Adaptation of Escherichia coli Spheroplasts to the Characterization of Antimicrobial Peptides (AMPs)

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Adaptation of *Escherichia coli* Spheroplasts to the Characterization of Antimicrobial Peptides (AMPs)

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Submitted in Partial Fulfillment of the Prerequisite for Honors in Biochemistry

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ABBREVIATIONS

AMP: Antimicrobial Peptide
HDAP: Histone Derived Antimicrobial Peptide
CPP: Cell Penetrating Peptide
RDA: Radial Diffusion Assay
PI: Propidium Iodide
TSB: Tryptic Soy Broth
BF2: Buforin II
BF1: Buforin I
HC: HipC
TM: Transmembrane Domain 1 of Mechano-sensitive Channel of Large Conductance Protein
HT: HipC + TM1
TH: TM1 + HipC
M: Magainin 2/Magainin
DP: Des1 + Parasin
PD: Parasin + Des1
GUV: Giant Unilamellar Vesicle
FITC: Fluorescein Isothiocyanate
CFU: Colony Forming Units
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ABSTRACT

With activity against a broad range of microorganisms and low susceptibility to the resistance mechanisms developed by multidrug resistant organisms, naturally-occurring antimicrobial peptides (AMPs) have the potential to serve as effective antimicrobial agents to combat the ever-increasing number of antibiotic-resistant illnesses. In addition to various spectroscopic measurements, antimicrobial activity assays, and molecular modeling, the mechanisms of action of novel AMPs can be further characterized using confocal microscopy. This imaging technique utilizes fluorescently-labeled peptides or fluorophore-containing peptide conjugates to examine the localization of antimicrobial peptides in bacterial cells, providing insight into a peptide’s mechanism of action. However, the small size and the rod shape of bacteria make it difficult to acquire clear and conclusive images to determine the localization of the peptide in the bacterial cell. The present study focuses on the adaptation of Escherichia coli spheroplasts, which are spherical and at least four times larger than normal bacteria, as a model for the characterization of the mechanisms of action of AMPs. The larger size of the spheroplasts increases the ease of focusing on the target and allows clearer images with increased resolution. The spherical shape of the spheroplasts allows for equivalent images to be taken from any angle of the sample. Together, the three aspects of optimization, enlargement of size, uniformity of shape, and addition of membrane dye, improved the resolution of the confocal images, allowing a more reliable and consistent determination of peptide localization in spheroplasts and their mechanism of action. This approach of confocal microscopy imaging in E. coli spheroplasts can then be applied to further characterize a wide variety of molecules, including AMPs and other cell-penetrating, non-antimicrobial peptides.
1. **INTRODUCTION**

1.1. *Antimicrobial peptides and their potential applications*

The ever-growing number of multi-antibiotics resistant bacterial strains led to a pressing demand for antimicrobial agents with novel mechanisms of action (Wang et al. 2015; Pavia et al. 2012; Hale and Hancock 2007; Hancock and Diamond 2000). With mechanisms of action that confer low susceptibility to the resistance mechanisms developed by multidrug resistant organisms and high activity against a broad range of microorganisms, naturally occurring antimicrobial peptides, or host defense peptides, show potential as a source for novel and more effective antimicrobial agents (Rotem and Mor 2009; Wang et al. 2015; Gaspar et al. 2013; Fernebro 2011). These AMPs can be found in a wide variety of species, ranging from prokaryotes to insects, plants, amphibians, and mammals including humans (Rotem and Mor 2009; Wang et al. 2015). In these organisms, the peptides serve as the first line of defense against pathogens as well as essential effectors of the host’s innate immune system (Jenssen et al. 2006; Rotem and Mor 2009; Wang et al. 2015; Birkemo et al. 2003; Birkemo et al. 2004; Xie et al. 2011; Bulet et al. 2004).

Antimicrobial peptides (AMPs) are usually short peptides containing less than 100 amino acid residues, multiple positive charges, and a number of hydrophobic residues (Wang et al. 2015; Pavia et al. 2012; Rotem and Mor 2009; Ganz 2003). These small and often amphipathic peptides are capable of interacting with the bacterial membranes through non-specific interactions with the lipid molecules (Gaspar et al. 2013; Seo et al. 2012). The diversity of antimicrobial peptides isolated leads to difficulty of peptide classification; as a result, the AMPs are only broadly grouped based on their secondary structures (Zasloff 2002; Hale and Hancock...
An underlying structural basis for all categories of AMPs is the amphipathic organization of residues in the peptides, where the peptide clusters the positively charged and hydrophobic residues in separate regions of the peptide (Zasloff 2002). Peptides are then differentiated into different categories based on additional modifications to this foundation and different secondary structures (Zasloff 2002).

For conventional antibiotics like penicillin and vancomycin, bacteria can develop and employ one or a combination of resistant mechanisms, such as the enzymatic degradation of antibiotics inside the cells, alteration of bacterial protein targets through mutations, and changes in membrane permeability to the antibiotics and/or systems of drug molecule efflux, leading to quick acquisition of resistance (Dever and Dermody 1991; Berger-Bächli 2002). For AMPs, the short time-frame of their interactions to the microbe membranes not only leads to rapid death of the microbes but also decreases the chance of resistance development (Gaspar et al. 2013; Fernebro 2011). Considering the span of time and the amount of energy necessary for bacteria to evolve a specific resistance mechanism to a particular antimicrobial agent, since the antimicrobial peptides target the bacterial membrane, the bacterial strain would have to re-design its bacterial membrane by changing the composition and organization of the lipid molecules, an option that is very energetically and temporally costly for most, if not all, microbial species (Zasloff 2002). A microbe species also faces more than one type of AMPs at a time so the development of one resistance mechanism effective against a single set of AMPs would likely not increase the survival chance of that microbe species significantly (Zasloff 2002).

