Supporting information

for

“Daptomycin pore formation is restricted by lipid acyl chain composition”

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Materials and Methods

Synthesis of NBD-labeled Daptomycin

NBD-daptomycin was prepared using a modification of the procedure described before[1]. Daptomycin (79.4 mg, 1.0 equiv.; generously provided by Jared Silverman, formerly of Cubist, Inc.) was dissolved in water (milli-Q, 4 mL) into a dual-opening 50 mL round bottom flask. Sodium bicarbonate ($NaHCO_3$, 12.3 mg) was added and both were stirred and brought to 55°C in an oil bath. A solution of NBD-Cl (10.75 mg, 1.1 equiv.) in methanol (HPLC grade, 4 mL) was added dropwise over 10 min. The mixture was stirred at 55oC for 1 hour under reflux. After the 1 hour was reached, the reaction was terminated by adding 1 M HCl to apH of ≈2-3. The reaction mixture was then evaporated to dryness and lyophilised.

Analytic RP-HPLC of the mixture showed two major peaks, corresponding to native daptomycin (t_r = 26 min) and NBD-daptomycin (t_r = 40 min), respectively. The two products were separated on a semi-preparative column (Higgins Clipeus, C-18, 10 µm, with a linear gradient of 65% H_2O (0.1% TFA)/35% acetonitrile to 55% H_2O (0.1% TFA)/45% acetonitrile, over 50 minutes. The NBD-daptomycin fractions were collected and concentrated to dryness. This gave pure NBD-daptomycin, a yellow powder, after lyophilisation (8.81 mg, 10.1 % yield).

The analytical RP-HPLC chromatogram (Higgins Clipeus analytical C-18 column, 5 µm, linear gradient of 65% H_2O (0.1% TFA)/35% acetonitrile to 55% H2O (0.1% TFA)/45% acetonitrile (50 minutes) of the pure NBD-daptomycin showed one peak with a t_r = 41 min (see Figure S1). The high-resolution mass spectrometry showed three peaks that are accounted for by a single molecular species (see Figure S2).

Antibacterial activity assay

Daptomycin and NBD-daptomycin were tested against *Bacillus subtilis* ATCC 1046 at a concentration of 5 mM $CaCl_2$. Each antibiotic was tested three times in duplicate; growth and sterility controls were run in parallel. The procedure used was as described before[2].

Liposome preparation

All lipids used to make liposomes were acquired from Avanti Polar Lipids (Alabaster, Alabama). Lipids included 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt; DMPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt; POPG), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt; DOPG).

Large unilamellar vesicles were prepared as described before[1]. Briefly, the requisite amounts of lipids – equimolar equimolar mixtures of PC and PG were used in each case – dissolved in chloroform/methanol (3:1) and dried under N_2 in a round-bottom flask. After evacuation, the lipid film was resuspended in the appropriate buffer, and the resulting lipid suspension extruded repeatedly through a 100 nm polycarbonate filter. Liposomes were used at a final total concentration of 250 μM total lipid in the various assays unless stated otherwise.

Kynurenine fluorescence

DMPC/DMPG, DOPC/DOPG or POPC/POPG liposomes were mixed with daptomycin (3 μM) and calcium chloride (0 to 100 mM final concentration, as stated in the Results section) in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4). Each sample was incubated for 3 minutes. Excitation spectra (emission wavelength: 445 nm) and emission spectra (excitation wavelength: 365 nm) were acquired on a PTI QuantaMaster 4 instrument. All trials were run in triplicates at 37ºC.

Permeabilization assays utilizing pyranine

DMPC/DMPG, DOPC/DOPG or POPC/POPG liposomes were prepared in 5 mM MES, 5 mM Tricine, 5 mM NaCl, 250 mM sucrose (MTS), pH 6.0 with 1 mM pyranine (Sigma-Aldrich). The resulting pyranine-containing LUVs were passed through a size-exclusion column (Bio-Rad P-6DG, Bio-Rad, Richmond, CA, USA) using MTS pH 6.0 without pyranine to remove the excess non-entrapped dye.

