Supplemental Information for Wei et al.

Materials and Methods

Preparation of E. coli Spheroplasts

Spheroplasts were prepared from *E. coli* strain Top 10 (containing a pET45b plasmid for ampicillin resistance) in steps similar to that described in Martinac et al. (1). An overnight culture grown (37˚C in shaking incubator, approximately 148 rpm) from one plate-picked colony was diluted 1:100 in TSB liquid media in the presence of ampicillin (25 μg/mL) and allowed to grow to OD_{600} of 0.5-0.7. 3 mL of this culture was diluted 1:10 into ampicillin-containing TSB media and cephalexin was added to reach a final concentration of 60 μg/ml. The culture was then shaken at 37˚C for 2-3 hours until single-cell filaments reached sufficient length observable under light microscope at 1000x oil immersion magnification; Martinac et al. noted that filaments from 50-150 μm should produce spheroplasts 5-10 μm in diameter (1).

Filaments were harvested by centrifugation at 1500 x g for 4 minutes, and the pellet was rinsed without resuspension by gentle addition of 1 mL of 0.8 M sucrose with 1 min incubation at room temperature and then re-suspended in 3 mL of 0.8M sucrose after supernatant has been removed via pipetting. The following reagents were added in order: 150 μL of 1 M Tris Cl (pH 7.8); 120 μL of lysozyme (5mg/ml); 30 μL of Dnase I (5mg/ml); and 120 μL of 0.125 M sodium EDTA (pH 8.0). This mixture was incubated at room temperature for 6 - 10 minutes to hydrolyze the peptidoglycan layer, and spheroplast formation was followed under microscope at 1000x. 1 mL of Solution A (20 mM $MgCl_2$, 0.7 M sucrose, 10 mM Tris Cl at pH 7.8) was gradually added over a 1 minute period while stirring, and the mixture was incubated for 4 minutes at room

temperature. The mixture was layered over two separate 7-mL aliquots of Solution B (10 mM $MgCl_2$, 0.8 M sucrose, 10 mM Tris Cl at pH 7.8) previously kept on ice. These mixtures were centrifuged for 2 minutes at 1000 x g to collect spheroplasts into a pellet, and the majority of the supernatant was removed via pipetting. Spheroplast pellets were re-suspended in about 300 μL of remaining liquid.

Confocal Microscopy Imaging of Spheroplasts

Spheroplasts were either prepared immediately before or thawed from frozen stock at -80˚C and diluted 1:2 in 0.8 M sucrose. Spheroplasts frozen for at least 1-2 weeks appeared to provide consistent results in these experiments. Diluted spheroplasts were then placed on a poly-L-lysine coated glass slide and incubated with equal volume of FITC-labeled peptide (peptide stock concentration of 1.1-6.2x10^{-4}M), giving an effective peptide concentration above the MIC for BF2, P11A BF2 and magainin 2 (HipC has effectively no antibacterial activity against *E. coli*). Peptides were typically incubated with spheroplasts for 1 minute, although some samples with HipC were allowed to incubate for 10-20 minutes. All peptides were synthesized at >95% purity by NeoScientific (Cambridge, MA) with a FITC group conjugated at the N-terminus. 1 μL of di-8-ANEPPS (Biotium, Hayward, CA) membrane dye was also added to membrane labeled samples. Spheroplasts were visualized with a Leica TCS SP5 laser scanning confocal microscope with excitation at 488 nm by an argon laser at 20% laser power output and 20% transmission and emission ranges of 499-532 nm (FITC) and 670-745 nm (di-8-ANEPPS). 8-bit, 512x512 images were collected at 63X magnification (Leica Plan-Apochromat oil objective; numerical aperture 1.40). Composite images were produced by Leica LAS AF software (Buffalo Grove, IL). Z-stacks composed of slices with 0.04-0.08 μm thickness were evaluated for localization of peptide

fluorescence within the spheroplast to prevent bias in the reading of the data. Data for each peptide was collected from a minimum of at least two independently prepared batches of spheroplasts characterized over a total of at least five separate imaging sessions for each peptide (Supplemental Tables 1-4). Data was generally consistent between different batches, although a few outliers, particularly one batch incubated with buforin II, emphasizes the need for sufficient replication to robustly characterize peptide mechanisms.

