The mechanism of action of lysobactin

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Supporting information

Materials and general methods

Heptaprenyl MurNAc pyrophosphate (MurNac-C35), Lys-Lipid I, Lys-Lipid II, and BDL were prepared as previously described.[1] Fmoc-D-Lys(biotinyl)-OH was purchased from VWR. Acetyl-D-Lys-D-Ala-D-Ala was purchased from Sigma Aldrich. Human blood was purchased from Innovative Research. All other chemicals were purchased from Sigma Aldrich unless otherwise stated. Prism (GraphPad) software was used for data analysis and curve fitting. *Staphylococcus aureus* was primarily maintained in tryptic soy broth (BD Bioscience) medium at 30℃.

Lysobactin purification

Lysobacter enzymogenes strain Lyso16, a producer of lysobactin was obtained from DSMG (German Type Culture Collection: DSM2043), used for the experiments has been described elsewhere.[2] For cultivation in liquid culture, medium CY with the following composition was used: casitone (Bacto Casitone, Becton Dickinson, USA) 0.3%(w/v); yeast extract (Fould Springer, France) 0.1% (w/v); $CaCl_2 \cdot 2H_2O$ (Roth, Karlsruhe) 0.1% (w/v); HEPES (Roth, Karlsruhe) 1.19% (w/v). The medium was adjusted to pH 7.2 with KOH. The strain LYSO16 was grown in 10 x 100 mL medium CY in 250 mL Erlenmeyer flasks in resin-free supernatend for 2 days at 30°C and 160 rpm. Afterwards, 20% of the precultures were transferred into sterile 10 x 200 mL medium CY in 2 L Erlenmeyer flasks containing respectively 2% adsorber resin (Amberlite XAD16, Rohm and Haas, Frankfurt) (v/v). After 6 days of cultivation the adsorber resin was harvested and extracted exhaustively with 30% aquous methanol and subsequently with pure methanol. The lysobactin containing extract were evaporated to dryness, desolved in ethyl acetate and partitioned with water. Between the organic and the aqueous phase we found an intermediate phase mainly containing lysobactin. Afterwards the remaining aqueous phase was filtered through a RP cartridge and then eluted with methanol. These two lysobactin containing extracts were combined and filtered over an open column filled with 2.2g silica gel (Si 100, 63-200µm, Merck, Darmstadt). Further purification by means of a dichloromethane /methanol gradient gave a 101 mg fraction containing lysobactin. As a final step we used a chromatography in two runs on a reversed phase column (Gemini C18 10µm 110A, 250x21 mm, Phenomenex, Aschaffenburg) flow rate 20 mL/min. eluent: acetonitrile / water gradient (A: 100% water + 0.5% formic acid, B: 100% acetonitrile + 0.5% formic acid, 25 %B →10 min. 25%B →33 min. up to 40 %B). Lysobactin eluted at approximately 12 min. In summary the freeze dryed lysobactin fraction gave 2.1 mg.

Determination of minimum inhibitory concentration (MIC) of lysobactin against *S. aureus, M. smegmatis, M. tuberculosis*

The MIC against *S. aureus* HG003 was determined using a 150 µL culture of *S. aureus* HG003 at OD_{600}=0.1 in tryptic soy broth (TSB) media and 1.5 µL of a serial dilution of lysobactin in a clear 96-well plate (Corning). The plate was shaken at

30 ℃ and the OD_{600} reading was taken after incubation for 18 hours (OD_{600} measurements were taken on Spectra Max plus384, Molecular Devices). The concentration of lysobactin that showed no bacterial growth was designated the MIC. Similarly, to determine MICs against *Mycobaceterium smegmatis*, a 200 μL culture of *M. smegmatis* MC2 155 at OD_{600}=0.001 in Middlebrook 7H9 supplemented ADC (albumin, dextrose, catalase) and 2 μL of a serial dilutions of lysobactin was added to a 96-well-plate. The plate was incubated at 37℃ for 60 hours with shaking. The MIC was determined as described above for *S. aureus*. To determine the MIC against *M. tuberculosis*, a colorimetric Alamar Blue assay was used as described.[3] A culture of *M. tuberculosis* H37Rv was grown in Middle brook 7H9 broth containing 0.2% glycerol, 10% OADC (oleic acid, albumin, dextrose, catalase) and 0.05% Tween 80 until it reached OD_{600} 0.6. Bacterial cultures were then diluted to 0.003 of OD_{600} in 200 μL 7H9 OADC and 2 μL of a serial dilution of lysobactin in a 96-well plate. Following a 5 day incubation at 37℃, 20μL of 0.02% Resazurin (Alamar Blue) was added to each well. After 24 hours incubation at 37℃, the MIC was determined as the lowest concentration at which the reagent color changed.

