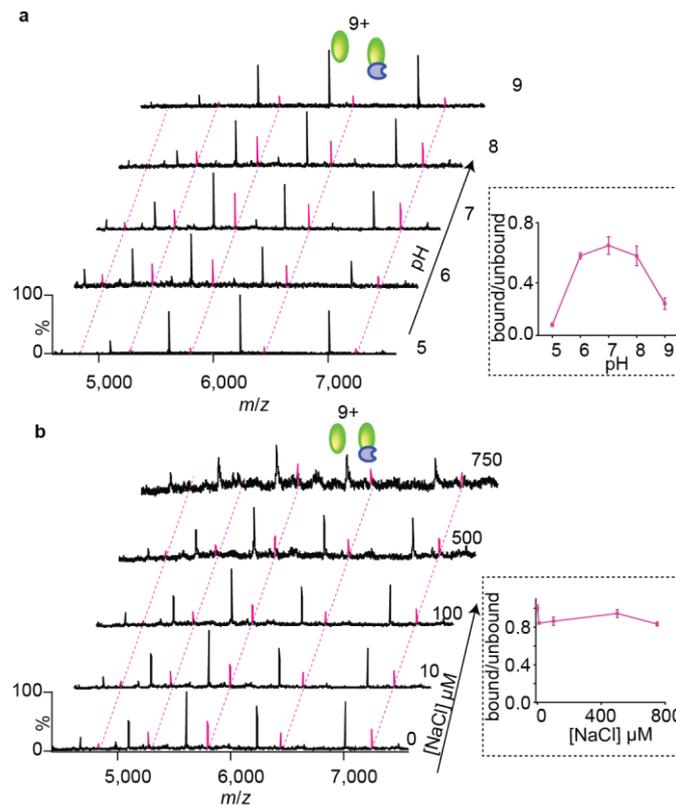


**Supplementary Fig. 2: Mass spectra of MurJ: lipid II complex after a few chromatographic steps.** Mass spectra of MurJ: lipid II complex obtained firstly, by incubating protein and lipid II in 1:1 ratio and performing a desalting purification step prior to MS analysis (left spectrum, corresponds to lane 3 in the SDS-PAGE). Secondly, incubated protein- and lipid II were subsequently purified using immobilised-metal affinity chromatography, followed by MS analysis (right spectrum, corresponds to lane 5 in the SDS-PAGE). In both cases the resulting mass spectra show that the lipid-protein complex survives, even under these conditions where the pool of available lipid II is depleted. Lane 1, 2 and 4 in the SDS-PAGE are marker, MurJ and lipid II respectively.