Daptomycin (2 μM), the proton carrier carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 5 nM), and calcium chloride (concentrations as stated in Results) were added into MTS buffer with 100 mM NaCl and at pH 8.0. At time 0, pyranine-loaded LUVs were added to a final concentration of 250 μM total lipid. Fluorescence (excitation

wavelength, 460 nm; emission wavelength, 510 nm) was monitored for 300 s. Triton X-100 (0.1% final concentration) was then added to solubilize the LUVs. The fluorescence intensity after Triton X-100 solubilization was used to normalize the entire curve. All experiments were run at 30ºC.

Since Zhang et al.[3] reported problems with liposome leakiness when attempting to use this assay, it may be useful to point out some experimental conditions that we have found to affect reproducibility:

- liposomes appear to be more stable when saccharose is included in the buffer;
- although we used 5 mM of pyranine in earlier studies[4,5], we now routinely use only 1 mM, since this also improves liposome stability. Note that only 0.5 mM were used in the study that first described this assay[6];
- with liposomes containing an equimolar mixture of DMPC and DMPG, we also found calcium to be required for stability; below 1 mM of calcium, liposomes become increasingly leaky, particularly when exposed to CCCP (even without daptomycin).

Ion-leakage assays utilizing PBFI

Ion-Leakage assays were performed as reported before[3] but with several added controls. PBFI, which was synthesized according to a previously published procedure[7], was dissolved in HEPES buffer (20 mM HEPES, pH 7.4) to a final concentration of 0.85 mM, using a molar extinction coefficient at 340 nm of 48 000 $M^{-1}cm^{-1}$. 1 ml of PBFI solution was added to a lipid film containing POPC/POPG (at equimolar ratio; total concentration of lipids, 8.2 mM) and sonicated for 45 minutes in a bath sonicator. The liposomes were then filtered through a 0.22 µm filter and subsequently run through a size-exclusion column (Sephadex G-50) to remove non-encapsulated PBFI, using a potassium buffer (20 mM HEPES, 200 mM KCl, pH 7.4) as the eluent.

Fluorescence (excitation wavelength, 338 nm; emission wavelength, 507 nm) was acquired on a PTI QuantaMaster 4 instrument. At time 0, PBFI-loaded liposomes (0.55 mM total lipid) were added to potassium buffer. Calcium chloride (0.6 mM) as well as daptomycin (4.8 µM), valinomycin (0.5 µM) or daptomycin-EW[8] (4.8 µM), were added after 60 s. At 300 s, the assay was terminated using Triton X-100 (0.1%) to solubilize the liposomes. The fluorescence intensity after Triton X-100 was added was used to normalize the entire curve. All assays were run at room temperature.

Daptomycin oligomer subunit stoichiometry experiments

These experiments were carried out exactly as described before[9]. Numbers given in the main text represent averages ± standard deviation of 4 experiments.

Determination of daptomycin membrane translocation

These experiments were conducted following the methodology described before[5] with several deviations. The deviations included using 1 mM dithionite (Sigma-Aldrich) instead of 10 mM, and shortening the incubation period from 5 mins to 4 mins. Calcium chloride was used at a final concentration of 25 mM unless stated otherwise.

Again in the interest of reproducibility, we point out that we noticed considerable batch-to-batch variation in the activity of dithionite; the batch that was used at 1 mM had been freshly purchased. When used at concentrations greater than 3-4 mM, this batch caused more extensive quenching of NBD-daptomycin. We speculate that this may be related to the introduction of sulfuric acid that forms through spontaneous chemical decay[10], but we have not systematically examined this possibility. The assay is also affected by the calcium concentration; we found less experimental variation at 25 mM than at lower concentrations.