Bactericidal assessment

An overnight culture of *S. aureus* HG003 was grown at 37℃. Cells were diluted to OD_{600}=0.1 in TBS media and further incubated at 37℃ until the cultures reached exponential phase (OD_{600}=0.5) or stationary phase (OD_{600}=1.0). To each 2 mL cultures, antibiotics at 2X MIC values (2 μg/mL vancomycin, 1.5 μg/mL lysobactin, and 2 μg/mL ramoplanin) were added. Bacterial viability was monitored by cfu counting hourly for 8 hours total. Experiments were carried with biological replicates.

Antagonism assay with exogenous cell wall substrates

The antibacterial activity of lysobactin was evaluated with the addition of exogenous cell wall precursors using a similar setup to the MIC assays above. A series dilution of lysobactin was mixed with potential antagonists at 5 μM: Acetyl-D-Lys-D-Ala-D-Ala (CAS #28845-97-8), Lys-Lipid I-C35, and heptaprenyl MurNAc pyrophosphate (MurNac-C35). *S. aureus* HG003 at OD_{600}=0.001 in TSB were added to 96-well plates with the above mixtures. The plates were incubated and shaked at 30℃ incubator for 18 hours. The bacterial growth was measured by OD_{600} and shifts in MICs were evaluated.

Electron microscopy imaging of *S. aureus* treated with lysobactin and ramoplanin

An overnight culture of *S. aureus* HG003 grown at 30℃ was diluted to OD_{600}=0.1 in fresh TSB and grown to OD_{600}=0.5. Cultures were then treated with antibiotics (1.5 μg/mL lysobactin and 2 μg/mL ramoplanin) for 30 minutes. Bacterial cells were prepared for EM as described.[4] Cells were fixed by adding a mixture of 1.25% (wt/vol) formaldehyde, 2.5% (wt/vol) glutaraldehyde, and 0.03% picric acid in 0.1M sodium cacodylate buffer (pH 7.4). Fixed cells were washed 3 times in sodium cacodylate buffer followed by incubation in 1% OsO_4/1.5% (wt/vol) $K_4Fe(CN)_6$ in H_2O for 1 hour. Cells were washed three times with H_2O and dehydration in ethanol. Cells were treated with propylenoxide for 1 hour, followed by 1:1

epon:propylene oxide for 2 hours. Cells were then embedded in Epon and sectioned. Finally, cells were stained with uranyl acetate, and images were taken using an electron microscopy (JEOL 1200EX-80kv, Harvard Medical School EM facility).

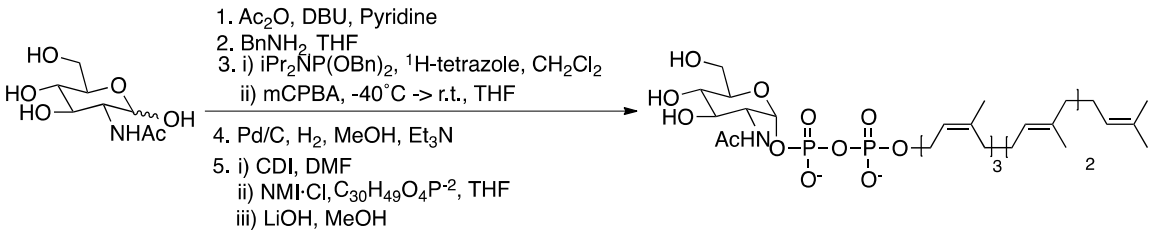
Assays to determine in vitro enzyme inhibitory activity of lysobactin

MurG: Lys-Lipid I was chemo-enzymatically prepared as previously described.[1b] MurG was prepared and enzyme kinetics was evaluated as described.[5] Lys-Lipid I, a 2x molar excess of N-Acetyl-D-[1-^{14}C]-glucosamine (0.2mCi/mL), 4 μM lysobactin, and MurG reaction buffer (50mM HEPES pH7.9, 5mM $MgCl_2$) was briefly incubated. MurG (0.5 mg/mL) was added to initiate the reaction. Reactions were stopped after 60 minutes by adding ice cold 180 μL H_2O. Control reactions were set-up with DMSO replacing the lysobactin. Products from the reaction were separated using pre-equilibrated Bakerbond^{TM}speOctadecyl Extraction Columns (J. T. Bakers) as described, and each fraction was quantified using a scintillation counter (LS650 Beckman). Product formation was calculated by comparing radioactivity from purified product fractions to the total amount of radioactivity on the reaction. A curve fit to the kinetic data assuming a 1:1 binding stoichemestry and using the following equation for substrate depletion.[6]

