SUPPORTING INFORMATION

Modified horseshoe crab peptides target and kill bacteria inside host cells

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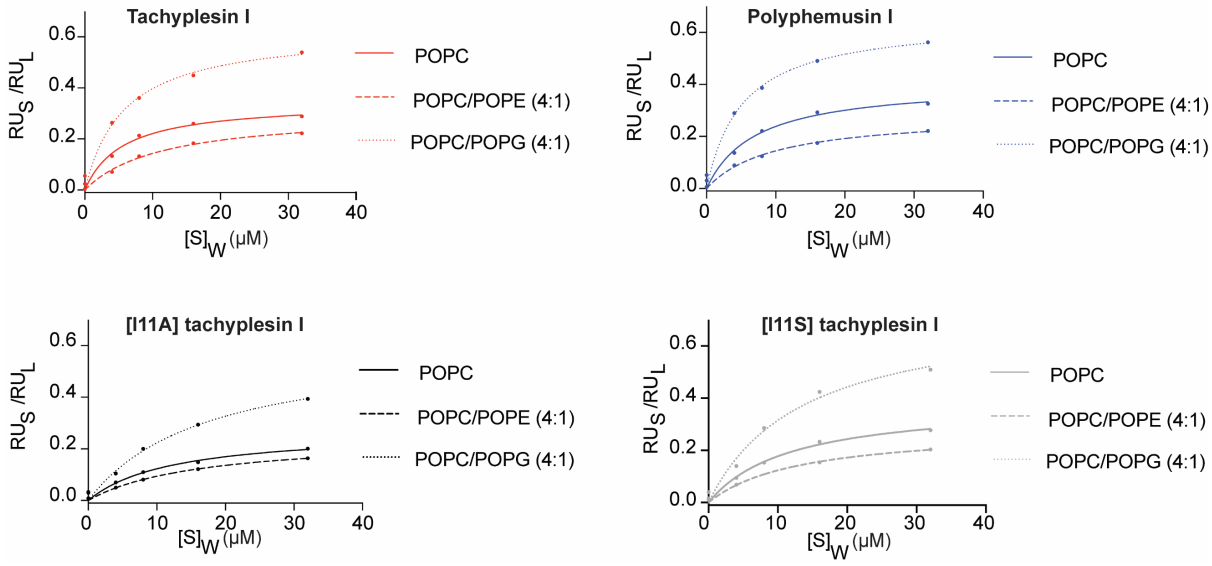
Supplementary Table 1 Average lipid mass of bilayers composed of cell membrane extracts.

	Cell membrane composition (%) [a]				
Lipid subtype [b]	***E. coli***	***S.* Typhimurium**	***Burkholderia***	**BMM**	**RBC**
Phospholalanine	-	-	-	-	1.1
Phosphocholine	-	-	-	30.1	29.3
Phosphoethanolamine	77	92	64.9	21.6	27.6
Phosphoglycerol	9	5	12	-	-
Phosphoinositol	-	-	-	6	0.6
Phosphoserine	-	-	-	6	14.9
Cardiolipin	14	3	9.9	-	-
Sphingomyelin	-	-	-	15.4	25.5
Other	-	-	13.2	20.9	1
Average mw (Da)	**831.1**	**743.5**	**706.1**	**752.2**	**734.9**

[a] Proportional representation of lipid subtypes was determined from previously reported cell membrane compositions for *Escherichia coli* (*E. coli*, represented by K-12) [1], *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) [2], *Burkholderia* (represented by *B. cenocepacia)* [3], murine bone marrow-derived macrophages (BMM) [4], and human red blood cells (RBC) [5].