Results

Figures S3 and S4 depict additional experiments to support the conclusion that the PBFI-based assay does not indicate permeabilization of POPC/POPG membranes. In Figure S3, it is shown that the derivative daptomycin-EW, which contains glutamate instead of methyl-glutamate in position 12 and tryptophan instead of kynurenine in position 13, resembles native daptomycin in its binding to and permeabilization of DMPC/DMPG membranes; this validates its use as a control in the PBFI assay (cf. Figure 3 in the main text). In Figure S4, the PBFI assay was carried out with native daptomycin as before, but emission was observed at two wavelengths. The intrinsic kynurenine emission of daptomycin is much higher at 445 than at 507 nm (A), whereas the incremental emission of PBFI in response to an increase in effective potassium concentration is similar at both wavelengths (B). The ratios of the changes in emission at both wavelengths that occur after addition of daptomycin and of Triton, respectively (C), indicate that adding daptomycin results in kynurenine rather than PBFI fluorescence; the latter increases only after the liposomes are solubilized with Triton-X100.

Legends to Figures

Figure S1: Analytical RP-HPLC chromatogram (at 220 nm) of the pure NBD-daptomycin.

Figure S2: ESI+-MS of NBD-daptomycin. The peaks with m/z = 892, 1190 and 1784 correspond to the doubly charged monomers, triply charged dimers, and singly charged monomers, respectively, of NBD-daptomycin.

Figure S3: Activity of the daptomycin-EW derivative on DMPC/DMPG liposomes, compared to native daptomycin. **A**: Membrane binding as a function of calcium concentration. Binding of native daptomycin is monitored using kyurenine fluorescence (E_{455}), whereas binding of daptomycin-EW is monitored by the blue-shift of tryptophan fluorescence (E_{339}/E_{360}). **B**: Pyranine fluorescence assay of membrane permeabilization. Lipopeptides (where present) were used at 3 μM.

Figure S4: Dual wavelength monitoring of the PBFI-based assay of permeabilization using native daptomycin. B: Emission spectra of daptomycin on POPC/POPG membranes at different concentrations of calcium. Solid and dashed arrows indicate changes in emission at 507 and 445 nm, respectively; these were used to calculate the characteristic emission ratio shown in D. **B**: Emission spectra of PBFI – in the presence of 0.1% Triton-X100, which was found to cause a significant spectral shift – at different potassium concentrations. Arrows have the same purpose as before. **C**: Dual wavelength time-based emission scan of daptomycin added to PBFI-loaded liposomes. Solid and dashed arrows indicate the emission changes after adding daptomycin (first step) and Triton (second step) at 507 and 445 nm that were used to calculate the emission ratios shown in D. **D**: Ratios of emission changes at 507 and 455 nm calculated from references (A,B; blue) and from the time-based scan (C; red). Adding daptomycin to PBFI-loaded liposomes causes a change that resembles an increased kyurenine emission, whereas adding Triton causes a change that resembles an increased PBFI emission.

Figure S5: “Mock PBFI assay”. All experimental conditions were as described in the Methods section above, except that liposomes did not contain PBFI. Daptomycin produces an instantaneous emission signal, whereas daptomycin-EW does not. Addition of Triton is without effect.

Figure S6: Fatty acid profiles of several bacterial species that are susceptible to daptomycin. Different sources were used for *Bacillus subtilis* and *megaterium*[11,12], *Enterococcus faecalis* and *faecium*[13], and *Staphylococcus aureus*[14,15]. Iso- and antiso-acyl chains are grouped together as “methylated”.

Figure S7: Structures of all lipid species used in this study.