$$rate = \text{Vmax} \times \left(\frac{((0.5 \times [\text{I}] - [\text{S}] + Kd)^2 + 4 \times Kd \times [\text{S}])^{0.5} - (0.5 \times [\text{I}] - [\text{S}] + Kd))}{2 \times (\text{Km} + \frac{((0.5 \times [\text{I}] - [\text{S}] + Kd)^2 + 4 \times Kd \times [\text{S}])^{0.5} - (0.5 \times [\text{I}] - [\text{S}] + Kd))}{2}} \right)$$

SgtB: The [^{14}C]GlcNAc-labeled heptaprenyl Lipid II analogue was chemo-enzymatically prepared as described.[7] *S. aureus* mono-transglycosylase, SgtB, was purified and used in a polymerization assay as previously described.[8] [^{14}C]GlcNAc-labeled heptaprenyl Lipid II (Specific activity= 10^6cpm/pmol), 4 or 8 μM lysobactin, and SgtB reaction buffer (12.5mM HEPES pH7.5, 2 mM $MnCl_2$, and 0.25mM Tween-80) was briefly incubated before the addition of SgtB (30 nM). Control reactions were set-up with DMSO replacing the lysobactin. Reactions were quenched after 30 minutes with 10 μL ice cold methanol, and subjected to paper chromatography (3MM Whatman) in isobutyric acid : 1N NH_4OH =5:3. The amount of starting material and polymerized product was measured using a scintillation counter (LS650 Beckman). Reaction rates were calculated by comparing radioactivity from the polymerized product to the total amount of radioactivity on the strip. A curve fit to the kinetic data assuming a 1:1 binding stoichemestry and using the equation used above in MurG kinetics for substrate depletion.

TagB: *Preparation of compounds 1b and [^{14}C]-CDP-glycerol for TagB enzymatic reactions:* Hexaprenyl GlcNac pyrophosphate 2 (4 mg) was prepared previously described synthetic routes.[1b,9] UDP-ManNac, purified TagA, and hexaprenyl pyrophosphate were used as we previously described to prepare TagB substrate, Lipid II_A^{WTA}.[9-10] [^{14}C]-CDP-glycerol was prepared as previously described using purified TagD and [^{14}C]-Glycerol-3-phosphate (specific activity=150 mCi/mmol).[10]



TagB reaction kinetics with ramoplanin and lysobactin: TagB was purified as previously described.[9-10] Lipid II_A^{WTA} was incubated with a 4X molar excess of [^{14}C]-CDP-glycerol (ranging from 4-80 μM), buffer (20 mM Tris (pH 8), 100 mM NaCl, 10 mM $MgCl_2$), and 4 μM of Ramoplanin or Lysobactin. TagB (10 nM) was used to initiate the reactions. Control reactions were set-up with DMSO replacing the antibiotic. TagB reactions were directly quenched with methanol following 5 minute incubation. Material was loaded onto a Bakerbond^{TM}spe Octadecyl Extraction Column. Excess [^{14}C]-CDP-glycerol was removed in four consecutive washes of 500 μl H_2O with 0.1% NH_4OH. TagB product was eluted with 2X 500 μl of MeOH with 0.1% NH_4OH. Radioactivity was quantified using a scintillation counter (LS650 Beckman). A curve fit to the kinetic data assuming a 1:1 binding stoichemetry for lysobactin (and 2:1 in the case of ramoplanin) using the equation used above in MurG kinetics for substrate depletion.