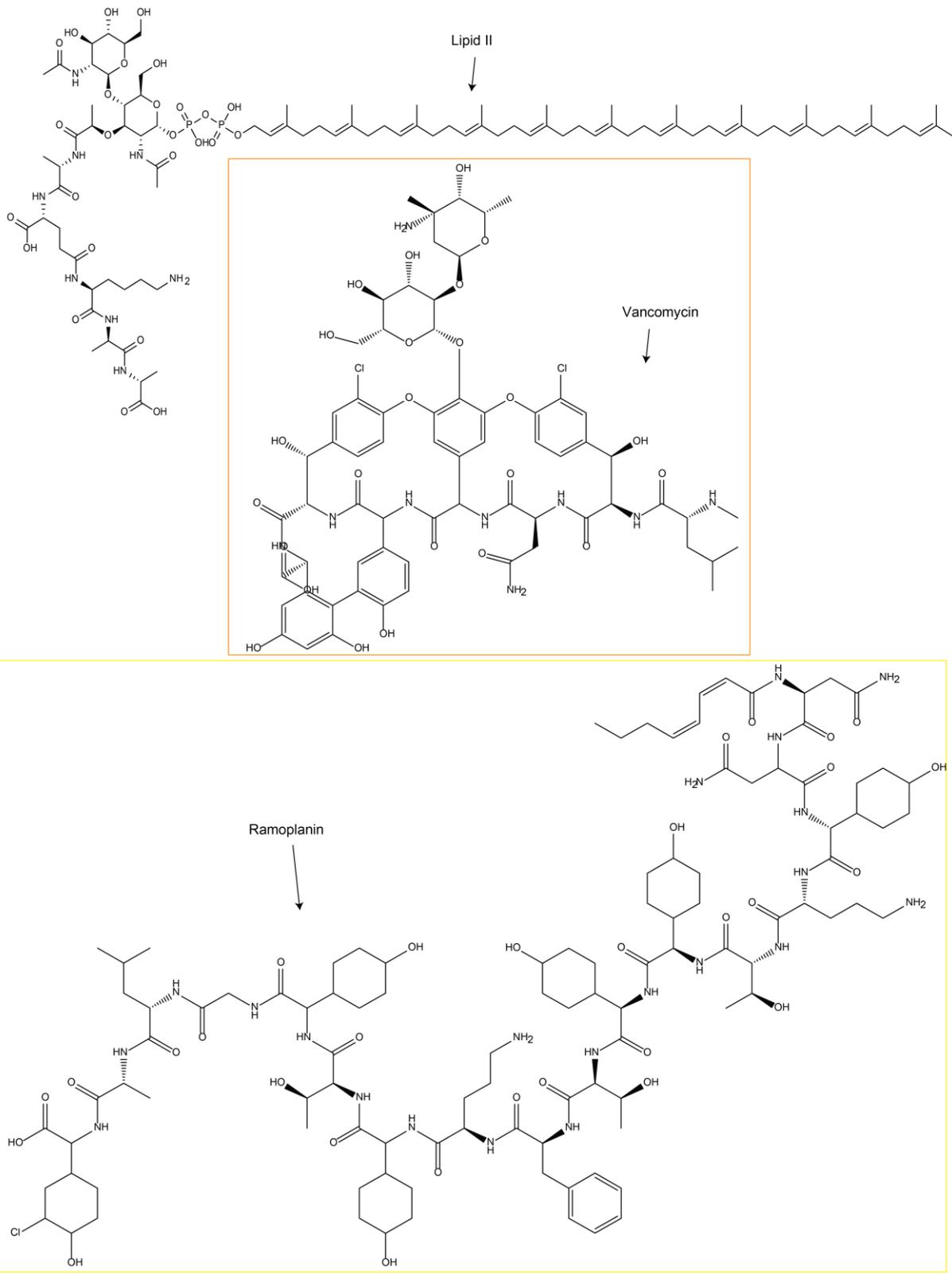


**Supplementary Fig. 3: Lipid II binding to MurJ is independent of the detergent used and is specific.** Mass spectra of MurJ and lipid II in 0.02% DDM (a) and 0.12% OGNG (b). Similar level of interaction between MurJ and lipid II (5  $\mu$ M and 10  $\mu$ M) is observed in these two detergents, suggesting that lipid II binding is independent of the detergent used. (c) Mass spectrum of *Pyrococcus furiosus* MATE

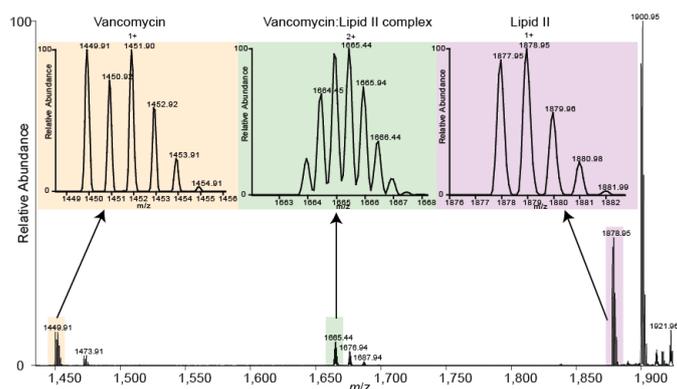
(5  $\mu\text{M}$ ) with lipid II (20  $\mu\text{M}$ ) in 0.05% LDAO. (d) Mass spectrum of MurJ with C55-P (30  $\mu\text{M}$ ) in 0.05% LDAO. No binding of C55-P was observed.