[b] Molecular weight of representative lipids was used for determining the average molecular weight (mw) of the cell membrane extracts. 1-palmitoyl-2-oleoyl-sn-glycero-3 (PO)-phosphate (POPA, 696.5 Da), PO-phosphocholine (POPC, 759.6 Da), PO-phosphoethanolamine (POPE, 717.5 Da), PO-phosphoglycerol (POPG, 770.5 Da), PO-inositol (POPI, 853.6 Da), PO-phosphoserine (POPS, 783.5 Da), Cardiolipin (1494.3 Da), sphingomyelin (18:0 SM, 730.6), and 'other' components not used in the calculation.

A



B

Peptide	POPC		POPC/POPE (4:1)		POPC/POPG (4:1)	
	σ (mol/mol)	K_p ($\times 10^4$)	σ (mol/mol)	K_p ($\times 10^4$)	σ (mol/mol)	K_p ($\times 10^4$)
Tachyplesin I	8.6 ± 1.3	2.71 ± 0.30	9.9 ± 3.3	1.18 ± 0.25	4.7 ± 1.4	4.77 ± 1.00
Polyphemusin I	7.9 ± 1.1	2.34 ± 0.24	11.4 ± 4.5	1.17 ± 0.30	5.0 ± 1.2	5.11 ± 0.96
[I11A]tachyplesin I	10.7 ± 1.7	1.00 ± 0.10	12.0 ± 6.5	0.67 ± 0.21	4.8 ± 1.3	1.59 ± 0.24
[I11S]tachyplesin I	7.6 ± 1.5	1.46 ± 0.18	10.4 ± 2.9	0.96 ± 0.17	3.9 ± 1.2	2.44 ± 0.45

Supplementary Fig. 1 Peptide-lipid binding with model membranes composed of synthetic lipids obtained with surface plasmon resonance. Peptide samples were injected over deposited lipid bilayers for 180 s (see examples of sensorgrams in Fig. 4C). (A) Dose-response curves obtained with tachyplesin I, polyphemusin I, [I11A]tachyplesin I, [I11S]tachyplesin I show the ratio of response units (RU) obtained with peptide (RU_S) at the end of the association phase and obtained for the respective lipid deposition (RU_L), plotted as a function of peptide concentration in aqueous solution ($[S]_W$). Curves were fitted with a membrane partition equation following a steady-state model and the partition constants (K_p) were determined by fitting equation 1 [6]:

$$\frac{RU_S}{RU_L} = \frac{\gamma_L K_p \frac{M_S}{M_L}[S]_W}{1 + \sigma\gamma_L K_p [S]_W} \qquad \text{(equation 1)}$$

Where γ_L is molar volume of the lipid, M_S is the molecular mass of the peptide, M_L is the average molecular mass of the lipid mixture, σ is the lipid-to-peptide ratio ($n_L/n_{S,L}$) when the binding reaches saturation [6]. (B) Fitted σ and K_p values and the respective error associated with the fitted parameters.

