Exogenous fatty acids protect *Enterococcus faecalis* from daptomycin induced membrane stress independent of the response regulator LiaR

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Running title: Fatty acids protect LiaR-deficient *E. faecalis* from damage

SUPPLEMENTAL MATERIALS AND METHODS

For supplemental materials and methods, refer to materials and methods in the main text.

Proton Nuclear Magnetic Resonance (^{1}H NMR) Profile

1.23 mM Daptomycin: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.65 (dd, J = 8.3, 1.5 Hz, 1H), 7.54 (dt, J = 7.9, 1.0 Hz, 1H), 7.32 (dd, J = 8.2, 0.9 Hz, 1H), 7.21 (ddd, J = 8.4, 6.9, 1.4 Hz, 1H), 7.15 (s, 1H), 7.07 (ddd, J = 8.1, 7.0, 1.1 Hz, 1H), 6.98 (ddd, J = 8.0, 7.0, 1.0 Hz, 1H), 6.70 (dd, J = 8.5, 1.1 Hz, 1H), 6.54 (ddd, J = 8.1, 6.9, 1.2 Hz, 1H), 5.45 – 5.39 (m, 1H), 4.72 (t, J = 6.7 Hz, 1H), 4.64 (t, J = 6.7 Hz, 1H), 4.63 – 4.58 (m, 2H), 4.54 (d, J = 3.4 Hz, 1H), 4.52 (d, J = 7.6 Hz, 1H), 4.48 (t, J = 5.4 Hz, 1H), 4.36 – 4.30 (m, 1H), 4.28 (t, J = 7.1 Hz, 1H), 3.99 (d, J = 4.3 Hz, 1H), 3.94 (d, J = 11.7 Hz, 2H), 3.86 (qd, J = 11.0, 9.9, 4.2 Hz, 3H), 3.51 (q, J = 7.0, 4.6 Hz, 1H), 3.24 (dd, J = 14.3, 7.8 Hz, 1H), 3.12 (dd, J = 14.3, 7.3 Hz, 1H), 2.98 – 2.79 (m, 5H), 2.74 (dt, J = 15.9, 7.5 Hz, 3H), 2.69 – 2.58 (m, 3H), 2.40 (dd, J = 15.6, 6.5 Hz, 1H), 2.30 – 2.12 (m, 5H), 1.97 (d, J = 0.7 Hz, 1H), 1.91 – 1.76 (m, 2H), 1.72 (q, J = 7.6 Hz, 2H), 1.50 (q, J = 7.6 Hz, 2H), 1.38 – 1.27 (m, 9H), 1.27 – 1.14 (m, 16H), 0.97 (d, J = 6.9 Hz, 3H), 0.89 (t, J = 7.0 Hz, 4H).

50 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 5.34 (ddd, J = 5.7, 4.4, 1.1 Hz, 2H), 2.27 (t, J = 7.4 Hz, 2H), 2.03 (q, J = 5.8 Hz, 4H), 1.59 (p, J = 7.3, 7.3 Hz, 2H), 1.39 – 1.24 (m, 20H), 0.89 (t, J = 6.9 Hz, 3H).

1.20 mM Daptomycin and 1.20 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.66 (d, J = 8.1 Hz, 1H), 7.55 (d, J = 7.9 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.21 (t, J = 8.0 Hz, 1H), 7.14 (s, 1H), 7.07 (t, J = 7.5 Hz, 1H), 6.98 (t, J = 7.5 Hz, 1H), 6.70 (d, J = 8.4 Hz, 1H), 6.54 (t, J = 7.6 Hz, 1H), 5.46 – 5.39 (m, 1H), 5.34 (t, J = 4.9 Hz, 1H), 4.70 (t, J = 6.7 Hz, 1H), 4.67 – 4.52 (m,