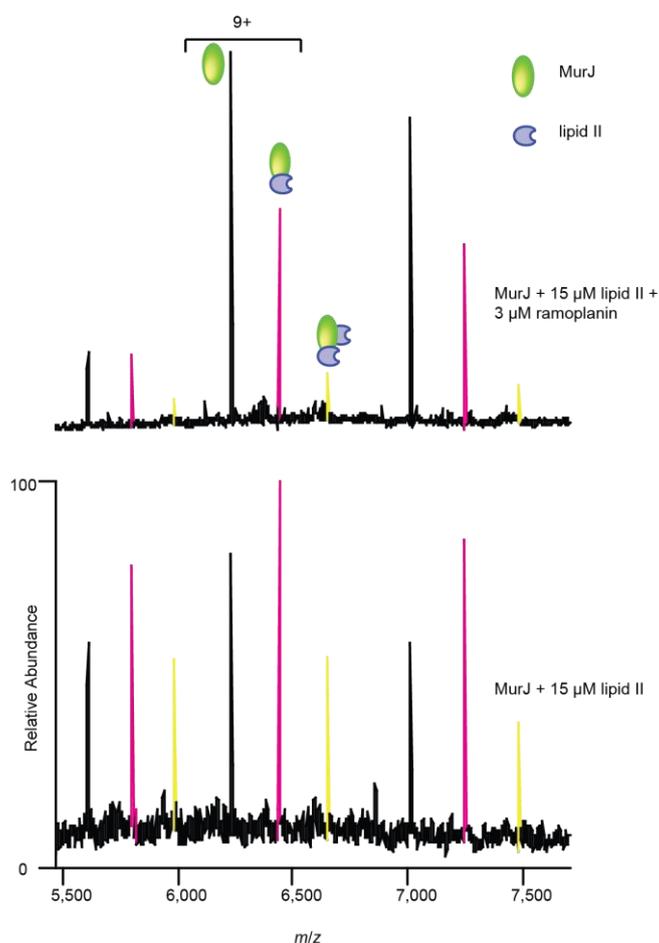
**Supplementary Fig. 4: Lipid II binding to MurJ is sensitive to change in pH.** (a) Mass spectra of MurJ:lipid II complex acquired at different solutions pHs and sodium ion concentrations. At pH 5 and 9, less lipid II binding to MurJ is observed compared to pH 6, 7 or 8. (b) Mass spectra of the MurJ:lipid II complex at various sodium chloride concentrations. The sodium ion concentration does not affect lipid II binding to MurJ. All experiments are repeated three times. Standard deviations were calculated from the four observed charge states in three independent experiments.