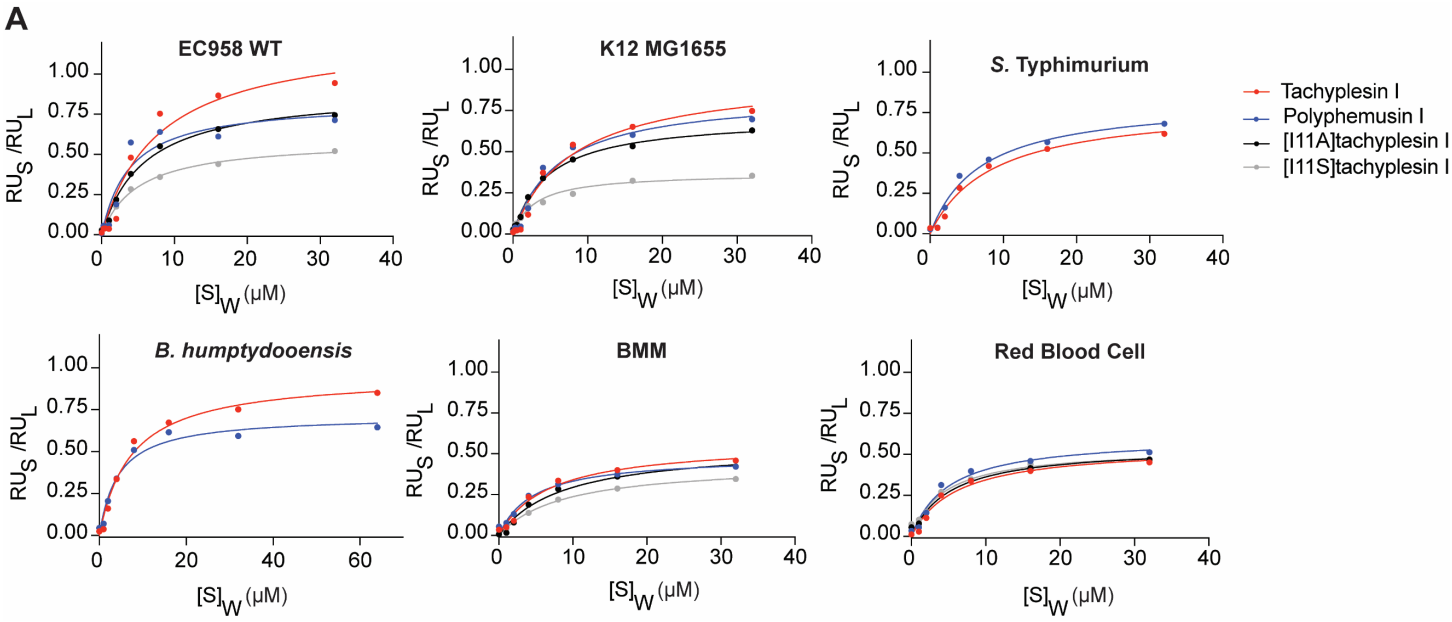
Supplementary Table 2. Peptide-to-lipid ratio when the binding reaches saturation obtained with synthetic lipids. Comparison of fitted values obtained using two approaches.

Peptide	POPC		POPC/POPE (4:1)		POPC/POPG (4:1)	
	P/L_{max} [a] (mol/mol)	$1/\sigma$ [b] (mol/mol)	P/L_{max} [a] (mol/mol)	$1/\sigma$ [b] (mol/mol)	P/L_{max} (mol/mol)	$1/\sigma$ [b] (mol/mol)
Tachyplesin I	0.13 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.10 ± 0.03	0.21 ± 0.02	0.21 ± 0.06
Polyphemusin I	0.11 ± 0.01	0.13 ± 0.02	0.08 ± 0.02	0.09 ± 0.03	0.21 ± 0.09	0.20 ± 0.05
[I11A]tachyplesin I	0.10 ± 0.04	0.09 ± 0.02	0.08 ± 0.03	0.08 ± 0.05	0.24 ± 0.05	0.21 ± 0.06
[I11S]tachyplesin I	0.10 ± 0.02	0.13 ± 0.03	0.13± 0.02	0.10 ± 0.03	0.19 ± 0.02	0.25 ± 0.08

[a] Values obtained by fitting dose-response curves in which the response units (RU) obtained with peptide and lipid were normalised to their molecular weight (i.e. P/L curves shown in Fig. 4).
[b] Values obtained by fitting dose-response curves with a membrane partition equation following a steady-state model described in Figueira et al (2017)[6] (see Supplementary Fig. 1)

Peptide-to-lipid ratio obtained when the binding reaches saturation obtained by fitting P/L dose response curves (P/L_{max}), or the membrane partition equation ($1/\sigma$), are consistent.