4H), 4.47 (s, 1H), 4.37 – 4.26 (m, 2H), 4.06 – 3.97 (m, 1H), 3.94 (d, *J* = 15.3 Hz, 2H), 3.88 (d, *J* = 16.6 Hz, 3H), 3.52 (s, 2H), 3.44 (p, *J* = 1.6 Hz, 2H), 3.16 (p, *J* = 1.7 Hz, 2H), 3.12 (dd, *J* = 14.4, 7.3 Hz, 1H), 2.97 – 2.91 (m, 2H), 2.89 – 2.66 (m, 4H), 2.66 – 2.58 (m, 2H), 2.39 (dd, *J* = 15.5, 6.6 Hz, 1H), 2.33 – 2.13 (m, 6H), 2.06 – 1.99 (m, 2H), 1.92 – 1.78 (m, 1H), 1.72 (q, *J* = 7.5 Hz, 2H), 1.65 – 1.56 (m, 1H), 1.52 – 1.45 (m, 2H), 1.38 – 1.27 (m, 20H), 1.23 (dd, *J* = 16.8, 5.5 Hz, 15H), 0.98 (d, *J* = 6.9 Hz, 4H), 0.89 (td, *J* = 6.9, 5.0 Hz, 5H).

1.10 mM Daptomycin and 5.50 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.66 (d, *J* = 8.1 Hz, 1H), 7.55 (d, *J* = 7.9 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.24 – 7.18 (t, *J* = 8.0 Hz, 1H), 7.14 (s, 1H), 7.07 (t, *J* = 7.5 Hz, 1H), 6.98 (t, *J* = 7.5 Hz 1H), 6.70 (d, *J* = 8.1 Hz, 1H), 6.54 (t, *J* = 7.6 Hz, 1H), 5.46 – 5.39 (m, 1H), 5.34 (ddd, *J* = 5.6, 4.4, 1.1 Hz, 10H), 4.71 (m, 1H), 4.62 (t, *J* = 6.4 Hz, 1H), 4.54 (s, 2H), 4.47 (s, 1H), 4.37 – 4.26 (m, 1H), 4.02 (d, *J* = 16.2 Hz, 1H), 3.94 (d, *J* = 15.0 Hz, 2H), 3.85 (d, *J* = 15.8 Hz, 3H), 3.52 (s, 2H), 3.44 (p, *J* = 1.7 Hz, 2H), 3.16 (p, *J* = 1.6 Hz, 2H), 2.94 (s, 3H), 2.89 – 2.67 (m, 9H), 2.63 (d, *J* = 15.3 Hz, 2H), 2.40 (dd, *J* = 15.5, 6.4 Hz, 1H), 2.25 (t, *J* = 7.5 Hz, 10H), 2.18 (t, *J* = 7.6 Hz, 3H), 2.07 – 1.99 (m, 20H), 1.76 – 1.69 (m, 2H), 1.64 – 1.55 (m, 10H), 1.49 (s, 3H), 1.38 – 1.27 (m, 100H), 1.27 – 1.19 (m, 12H), 0.98 (d, *J* = 6.9 Hz, 4H), 0.89 (td, *J* = 6.9, 5.3 Hz, 18H).

1.21 mM Daptomycin and 1.64 mM Calcium Chloride:^{1}H NMR (500 MHz, Methanol-d_4) δ 7.65 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.53 (s, 1H), 7.30 (s, 1H), 7.24 (s, 1H), 7.15 (s, 1H), 7.07 (t, J = 7.6 Hz, 1H), 6.98 (t, J = 7.5 Hz, 1H), 6.75 (d, J = 10.6 Hz, 1H), 6.58 (s, 1H), 5.45 – 5.39 (m, 1H), 4.72 (t, *J* = 6.7 Hz, 1H), 4.64 (t, *J* = 6.7 Hz, 1H), 4.63 – 4.58 (m, 2H), 4.54 (d, *J* = 3.4 Hz, 1H), 4.52 (d, *J* = 7.6 Hz, 1H), 4.49 (s, 1H), 4.36 – 4.30 (m, 1H), 4.29 (s, 1H), 4.02 (s, 1H), 3.94 (m, 2H), 3.86

(m, 3H), 3.53 (m, 1H), 3.31 (dd, J = 7.3, 2.2 Hz, 1H), 3.12 (m, 1H), 2.96-2.84 (s, 5H), 2.77 (s, 3H), 2.69 – 2.58 (m, 3H), 2.38 (d, J = 18.0 Hz, 1H), 2.30 – 2.12 (m, 5H), 1.95 (s, 1H), 1.77 (s, 2H), 1.72 (m, 2H), 1.45 (s, 2H), 1.33 (m, 9H), 1.29 – 1.13 (m, 16H), 0.95 (s, 3H), 0.89 (t, J = 7.1 Hz, 4H).