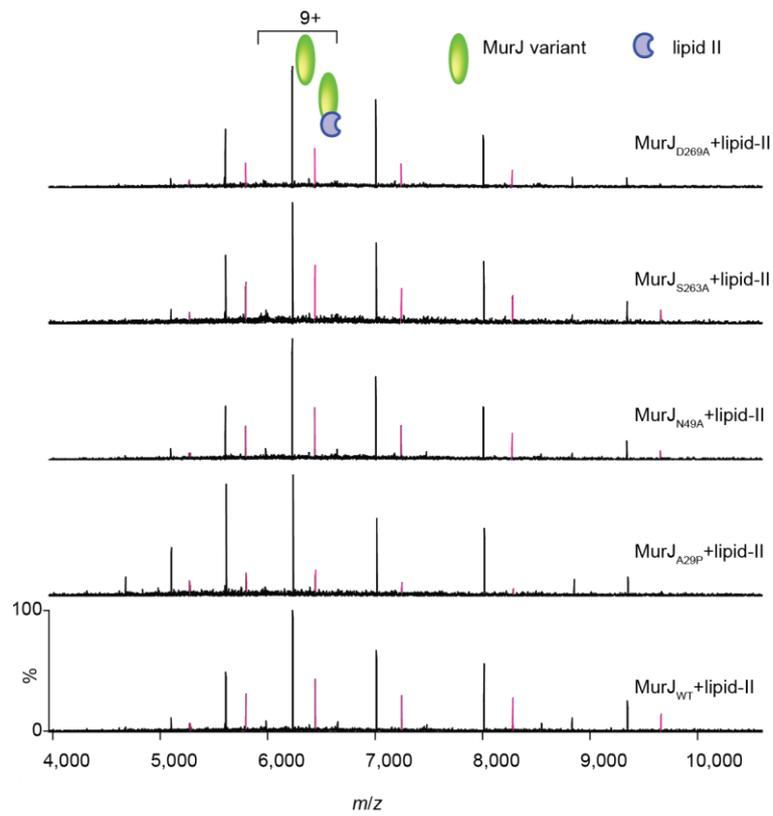
Supplementary Fig. 5: Chemical structures of lipid II, vancomycin, and ramoplanin.



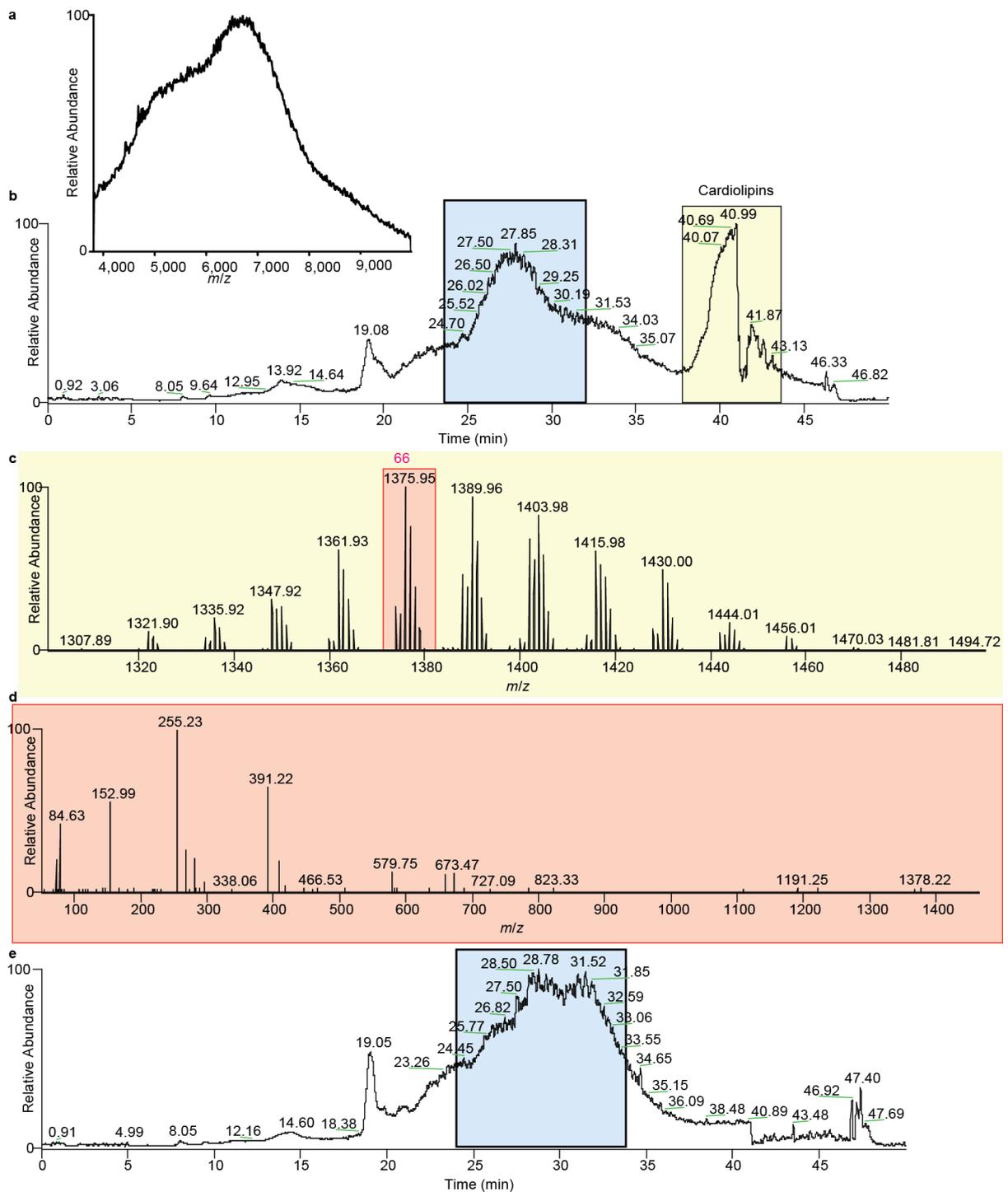
**Supplementary Fig. 6: Mass spectrum of lipid II (5  $\mu\text{M}$ ) with vancomycin (5  $\mu\text{M}$ ).** Vancomycin:lipid II complex is observed as doubly charged (green), while vancomycin (orange) and lipid II (pink) are observed singly charged. This suggests that the stoichiometry of vancomycin and lipid II interaction is 1:1.