Supplemental Tables

Table 1: Percentages of imaged spheroplasts showing translocation and membrane localization of buforin II (BF2) for different spheroplast batches

	Batch 1	Batch 2	Batch 3	Batch 4
% translocating	83	11	78	85
% membrane localized	17	89	22	15
n of spheroplasts imaged	6	19	9	33

Table 2: Percentages of imaged spheroplasts showing translocation and membrane localization of P11A buforin II (BF2) for different spheroplast batches

	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
% translocating	60	22	42	21	15
% membrane localized	40	78	58	79	85
n of spheroplasts imaged	5	27	19	24	26

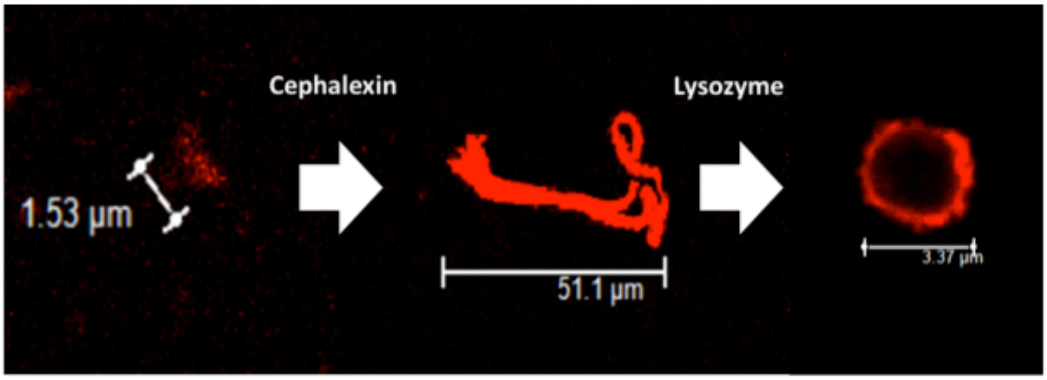
Table 3: Percentages of imaged spheroplasts showing translocation and membrane localization of magainin 2 for different spheroplast batches

	Batch 1	Batch 2
% translocating	17	21
% membrane localized	83	78
n of spheroplasts imaged	46	14

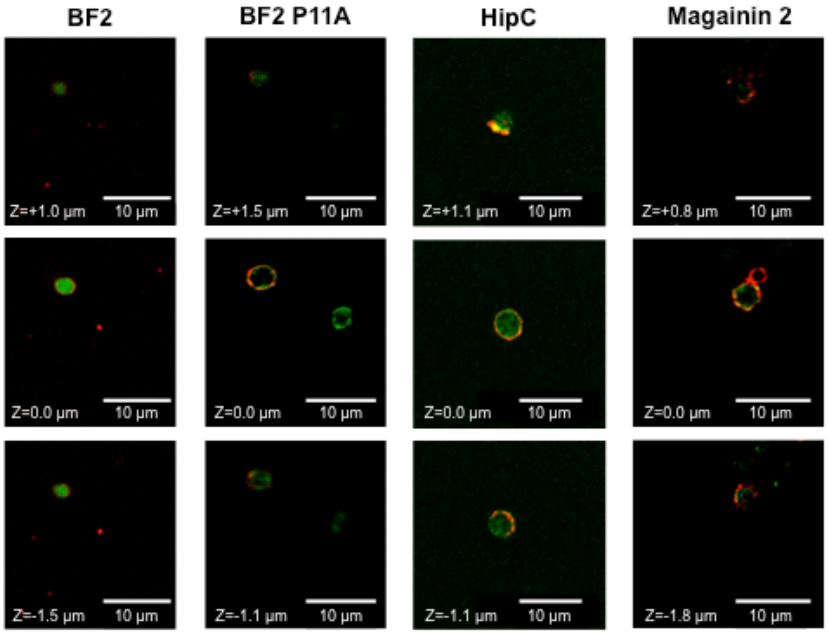
Table 4: Percentages of imaged spheroplasts showing translocation and membrane localization of HipC for different spheroplast batches

	Batch 1	Batch 2	Batch 3	Batch 4
% translocating	71	100	100	100
% membrane localized	28	0	0	0
n of spheroplasts imaged	7	2	22	36

Supplemental Figures



Supplemental Figure 1: Representative confocal microscopy images of *E. coli* at different stages during the formation of spheroplasts. Fluorescence shown is from the membrane dye di-8-ANEPPS.



Supplemental Figure 2: Confocal images of representative *E. coli* spheroplasts incubated with FITC labeled peptides (BF2, P11A BF2, HipC or magainin 2) and di-8-ANEPPS. The merged fluorescence of FITC (green) and di-8-ANEPPS (red) is shown. Images from three different positions in a single z-stack are shown for each peptide; z-positions are given relative to the middle image of each stack.

Supplemental Information References

1. **Martinac, B., M. Buechner, A.H. Delcour, J. Adler, and C. Kung**, 1987 Pressure-sensitive ion channel in Escherichia coli. Proc Natl Acad Sci U S A **84**:2297-301.