1.20 mM Daptomycin, 1.60 mM Calcium Chloride, and 1.20 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.71 (s, 1H), 7.53 (s, 1H), 7.30 (s, 1H), 7.24 (s, 1H), 7.07 (t, *J* = 7.6 Hz, 1H), 6.98 (t, *J* = 7.5 Hz, 1H), 6.74 (s, 1H), 6.58 (s, 1H), 5.46 – 5.39 (m, 1H), 5.34 (m, 1H), 4.70 (t, *J* = 6.7 Hz, 1H), 4.67 – 4.52 (m, 4H), 4.49 (s, 1H), 4.29 (s, 2H), 4.01 – 3.92 (m, 3H), 3.79 (s, 3H), 3.54 – 3.12 (m, 7H), 2.96 (s, 2H), 2.87 – 2.80 (m, 4H), 2.66 – 2.58 (m, 2H), 2.38 (d, *J* = 18.2 Hz, 1H), 2.34 – 2.13 (m, 6H), 2.06 – 1.97 (m, 2H), 1.94 – 1.78 (m, 1H), 1.77 (s, 2H), 1.60 – 1.45 (m, 3H), 1.35 – 1.25 (m, 20H), 1.24 (s, 15H), 1.19 (s, 2H), 1.13 (s, 2H), 0.89 (t, *J* = 7.1 Hz, 4H).

1.05 mM Daptomycin, 1.45 mM Calcium Chloride, and 5.8 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.70 (s, 1H), 7.53 (s, 1H), 7.30 (s, 1H), 7.24 (s, 1H), 7.14 (s, 1H), 7.07 (t, *J* = 7.6 Hz, 1H), 6.98 (t, *J* = 7.5 Hz, 1H), 6.74 (s, 1H), 6.58 (s, 1H), 5.34 (ddd, *J* = 5.7, 4.5, 1.1 Hz, 10H), 4.71 (m, 1H), 4.63 (t, *J* = 6.4 Hz, 1H), 4.54 (m, 2H), 4.48 (s, 1H), 4.29 (s, 1H), 4.02 (d, *J* = 16.5 Hz, 1H), 3.79 – 3.40 (m, 7H), 3.21 (td, *J* = 5.0, 2.4 Hz, 2H), 2.96 (s, 3H), 2.87 – 2.62 (m, 11H), 2.40 (s, 1H), 2.27 – 2.11 (m, 10H), 2.03 – 1.95 (m, 20H), 1.77 (s, 2H), 1.63 – 1.56 (m, 10H), 1.45 (s, 2H), 1.36 – 1.26 (m, 100H), 1.24 (s, 15H), 1.19 (s, 3H), 1.13 (s, 2H), 0.95 (s, 4H), 0.89 (td, *J* = 7.0, 4.8 Hz, 15H).

SUPPLEMENTARY TABLE S1. Membrane analysis of wild type and mutant strains during log phase growth.