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Figure S1

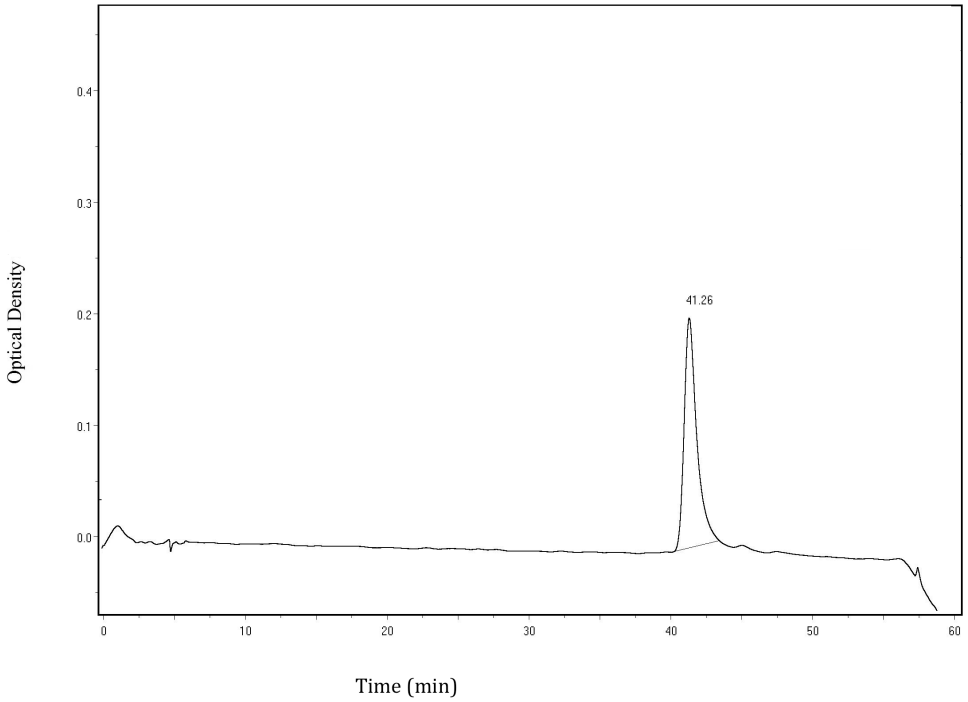

Figure S2

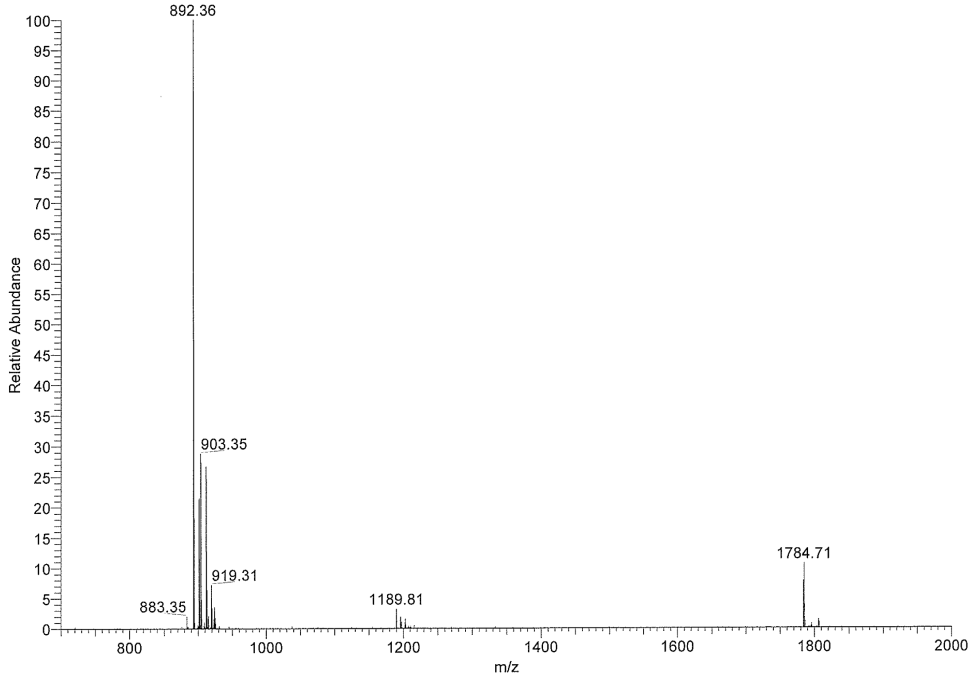

Figure S3

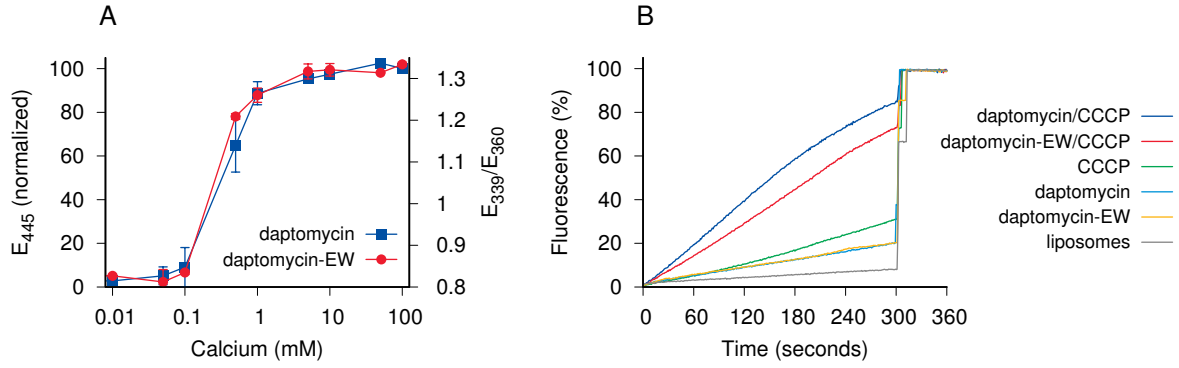