A



B

Peptide	EC958 WT		K12 MG1655		*S.* Typhimurium		*B. humptydooensis*		BMM		RBCs	
	σ (mol/mol)	K_p (× 10^4)	σ (mol/mol)	K_p (× 10^4)	σ (mol/mol)	K_p (× 10^4)	σ (mol/mol)	K_p (× 10^4)	σ (mol/mol)	K_p (× 10^4)	σ (mol/mol)	K_p (× 10^4)
Tachyplesin I	2.2 ± 0.7	7.73 ± 1.80	2.8 ± 0.7	5.84 ± 1.03	3.8 ± 0.9	4.07 ± 0.65	3.4 ± 0.6	5.31 ± 0.74	5.6 ± 1.4	3.48 ± 0.60	6.0 ± 1.5	3.65 ± 0.63
Polyphemusin I	3.6 ± 1.4	9.57 ± 2.88	3.5 ± 0.8	6.24 ± 1.06	4.0 ± 0.9	4.82 ± 0.78	4.9 ± 1.1	6.19 ± 0.11	7.1 ± 1.6	3.96 ± 0.65	5.9 ± 1.5	4.51 ± 0.82
[I11A]tachyplesin I	3.0 ± 0.4	7.53 ± 0.65	3.8 ± 0.4	7.51 ± 0.52					5.7 ± 1.3	2.64 ± 0.39	5.9 ± 1.1	4.30 ± 0.61
[I11S]tachyplesin I	4.6 ± 0.5	5.84 ± 0.49	7.3 ± 1.6	6.36 ± 1.09					7.1 ± 1.2	2.12 ± 0.23	6.0 ± 1.7	4.95 ± 0.98

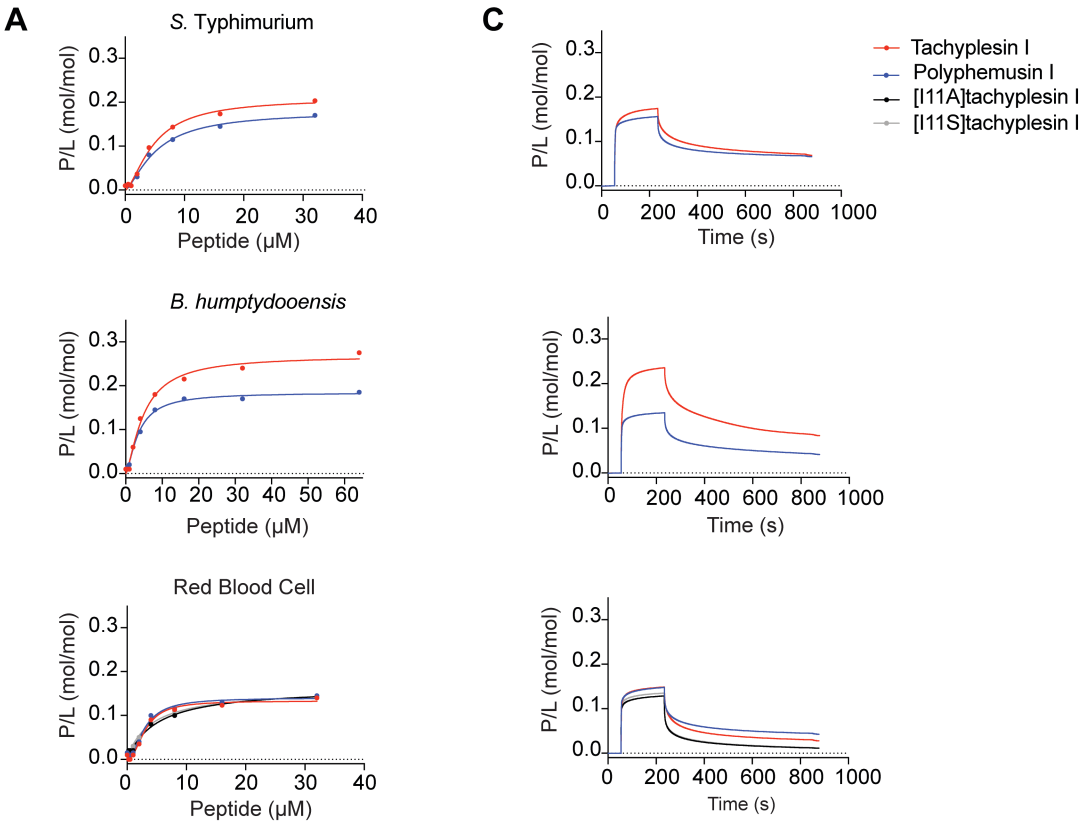
Supplementary Fig. 2 Peptide–lipid binding curves of bacterial and mammalian cell membrane extracts obtained with surface plasmon resonance. Membrane extracts from uropathogenic *Escherichia coli* (UPEC) strain EC958 wildtype (WT), *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), *Burkholderia humptydooensis* (*B. humptydooensis*), bone marrow macrophages (BMM) and red blood cells (RBCs) were used to create a lipid bilayer on an L1 chip. Peptide samples were injected over deposited lipid bilayers for 180 s (see examples of sensorgrams in Fig. 5C). (A) Curves show the ratio of response units (RU) obtained from sensorgram with peptide at the end of the association phase and the respective lipid deposition (RU_L), plotted as a function of peptide concentration in aqueous solution ($[S]_W$). Curves were fitted with a membrane partition equation following a steady-state model (see equation 1) [6]. The average molecular mass of the lipid mixture (M_L) were determined as indicated in the Suplemmentary Table 1. (B) Fitted parameters (σ and K_p, see equation 1) and respective error.