	Percent of total membrane content for indicated strain and supplement (avg ± SD)[a]		
Strain	WT	Δ*liaR*	Δ*liaR::liaR*
Fatty Acid	Ethanol[b]	Ethanol[b]	Ethanol[b]
$C_{12:0}$	1.4 ± 1.1	1.3 ± 0.9	0.9 ± 0.1
$C_{14:0}$	4.6 ± 0.4	4.2 ± 0.2	6.4 ± 0.1
$C_{16:1}$	5.7 ± 0.3	5.5 ± 0.8	8.7 ± 0.1
$C_{16:0}$	39.8 ± 0.5	42.7 ± 1.8	37.5 ± 0.1
$C_{17:1}$	ND	ND	ND
$C_{17:0\ 2OH}$	7.5 ± 1.0	5.3 ± 3.1	5.9 ± 0.3
$C_{18:1\ cis\ 9}$	0.3 ± 0.6	0.7 ± 1.3	0.6 ± 0.5
$C_{18:1\ cis\ 11}$	34.0 ± 1.0	32.6 ± 1.1	35.7 ± 0.1
$C_{18:0}$	5.6 ± 0.1	6.9 ± 0.8	3.2 ± 0.1
$C_{20:0}$	ND	ND	ND
$C_{18:2}$	ND	ND	ND
Others[c]	0.7 ± 0.3	0.4 ± 0.4	0.7 ± 0.2
Sat:Unsat	1.3 ± 1.0	1.4 ± 1.1	1.1 ± 0.6
C_{10}-C_{17}:C_{18}-C_{20}[d]	1.5 ± 0.3	1.5 ± 0.03	1.5 ± 0.1

[a] Membrane content determined by GC-FAME analysis by Microbial ID, Inc. Values represent average and standard deviations of three independent cultures. ND indicates fatty acid was not detected.

[b] Ethanol solvent control was added to a final concentration of 0.2%.

[c] Others indicates fatty acids comprised <1% of the total membrane content.

[d] Total fatty acid length ratios including both saturated and unsaturated fatty acids.

SUPPLEMENTARY TABLE S2. Membrane fatty acid analysis of clinical isolates during log phase growth.