Figure S4

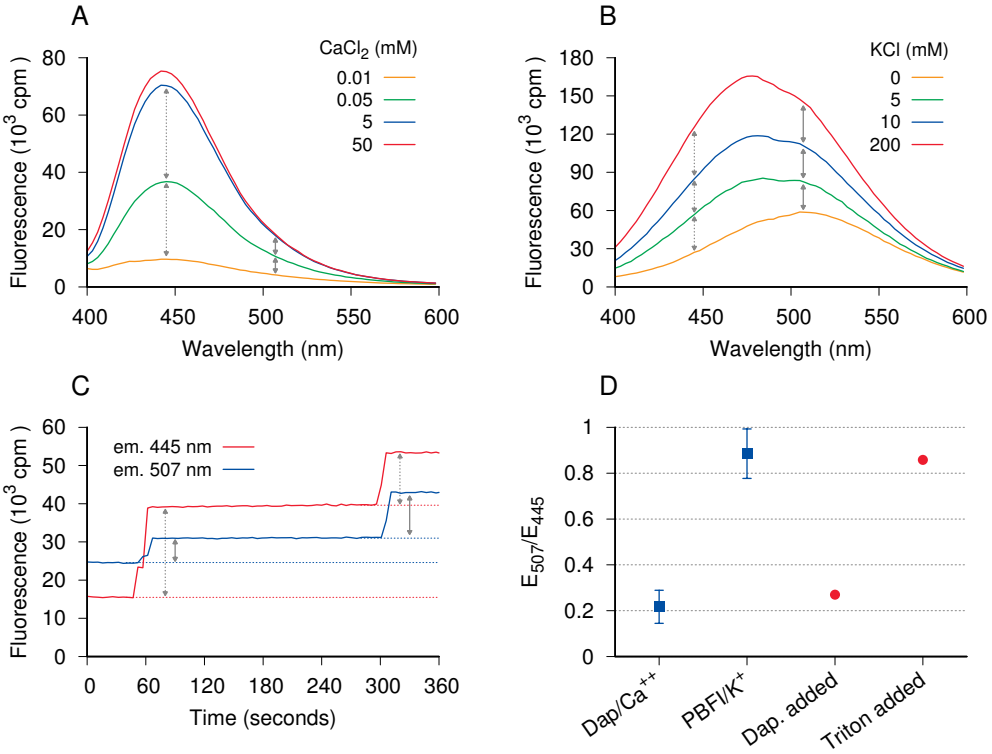

Figure S5

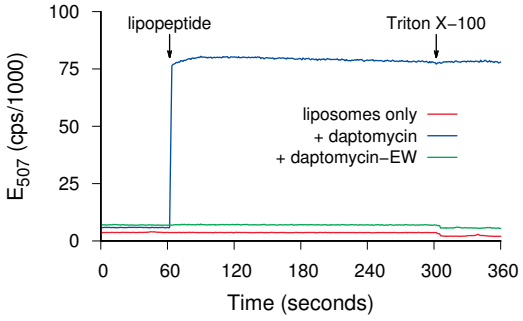

Figure S6

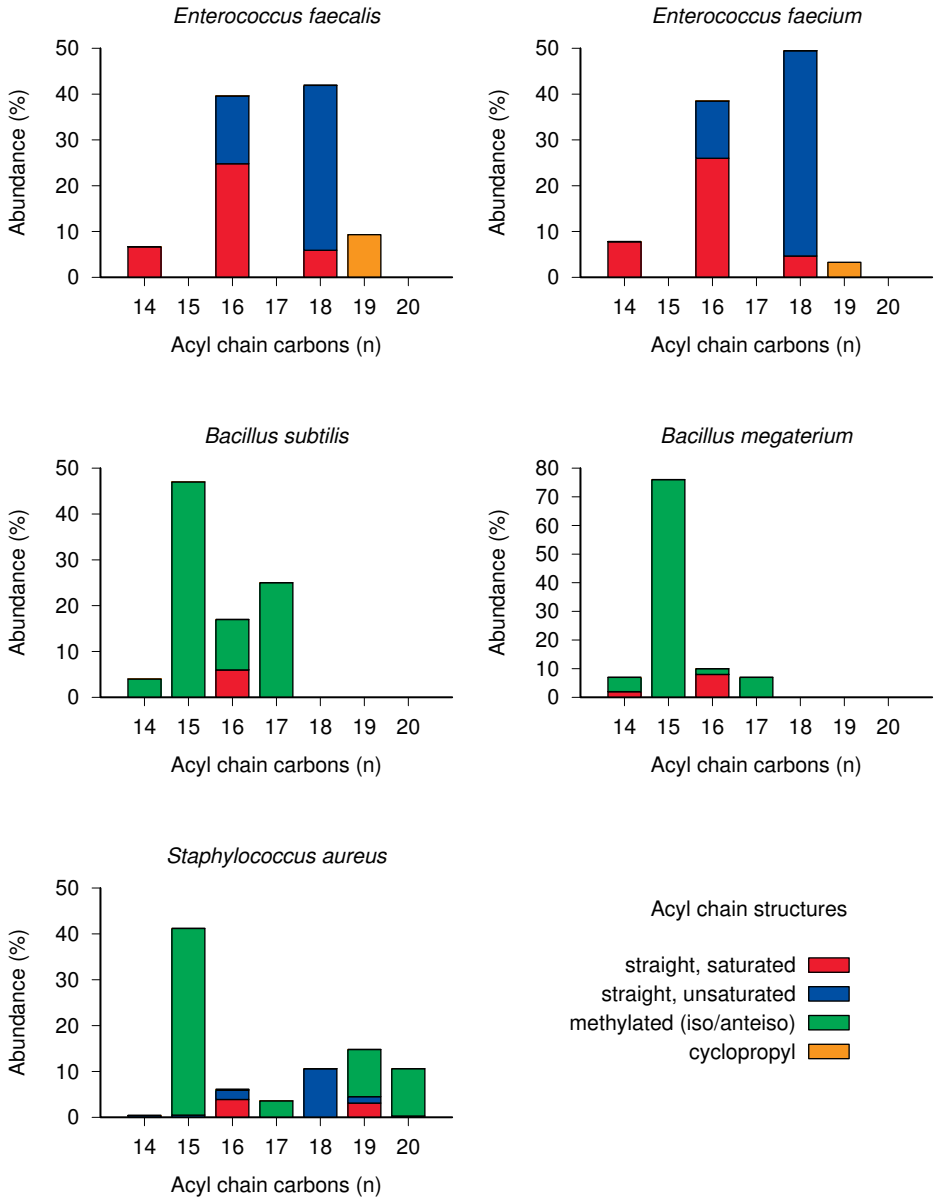
Enterococcus faecalis
Enterococcus faecium
Bacillus subtilis
Bacillus megaterium
Staphylococcus aureus
Abundance (%)
Acyl chain carbons (n)
Acyl chain structures
straight, saturated
straight, unsaturated
methylated (iso/anteiso)
cyclopropyl

Figure S7