**Supplementary Fig. 7: Mass spectra of the MurJ:lipid II complex at high concentrations of lipid II (15  $\mu\text{M}$  – a three-fold excess over the protein MurJ) to probe the effects of ramoplanin.** Mass spectra recorded under these conditions reveal binding of one and two lipid II molecules (pink and yellow respectively). In presence of ramoplanin (top panel) a reduction in lipid II binding is indicated for both peaks confirming that ramoplanin competes with MurJ for lipid II binding.

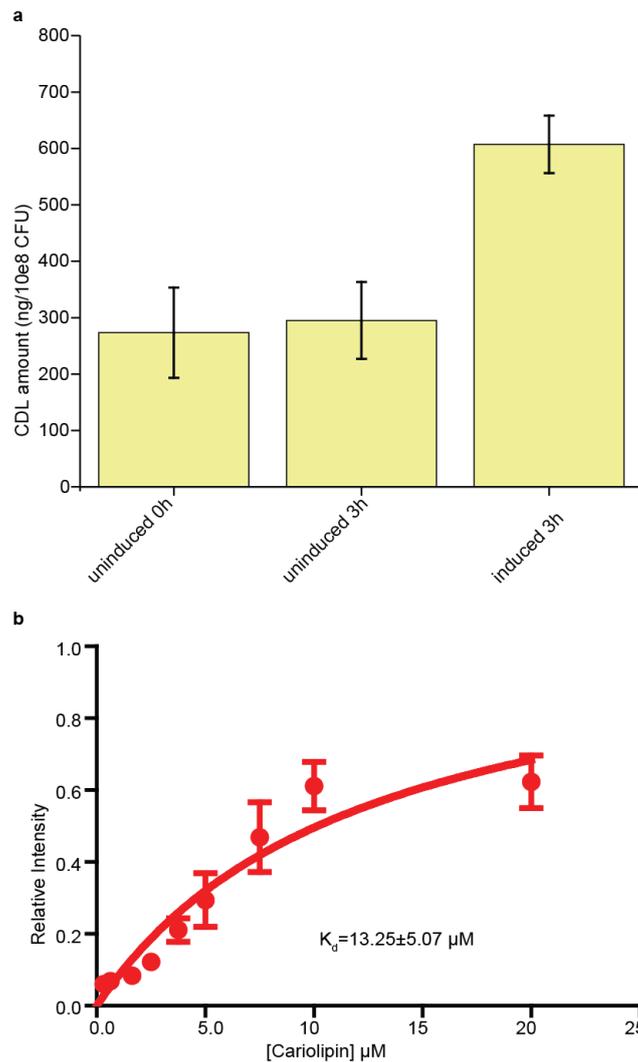


**Supplementary Fig. 8: Comparison of the mass spectra of MurJ variant with wild type in the presence of lipid II.** Comparing the extent of binding indicates that the effect of mutation is greatest for MurJ<sub>A29P</sub> > MurJ<sub>D269A</sub> > MurJ<sub>N49A</sub> ≈ MurJ<sub>S263A</sub> ≈ WT.

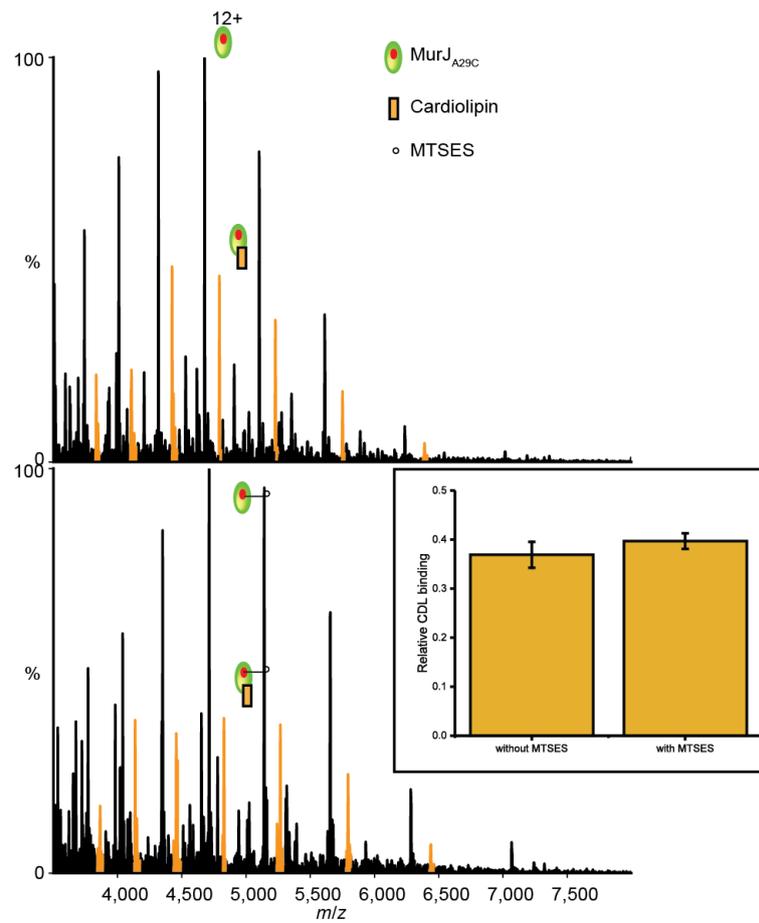


**Supplementary Fig. 9: Mass spectra of MurJ without delipidation (proteins extracted using DDM from membranes) and lipidomics experiments to identify bound lipids.** (a) Without delipidation mass spectrum could not be resolved due to the presence of endogenous lipids. (b) Total ion chromatogram of the MurJ preparation following separation on C18 column. The retention time of the highlighted light blue region corresponds to phospholipids and light yellow region corresponds to cardiolipins. (c) Mass spectrum recorded for the cardiolipin fraction highlighted (light yellow) in (b), showing the distribution of acyl carbon chain lengths in the cardiolipins ranging from 62 to 72. (d) Fragmentation pattern of the ion 1375.95 *m/z* (highlighted in red box, with chain length of 66) (e)