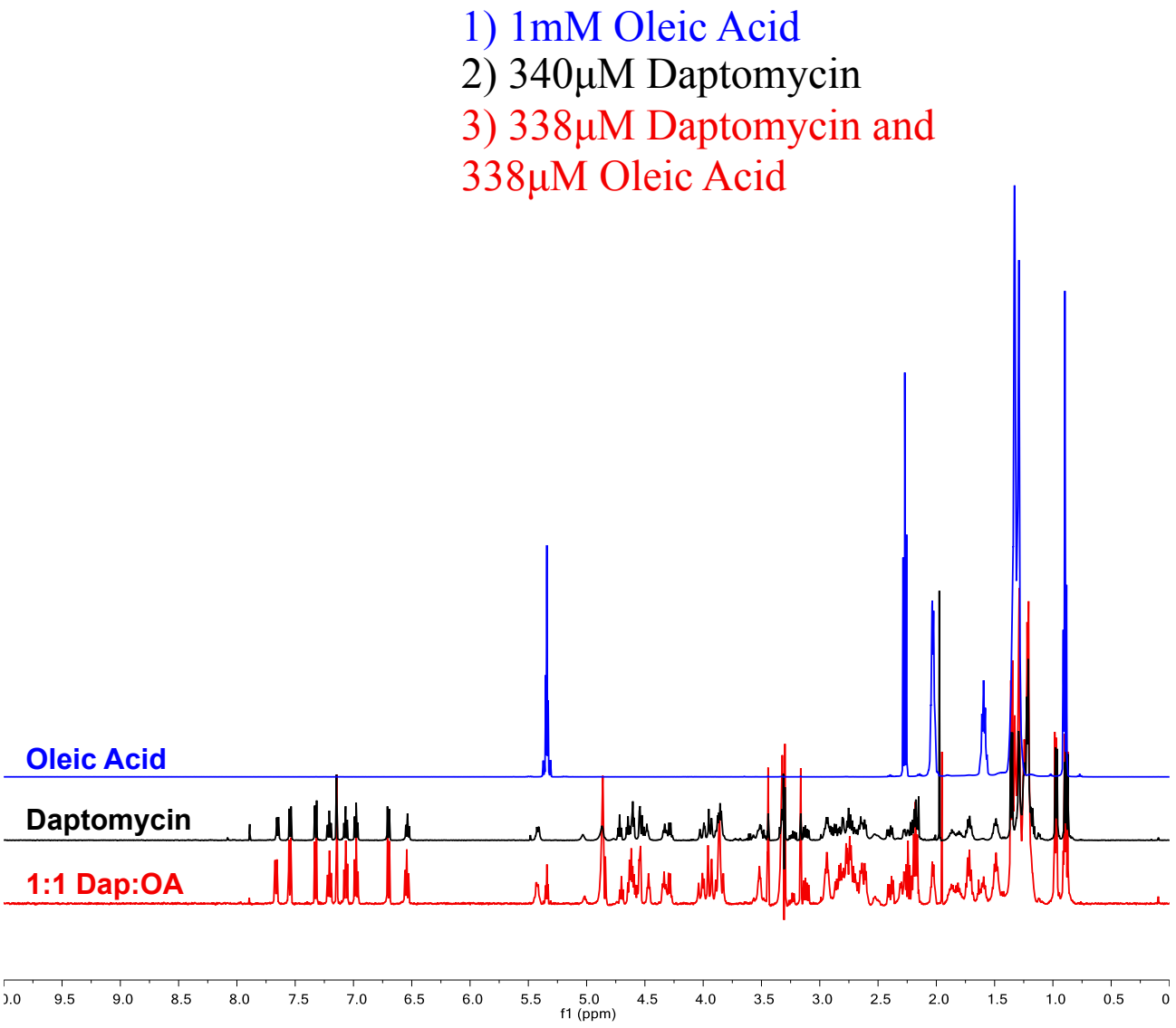
	Percent of total membrane content for indicated strain and supplement (avg ± SD)[a]	
Strain	S613	R712
Fatty Acid	Ethanol[b]	Ethanol[b]
$C_{12:0}$	1.0 ± 0.0	0.7 ± 0.1
$C_{14:0}$	5.5 ± 0.1	3.8 ± 0.2
$C_{16:1}$	8.0 ± 0.1	7.8 ± 0.4
$C_{16:0}$	36.4 ± 0.2	29.3 ± 1.0
$C_{17:0\ 2OH}$	4.4 ± 0.3	4.5 ± 3.9
$C_{18:1\ cis\ 9}$	0.9 ± 0.1	ND
$C_{18:1\ cis\ 11}$	39.1 ± 0.4	50.0 ± 2.1
$C_{18:0}$	3.8 ± 0.1	3.5 ± 0.1
$C_{20:0}$	ND	ND
$C_{18:2}$	ND	ND
$C_{20:4}$	ND	ND
Others[c]	0.4 ± 0.7	ND
Sat:Unsat	1.0 ± 0.4	0.6 ± 0.6
C_{10}-C_{17}:C_{18}-C_{20}[d]	1.3 ± 0.02	0.9 ± 0.08

[a] Membrane content determined by GC-FAME analysis by Microbial ID, Inc. Values represent average and standard deviations of three independent cultures. ND indicates fatty acid was not detected.