Supplementary Table 3. Peptide-to-lipid ratio when the binding reaches saturation obtained with membrane extracts. Comparison of fitted values obtained using two approaches.

	EC958 WT		**K12 MG1655**		***S.* Typhimurium**		***B. humptydooensis***		**BMM**		**RBCs**	
Peptide	P/L_{max} [a] (mol/mol)	$1/\sigma$ [b] (mol/mol)	P/L_{max} [a] (mol/mol)	$1/\sigma$ [b] (mol/mol)	P/L_{max} [a] (mol/mol)	$1/\sigma$ [b] (mol/mol)	P/L_{max} [a] (mol/mol)	$1/\sigma$ [b] (mol/mol)	P/L_{max} [a] (mol/mol)	$1/\sigma$ [b] (mol/mol)	P/L_{max} [a] (mol/mol)	$1/\sigma$ [b] (mol/mol)
Tachyplesin I	0.34 ± 0.02	0.46 ± 0.16	0.28 ± 0.02	0.36 ± 0.09	0.21 ± 0.01	0.26 ± 0.06	0.27 ± 0.02	0.30 ± 0.05	0.14 ± 0.02	0.18 ± 0.04	0.13 ± 0.01	0.17 ± 0.04
Polyphemusin I	0.23 ± 0.01	0.28 ± 0.11	0.23 ± 0.01	0.29 ± 0.07	0.18 ± 0.01	0.25 ± 0.06	0.18 ± 0.01	0.21 ± 0.05	0.12 ± 0.01	0.14 ± 0.03	0.14 ± 0.01	0.17 ± 0.04
[I11A]tachyplesin I	0.30 ± 0.01	0.34 ± 0.04	0.27 ± 0.02	0.27 ± 0.03					0.18 ± 0.01	0.18 ± 0.04	0.15 ± 0.01	0.17 ± 0.03
[I11S]tachyplesin I	0.22 ± 0.02	0.22 ± 0.03	0.20 ± 0.05	0.14 ± 0.03					0.13 ± 0.01	0.14 ± 0.02	0.15 ± 0.01	0.17 ± 0.04

[a] Values obtained by fitting dose-response curves in which the response units of peptide and lipid were normalised to their molecular weight (i.e. P/L curves shown in Figure 5 and in supplementary Figure 3).
[b] Values obtained by fitting dose-response curves with a membrane partition equation following a steady-state model described in Figueira *et al* (2017)[6], see fitted curves in Supplementary Fig. 2.