Bacterial lipid extraction and detection by western blot

The bacterial cellular lipids were extracted as described.[1a] Overnight cultures of *S. aureus* RN4220 were re-inoculated to an OD_{600} 0.1 and grown to exponential phase (OD_{600}= 0.4~0.5) at 37℃. 2mL of the culture were then treated with the following antibiotics for 10min at 37℃: 0.3 μg/mL moenomycin, 4 μg/mL vancomycin, 2 μg/mL targocil, 500 μg/mL bacitracin, 2 μg /mL ramoplanin, 2 (or 4) μg/mL CDFI, 16 (or 32) μg/mL DMPI. After normalizing biomass based on OD_{600}, cells were harvested immediately by centrifugation. Cell pellets were resuspended in 300 μL PBS (pH 7.5 for all cultures treated with antibiotics except ramoplanin; pH 12 for ramoplanin treated culture), and 1.5 mL of 2:1 methanol:chloroform. The resuspended mixture was vortexed repetitively for 10 minutes at room temperature. Mixtures were subsequently centrifuged for 10 minutes at 4000 x g at room temperature. The supernatant was transferred to a new glass tube and mixed with 500 μL $CHCl_3$ and 500 μLPBS (pH 7.5-12 as described above). Following 10 minutes vortexing at room temperature, the mixture was centrifuged for 10min at 4000 x g to separate the organic and aqueous layers. The organic layer was collected, dried *in vacuo*, and then were resuspended in 20μL DMSO.

Cellular Lipid II in the lipid extract was PBP4-labeled with biotin-D-lys(BDL) as described.[1a] In a total reaction volume of 10 μL, 2 μL of the lipid extracts was incubated with 10 μM of *S. aureus* PBP4 and 4mM BDL in the PBP4 reaction buffer (12.5mM HEPES pH7.5, 20mM $MnCl_2$, and 2.5mM Tween-80) for 60 minutes at room temperature. Reactions were stopped by adding equal amount of 2x SDS loading buffer, and then resolved on a 4-20% gradient SDS polyacrylamide gel. To detect BDL-labeled Lipid II, the products were transferred to immunoblot PVDF membrane (BioRad) and blotted with

streptavidin-HRP (1:10000 dilution, Pierce). Labeled Lipid II was visualized using ECL Prime Western Blotting Detection Reagent (GE Ambersham) and Biomax Light Film (Kodak).

Red blood cell (RBC) lysis

As previously described,[11] hemolytic activity of ramoplanin and lysobactin was measured using red blood cells (RBCs). Defibrinated human blood was washed 3 times with buffer (10mM Tris-HCl pH7.4, 0.9% NaCl), and diluted to a final concentration of 5% RBCs. 60 µL of the RBCs was added to 240 µL buffer with various concentrations of ramoplanin and incubated for 30 minutes at room temperature. Following centrifugation at 1300 x g for 5 min, 250 µL of the supernatant was transferred into 96 well plates and the released hemoglobin was measured at OD_{540} using a plate reader (SpectraMax, Molecular Dyanmics). H_2O was used as a positive control for 100% lysis of RBCs.

Supplementary figures

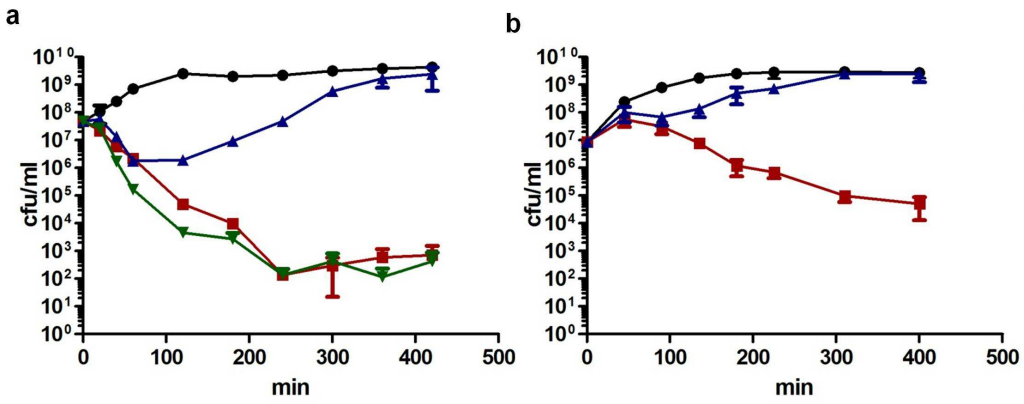


Figure S1. The growth of *S. aureus* treated with lysobactin is compared to ramoplanin treatment. (a) *S. aureus* cultures in log phase (OD_{600}=0.5) and (b) stationary phase (OD_{600}=1.0) were diluted to OD_{600}=0.5 and treated with 2x MIC: 1.5μg/mL lysobactin(red), 2 μg/mL vancomycin(blue), 2 μg /mL ramoplanin(green), no treatment(black). For the comparison of killing curves side by side, data in Figure 3b was used in panel a. Viable cells were monitored by CFU assay for 8 hours. Graph points are determined from 2 independent experiments, each performed in triplicate (± standard deviation).