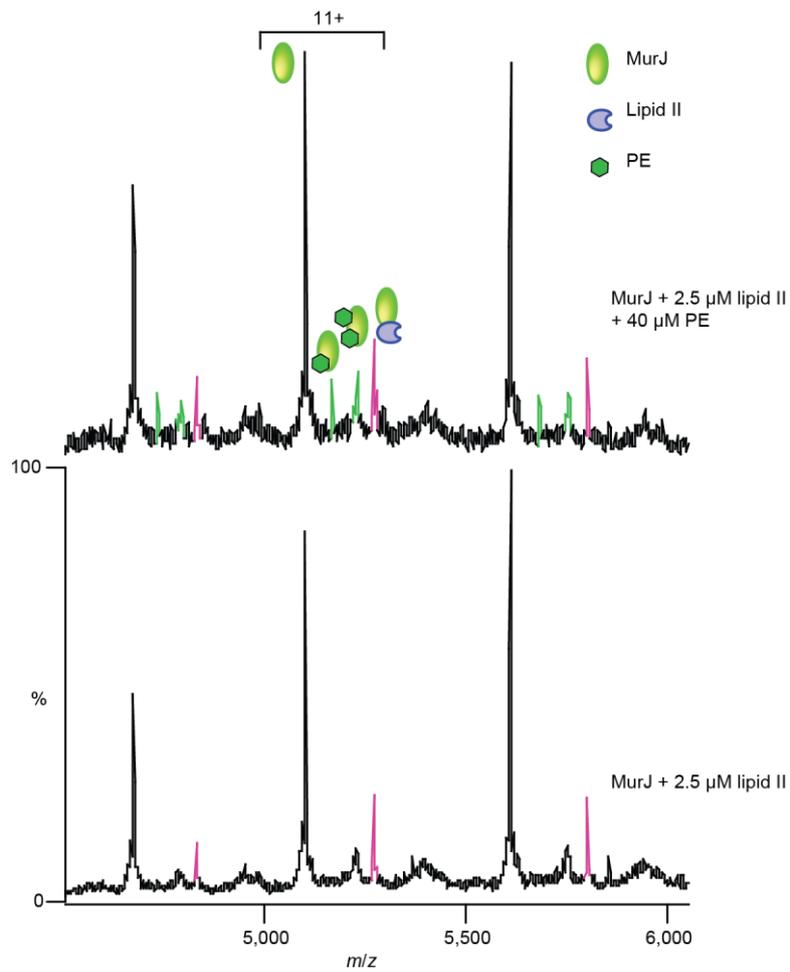
Lipidomics analysis of a non-delipidated FtsW preparation, indicating the presence of phospholipids (highlighted in light blue) and no cardiolipins.



**Supplementary Fig. 10: Cardiolipin quantification and the binding isotherm.** (a). Quantification of cellular levels of cardiolipins. When protein expression was induced a large increase in the amount of cardiolipins was observed (column 3 compared 1 and 2). (b) Binding isotherm of cardiolipins to MurJ (measured in the same way as for lipid II binding), indicating a micromolar range  $K_d$ . All experiments are repeated three times. Standard deviations were calculated from the four observed charge states in three independent experiments.



**Supplementary Fig. 11: Cardiolipin offers allosteric control to lipid II binding to MurJ.** Mass spectra of MurJ<sub>A29C</sub> variant with cardiolipin (top panel) and MTSES treated MurJ<sub>A29C</sub> with cardiolipin (bottom panel). Similar amounts of CDL binding is observed with and without MTSES (inset at the bottom). This suggests that cardiolipin may offer allosteric control to lipid II binding (see **Figure 6**).



**Supplementary Fig. 12: Effect of phospholipids (PE) on MurJ:lipid II complex.** No change in lipid II binding (relative intensity of bound vs unbound) is observed even in presence of 16-fold molar excess of phospholipid, indicating that PE does not compete with lipid II for MurJ binding sites.