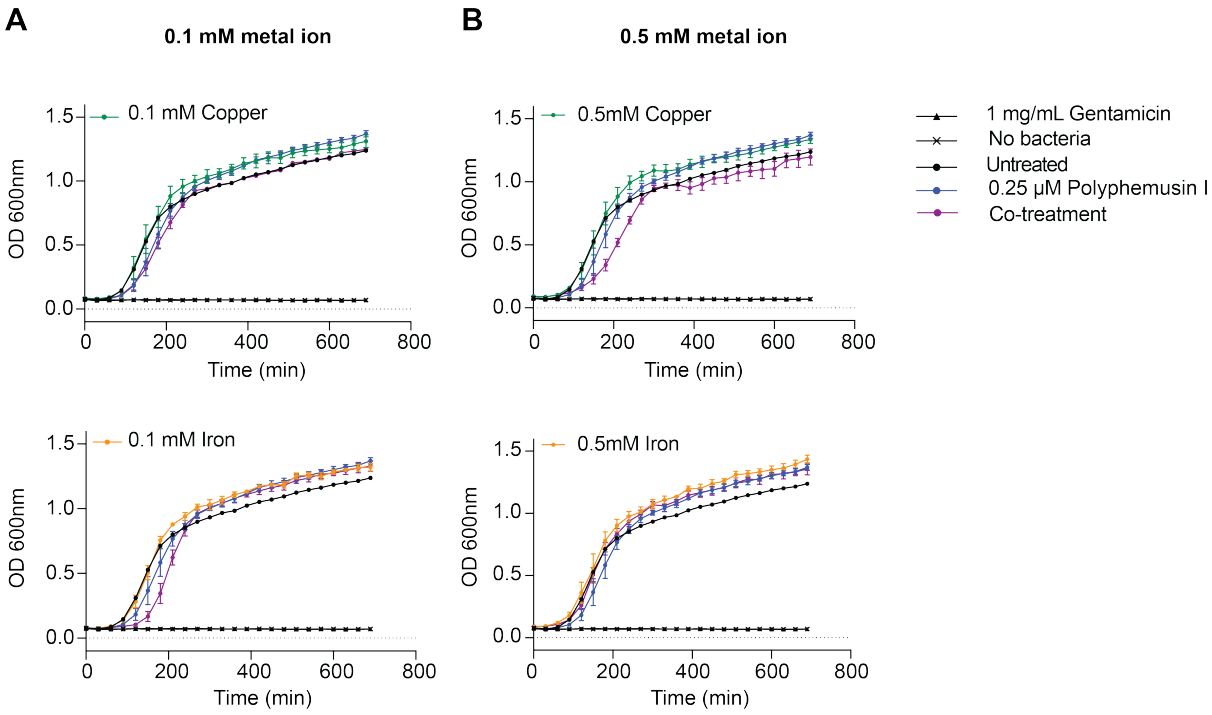
Peptide-to-lipid ratio obtained when the binding reaches saturation obtained by fitting P/L dose response curves (P/L_{max}), or the membrane partition equation ($1/\sigma$)), are consistent.