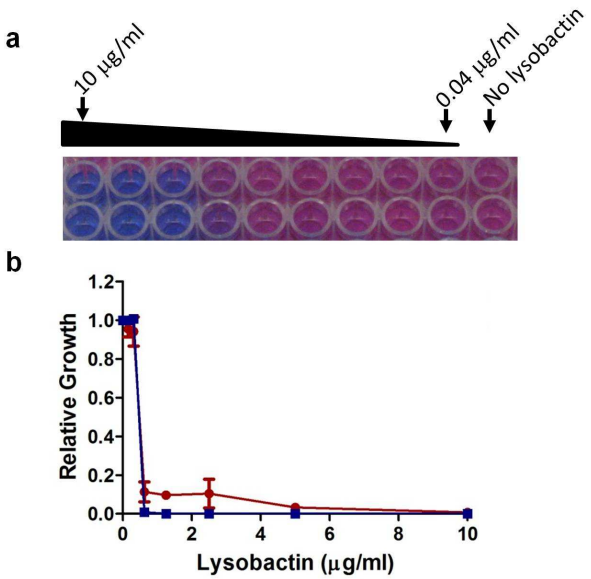


Figure S2. Lysobactin inhibits mycobacterial growth. (a) *M. tuberculosis* H37Rv culture was inoculated with a series dilution of lysobactin ranging from 10µg/mL to 0.04 µg/mL. After a 5 day incubation at 37 ℃, bacterial growth was determined by using resarzurin. A color change after 24 hours from blue to pink is indicative of bacterial viability. Each row represents independent replicates. (b) Parallel MICs of lysobactin against *S. aureus* (blue) and *M. smegmatis* (red) are represented. The MIC of lysobactin against *M. smegmatis* MC2 was determined using a 96-well plate assay. *M. smegmatis* MC2 culture was treated with a series dilution of lysobactin in the range from 10µg/mL to 0.15 µg/mL. After 60 hours incubation at 37 ℃, bacterial growth was measured using OD_{600} and compared to untreated culture. The graph points represent two biological replicates.

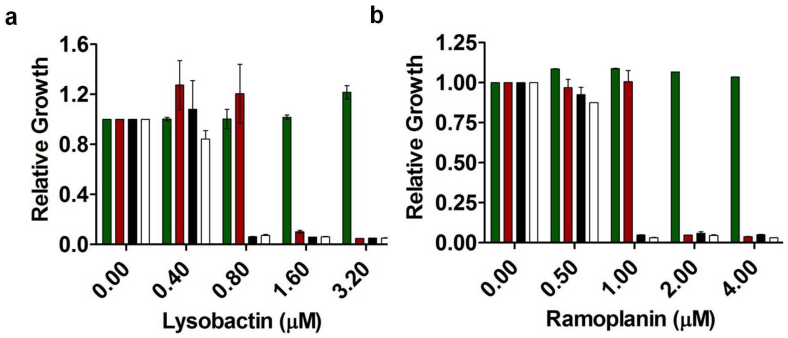


Figure S3. Lys-Lipid I-C35 and MurNAc-C35 antagonize lysobactin and ramoplanin. Compared to addition of DMSO (white), addition of 5μM exogenous Lys-Lipid I-C35 (green) or 5 μM MurNAc-C35 (red) protected *S. aureus* from lysobactin (a) and ramoplanin (b). 5μM Acetyl-D-Lys-D-Ala-D-Ala (black) had no effect on cultures treated with lysobactin and ramoplanin. Bacterial growth was measured following 18 hours of treatment. Relative growth is determined as the percentage of OD_{600} following treatment compared to the OD_{600} of untreated cultures. Each experiment was performed in biological replicates.