### 3. Supplementary Tables

**Supplementary Table 1: Summary constructs used and their expression levels.** ▲ Indicates protein expression while ▲ indicates poor to no expression. Note that C-terminal 6×His constructs were derived from C-terminal GFP constructs by using SLIM protocol.

Constructs used in this study	Primers	Expression level
MurJ <sub>WT</sub> (C-terminal GFP tag)	5' AAGGAGATATACATATGATGAATTTATTTAAAATCGCTGG 3' 3' GCCCGCCGGACGGTGTAAGCTAGCGGTGAAAACC 5'	▲▲
MurJ <sub>WT</sub> (C-terminal 6×His tag)	5'GAATTTGCCCGCCGGACGGTGCAACCACCACCACCACCTG 3' 3'GGCTTCAAAGTTAAAGAATTTGCCCGCCGGACGGTG 5'	▲▲▲▲
MurJ <sub>WT</sub> (N-terminal 6×His tag)	5' GTATTTTCAGGGATCCATGAATTTATTTAAAATCGCTGG 3' 3' GCCCGCCGGACGGTGTAAGCTAGCGGTGAAAACC 5'	▲
FtsW (C-terminal GFP tag)	5' AAGGAGATATACATATGCGTTTATCTCTCCCTCGC 3' 3' GTTTGTACGAGGTTACGAGCTAGCGGTGAAAACC 5'	▲
FtsW (C-terminal 6×His tag)	5'GCGTTTGTACGAGGTTACGACACCACCACCACCACCTG 3' 3' CGTCTGGAGAAAGCGCAGGCGTTTGTACGAGGTTACGA 5'	▲
FtsW (N-terminal 6×His)	5' GTATTTTCAGGGATCCCCTTTATCTCTCCCTCGC 3' 3' GTTTGTACGAGGTTACGACTCGAGCACCACCACC 5'	▲▲
MurJ <sub>A29C</sub>	5'GCAATTGTCTGCAGAATCTTTGGCGCAGGGATGGCAAC 3' 3' CTTGGCTTCGCACGAGACGCAATTGTCTGCAGAATCTT 5'	▲▲▲▲
MurJ <sub>A29P</sub>	5'GCAATTGTCCCAGAATCTTTGGCGCAGGGATGGCAAC 3' 3'CTTGGCTTCGCACGAGACGCAATTGTCCCAGAATCTT 5'	▲▲▲▲
MurJ <sub>N49A</sub>	5'AAACTTCCTGCCTTGTTACGCCGTATCTTTGCCGAAGG 3' 3'CCTTTTTCGTCGCTTTTAAACTTCCTGCCTTGTTACG 5'	▲▲▲▲
MurJ <sub>S263A</sub>	5'GGTTCGGTGGCTTGGATGTATTACGCCGACCGCTTAATG 3' 3'GCCTCGTTTCTTGCTCCGGTTCGGTGGCTTGGATGTA 5'	▲▲▲▲
MurJ <sub>D269A</sub>	5'GTATTACGCCGCCGCTTAATGGAGTTTCCGTCGGTG 3' 3'CGGTTTCGGTGTCTTGGATGTATTACGCCGCCGCTTAAT 5'	▲▲▲▲

**Supplementary Table 2: Summary of final concentrations of proteins and lipid II used in the various experiments presented in the different figures shown in this study.**

Proteins used in this study	[protein] μM	[lipid II] μM	Figures
MurJ <sub>WT</sub>	5	5	2, S4
FtsW	5	10	2
MurJ <sub>WT</sub>	5	0, 2.5, 5.0, 7.5, 10, 15, 20, 25	3
MurJ <sub>WT</sub>	5	3	4, 5, 6, S3, S8, S12
MurJ <sub>A29C</sub>	5	3	4, S8, S11
MurJ <sub>A29P</sub>	5	3	4, S8
MurJ <sub>N49A</sub>	5	3	4, S8
MurJ <sub>S263A</sub>	5	3	4, S8
MurJ <sub>D269A</sub>	5	3	4, S8

### 4. Supplementary References

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