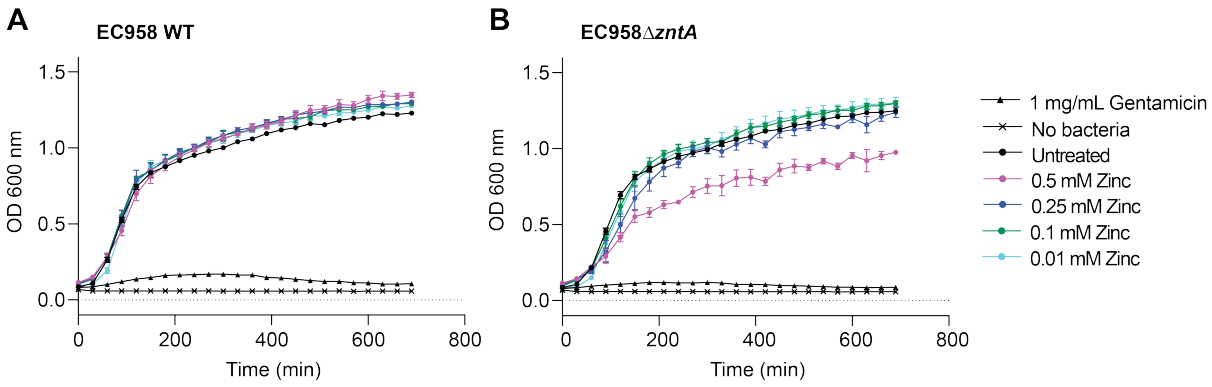
B

Peptide	*S.* Typhimurium		*B. humptydoensis*		Red blood cells	
	P/L_{max}	K_p (x10^4)	P/L_{max}	K_p (x10^4)	P/L_{max}	K_p (x10^4)
Tachyplesin I	0.21 ± 0.01	4.07 ± 0.65	0.27 ± 0.02	5.31 ± 0.74	0.13 ± 0.01	3.65 ± 0.63
Polyphemusin I	0.18 ± 0.01	4.82 ± 0.78	0.18 ± 0.01	6.19 ± 1.09	0.14 ± 0.01	4.51 ± 0.82
[I11A] tachyplesin I					0.15 ± 0.01	4.30 ± 0.61
[I11S] tachyplesin I					0.15 ± 0.01	4.95 ± 0.98

Supplementary Fig. 3 Peptide–lipid binding of tachyplesin I, polyphemusin I, [I11A] tachyplesin I and [I11S] tachyplesin I toward membrane extracts. Membranes from *S.* Typhimurium, *B. humptydooensis* and red blood cells were extracted and used to create a lipid bilayer on an L1 chip. SPR sensorgrams were obtained for peptides injected over lipid bilayers for 180 s, with dissociation monitored for 600 s. The response units at the end of the association phase were converted to peptide-to-lipid ratios (P/L (mol/mol)). (A) Dose-response curves allow comparison of peptide binding to the membrane extracts. (B) The maximum peptide-to-lipid ratio (P/L_{max}) was determined by fitting P/L dose response curves (saturation binding with Hill slope, GraphPad Prism 8). The partition coefficient (K_p) was determined from peptide response units at the end of the association phase and the respective lipid deposition response units, as above (Supplementary Fig. 2). (C) Representative sensorgrams for 16 μM peptide show peptide–lipid association and dissociation.



Supplementary Fig. 4: The effect of zinc on the growth on EC958. EC958 WT (A) and EC958Δ*zntA* (B) were treated with a range of of zinc sulfate concentrations. Samples were incubated at 37°C and absorbance readings at 600 nm were measured with a PolarStar Omega. Readings were taken every 30 min for a total of 12 h. Data represent the mean ± SEM from a minimum of three independent experiments.



Supplementary Fig. 5: Co-treatment of EC958 WT with 0.25 µM polyphemusin I with copper or iron. Using a sublethal concentration of polyphemusin I against EC958 WT (from MIC determined in the peptide susceptibility screen, see Table 1). (A) EC958 WT cells were treated with 0.25 µM polyphemusin I ± 0.1 mM of copper sulfate or iron sulfate. (B) EC958 WT cells were treated with 0.25 µM polyphemusin I ± 0.5 mM of copper sulfate or iron sulfate Samples were incubated at 37°C and absorbance readings at 600 nm were measured with a PolarStar Omega. Samples were shaken prior to absorbance readings at 600 nm. Readings were taken every 30 min for a total of 12 hrs. Data was plotted using GraphPad Prism 8.0 and represents the mean ± SEM from a minimum of three independent experiments.

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