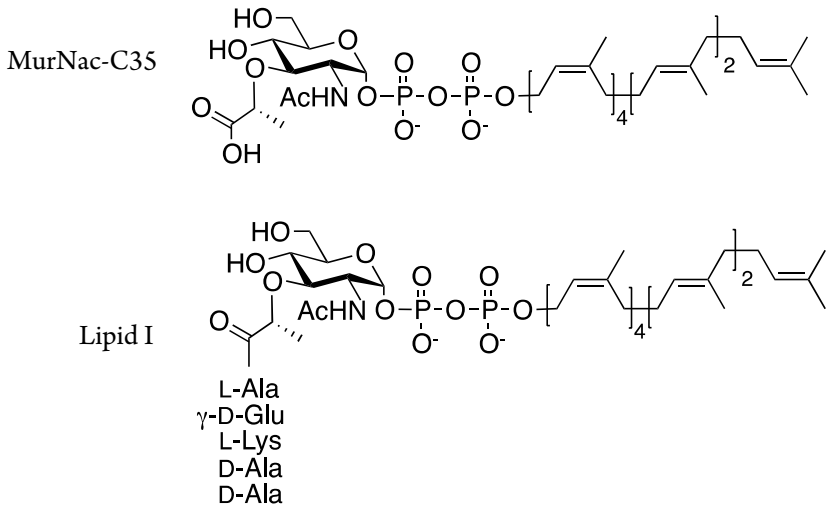


Figure S4. Structures of Lipid I-C35 and MurNac-C35

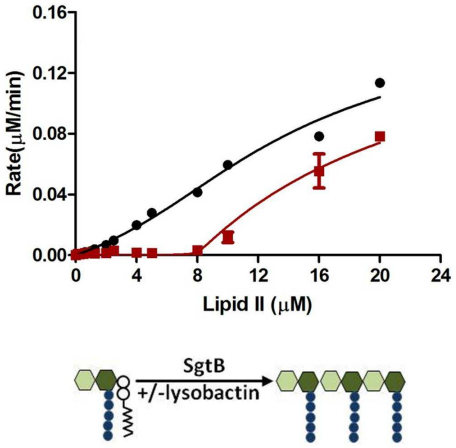


Figure S5. Lysobactin inhibits SgtB by binding Lipid II. Kinetics of Lipid II polymerization (black) by SgtB and its inhibition using 8μM lysobactin (red) show that lysobactin binds Lipid II with a stoichiometry of 1:1.

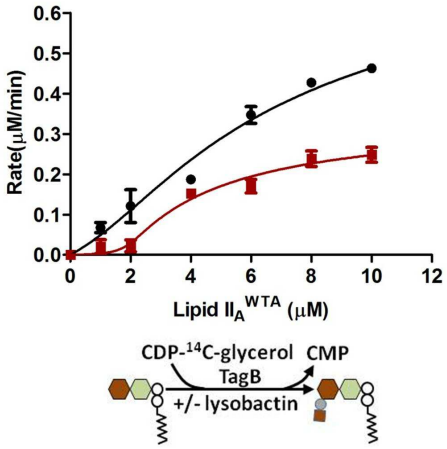


Figure S6. Ramoplanin inhibits TagB by binding Lipid II $_A^{WTA}$. Kinetics of Lipid II$_B^{WTA}$ formation (black) by TagB and its inhibition by 4μM ramoplanin (red) show that ramoplanin binds Lipid II $_A^{WTA}$ with a stoichiometry of 2:1.

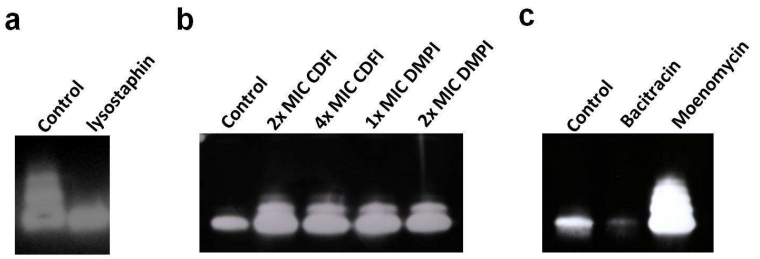


Figure S7. Cellular Lipid II levels following antibiotic treatment of *S. aureus* cultures. (a) Accumulation of Lipid II levels leads to multiple western blot bands corresponding to cross-linked Lipid II. Accumulation of Lipid II following moenomycin treatment showed multiple bands in the western blot analysis (control). Treatment of lysostaphin for 1 hour following BDL-labeling of a replicate sample showed only one band, indicating that that the multiple bands are a result of PBP4-crosslinking and PBP4-labeling with BDL. (b) Lipid II extracted from *S. aureus* RN4220 cells after treatment with CDFI and DMPI at 1-4X MIC values. (c) Lipid II levels extracted from cells and PBP4-labeled with BDL for cultures not treated with antibiotic (control), treated with bacitracin, and treated with moenomycin.