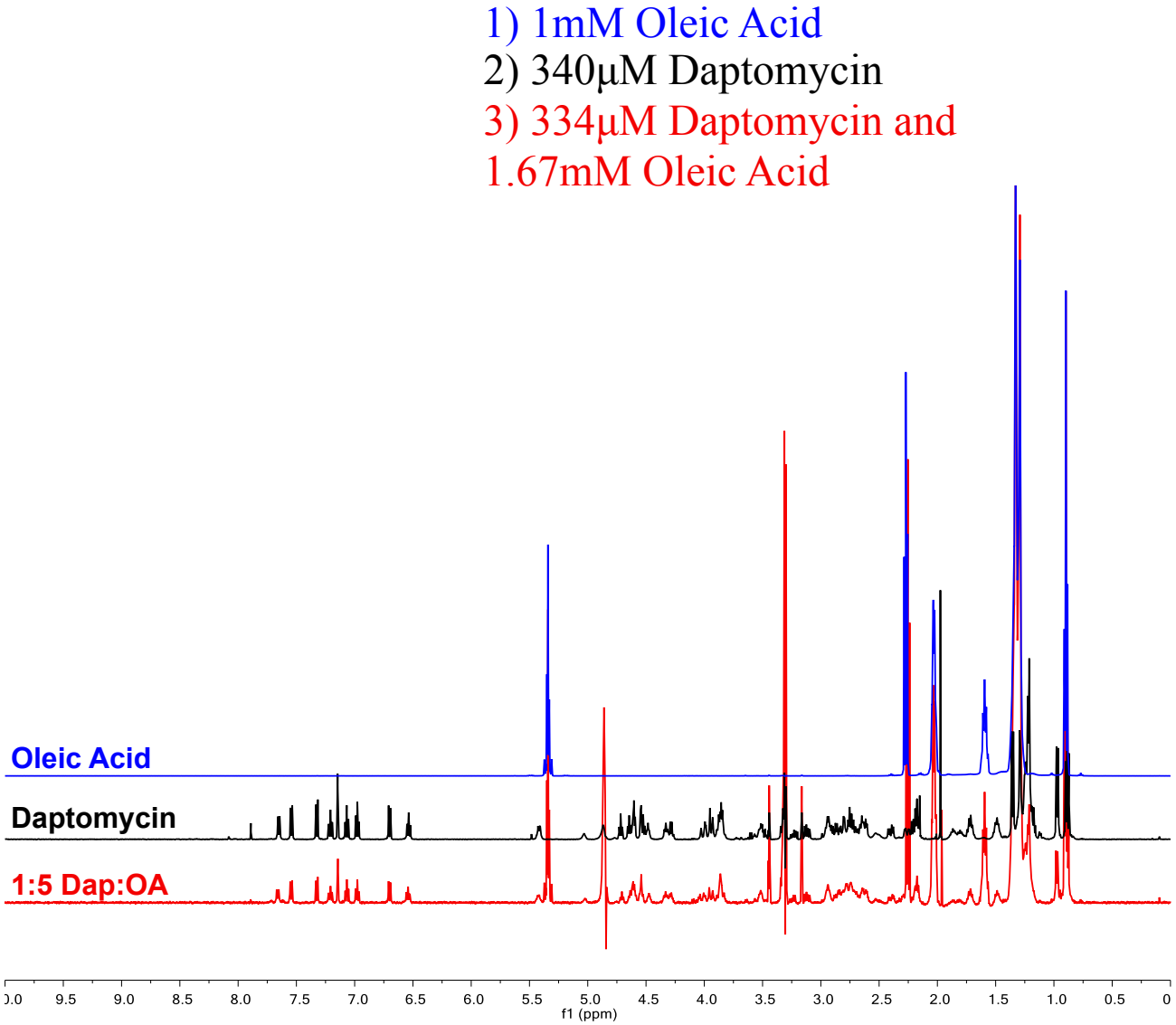
[b] Ethanol solvent control was added at a final concentration of 0.2%.

[c] Others indicates fatty acids comprised <1% of the total membrane content.

[d] Total fatty acid length ratios including both saturated and unsaturated fatty acids.



Supplemental Fig S1. Mixing 1:1 daptomycin and oleic acid reveals no observable interaction. Visual comparison of three individual ^{1}H NMR spectra, between 0.0 to 10.0 ppm, shows no interaction between daptomycin and oleic acid. The top spectra (blue) represents a 1mM solution of oleic acid, the middle spectra (black) represents a 340µM solution of daptomycin, and the bottom spectra (red) represents a 1:1 mixture of daptomycin:oleic acid. All solutions were made using methanol-d*4*, and spectra were generated using a VNMRS 500 MHz instrument. Spectra were superimposed using MestReNova software.



Supplemental Fig S2. Excess oleic acid reveals no observable interaction when mixed with daptomycin. The overlap of three individual ^{1}H NMR spectra, between 0.0 to 10.0 ppm, shows no spatial interaction with daptomycin in the prescence of excess oleic acid. The top spectra (blue) represents a 1mM solution of oleic acid, the middle spectra (black) represents a 340µM solution of daptomycin, and the bottom spectra (red) represents a 1:5 mixture of daptomycin:oleic acid. All solutions were made using methanol-d*4*, and spectra were generated using a VNMRS 500 MHz instrument. Spectra were superimposed using MestReNova software.