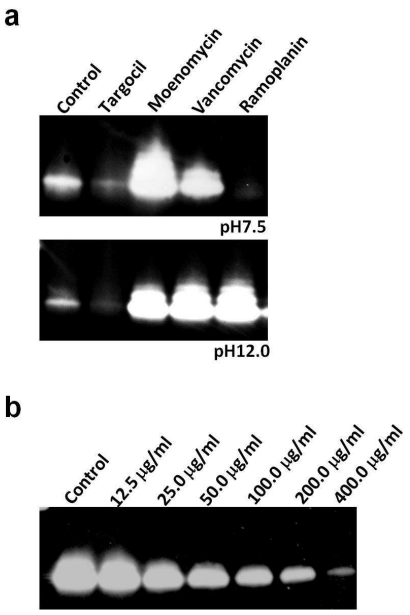
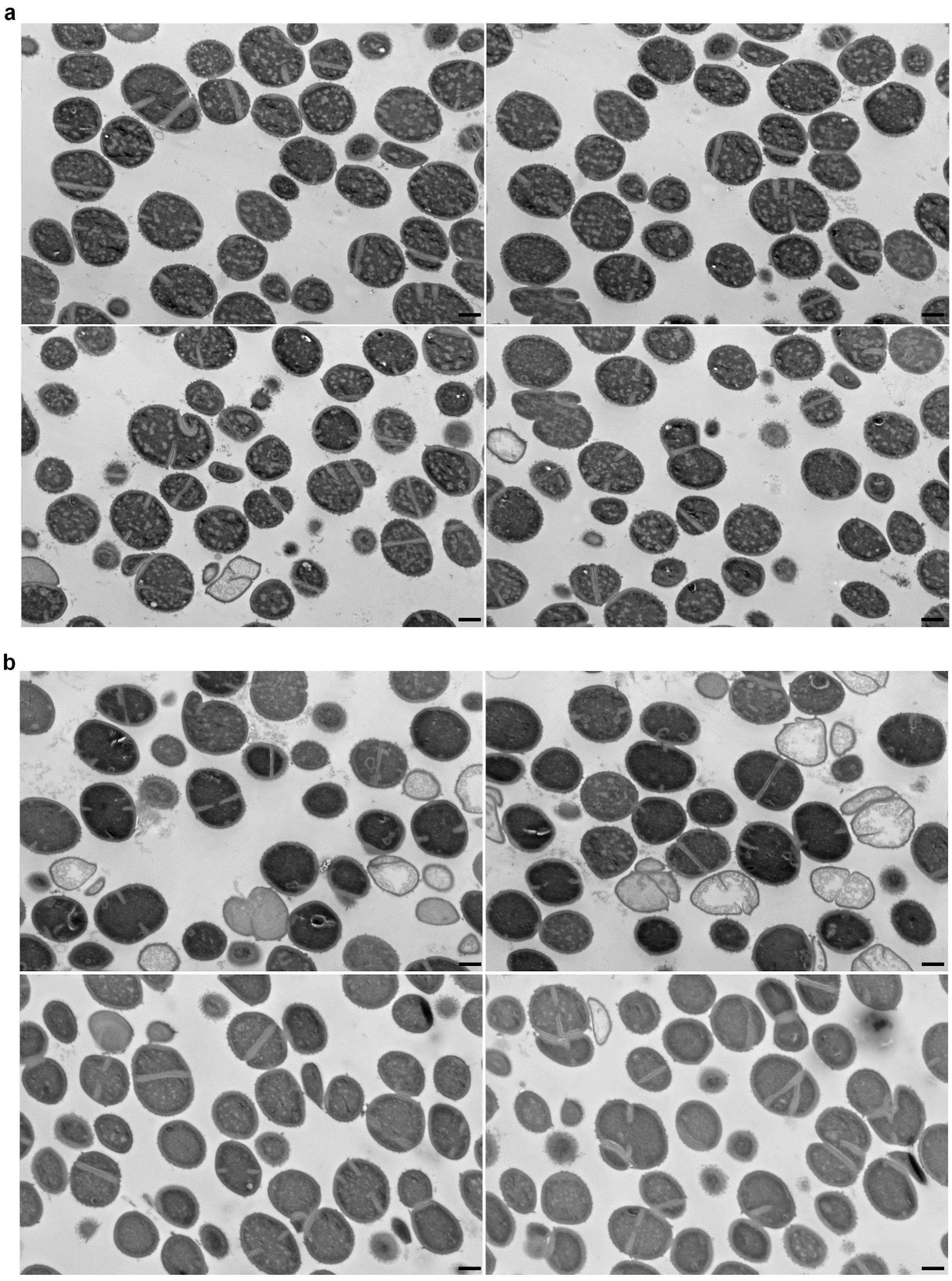


Figure S8. Lipid II extraction is pH dependent for ramoplanin. (a) At pH 12, an accumulation of Lipid II was observed in the organic phase, due to release of the Lipid II from the ramoplanin:Lipid II complex. For cultures treated with other antibiotics (targocil, moenomycin, vancomycin), extraction with PBS (pH 12) appeared no significant difference in Lipid II levels collected from the organic phase relative to extraction with PBS (pH 7.5). (b) Cellular Lipid II was extracted from 100 mL of *S. aureus* RN4220 treated with 0.3 μg/mL moenomycin treatment for 10 minutes. 2μLof the extracted lipids was mixed with 100 μL PBS (pH 7.5) containing ramoplanin at concentrations ranging from 12.5 μg/mL to 400 μg/mL. Control indicates no ramplanin addition. Following a 30 minute incubation at room temperature, the mixture was re-extracted and the Lipid II collected from the organic phase was labeled with BDL as described above. At pH 7.5, addition of increasing amounts of ramoplanin causes complex ramoplanin:Lipid II formation in the aqueous phase that results in increasing depletion of Lipid II upon re-extraction.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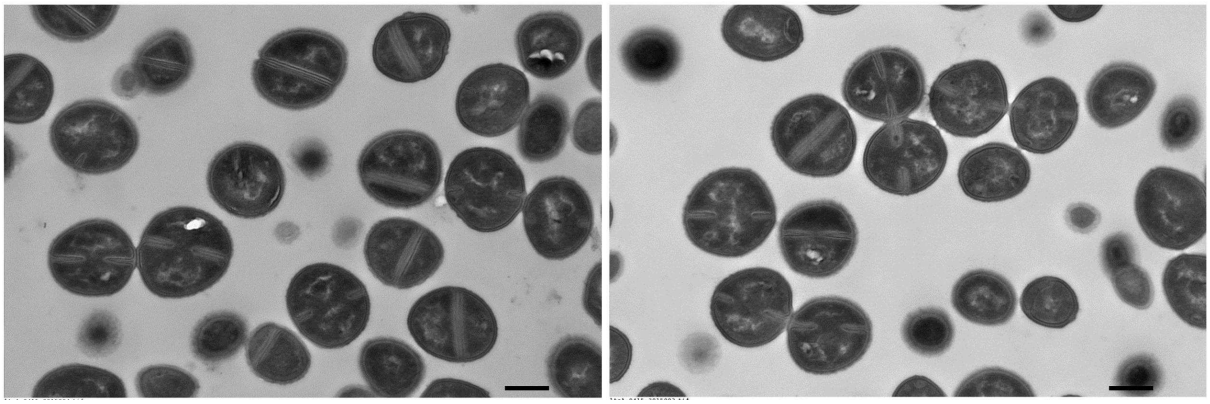

Figure S9. Electron micrographs of *S. aureus* HG003 treated with lysobactin revealed significant changes in cytoplasm and septal defects. *S. aureus* HG003 was treated with lysobactin (a) and ramoplanin (b) at 2x MIC value for 30 min, and untreated (c) at 30℃. Both lysobactin and ramoplanin treatments caused septal defects of 30% and 24% cells respectively (Scale bars: 500nm).

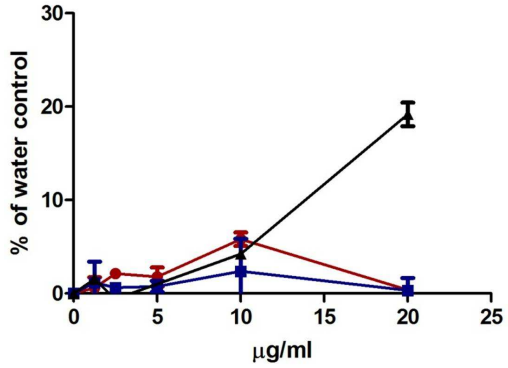

Figure S10. Hemolytic activity of ramoplanin and lysobactin. Defibrinated human red blood cells (RBCs) were challenged with various concentrations of the antibiotics for 30 minutes at room temperature. RBC lysis by antibiotics was determined by measuring released hemoglobin from lysed RBCs at an absorbance of 540 nm using a plate reader (SpectraMax, Molecular Dyanmics). Hemolytic activity of antibiotics is presented as the percent lysis compared to treatment of RBCs with water (100 %): ramoplanin (black), lysobactin(red), vancomycin(blue).

Antibiotic	MIC (μg/ml)
Lysibactin	0.75
Vancomycin	1
Ramoplanin	1
Moenomycin	0.125
Targocil	1
Bacitracin	250
CDFI	2
DMPI	16

Figure S11. MICs of compounds used in this study against *S. aureus*.

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