Together, these factors make naturally occurring antimicrobial peptides and their semisynthetic and synthetic derivatives a promising source for tools to combat antibiotic resistance illnesses.
As part of therapeutics development, other applications of AMPs have been explored, leading towards the development of antimicrobial peptide surface coatings with anti-biofilm properties, establishing nanoparticle-based drug delivery systems, and using antimicrobial peptides as biosensors and detectors of bacterial pathogens (Wang et al. 2015; Gaspar et al. 2013; Weinberg et al. 1998; Seo et al. 2012). Human antimicrobial peptide, LL-37, has been re-engineered to increase its effectiveness in inhibiting bacterial attachment and the formation and growth of biofilms in community-associated and hospital isolated methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Mishra et al. 2015). Majority of the current clinical trials exploring antimicrobial peptide as a treatment has been focusing on topical applications, such as treatment of diabetic foot ulcers and catheter-related infections (Fernebro 2011). Development of AMPs into drugs faces issues of toxicity during internalization, high cost of peptide synthesis, and instability and susceptibility towards protease degradation (Fernebro 2011). However, the dependence of AMP function on the biochemical properties rather than specific amino acid sequences has presented opportunities in developing synthetic peptide mimics for therapeutic applications (Fernebro 2011).

In addition to their antibacterial properties, AMPs also show activity against fungi and viruses, playing a role in combating other infectious diseases (Jenssen et al. 2006; Wang et al. 2015). The potential of AMPs to be used as anticancer peptides in cancer therapy either alone or with other conventional drugs has also been explored in an effort to develop drugs that are more selective towards cancerous cells and more effective in activity (Gaspar et al. 2013). Antimicrobial peptides (AMPs) present a hopeful arsenal for combating various infectious diseases and cancer.
1.2. Structures and mechanisms of antimicrobial peptides

AMPs can be classified into four categories based on their secondary structures: 1) α-helical peptides; 2) β-sheet peptides; 3) extended peptides; and 4) loop peptides (Seo et al. 2012; Jenssen et al. 2006). However, there are many peptides that do not belong in any one of the four categories; instead, they may have both an alpha-helical domain and a beta-sheet structure or other combinations of structures (Seo et al. 2012; Jenssen et al. 2006). Some peptides exhibit these secondary structures when interacting with membranes but adopt a different structure or remain unstructured in an aqueous environment (Jenssen et al. 2006). Structural stabilization of the membrane-bound forms of AMPs via covalent bonding and/or disulfide bond introduction has improved potency of some AMPs against bacterial strains and decreased their susceptibility against protease activity (Jenssen et al. 2006).

As noted previously, AMPs are short peptides with multiple positively charged residues and hydrophobic residues, giving them an amphipathic characteristic that is essential to their mechanisms of action. These cationic AMPs target the bacterial cell membrane, which has an outermost leaflet that is heavily populated by negatively charged lipid molecules. This difference in composition of lipid molecules in bacterial cells and eukaryotic cells, whose outer leaflet of the lipid bilayer are populated by phospholipids with no net charge, leads to the selectivity of AMPs towards bacterial cells (Zasloff 2002).

AMP mechanisms of action can be categorized into two groups, membrane permeabilization and translocation (Scheme 1). Membrane permeabilizing peptides rely on the disruption of bacterial cell membrane integrity and cause cell death through cell leakage and/or disruption of the normal functions of the cell membrane. This interaction begins with the initial
electrostatic attraction between the cationic peptide and the negatively charged components of the lipid molecules in the outer leaflet of the cell membrane, for instance, the phosphate head groups of the lipopolysaccharides in Gram-negative bacteria or the lipoteichoic acids on the membranes of Gram-positive bacteria (Jenssen et al. 2006; Scott et al. 1999). With Gram-negative bacteria, peptides integrate into the outer membrane structure via hydrophobic interactions, sometimes, requiring the peptides to adopt their respective membrane-associated conformations (Jenssen et al. 2006). The insertion of peptides permeabilizes this outer membrane, allowing other peptides to arrive and interact with the cytoplasmic membrane through a combination of electrostatic and hydrophobic interactions (Jenssen et al. 2006). Various models of the exact interaction of the peptide at the membrane surface have been proposed, the barrel-stave, carpet, detergent, toroidal pore, and aggregate models (Jenssen et al. 2006; Hale and Hancock 2007). Together, these models describe the pathways that peptides may take to arrive at one of the following outcomes: dissolution of the membrane, formation of a transient channel, or translocation across the membrane (Jenssen et al. 2006). Magainin, an antimicrobial peptide isolated from the skin of *Xenopus laevis*, has been found to permeabilize cell membrane through pore formation (Ludtke et al. 1996). Peptides that employ translocation mechanism can cross the cell membrane without significantly permeabilizing the membrane and accumulate inside the cell, where they interact with intracellular targets and interfere with essential cellular processes, resulting in cell death (Jenssen et al. 2006). Buforin II, an antimicrobial peptide derived from toad stomach tissue, has been found to cause cell death by translocating across bacterial membrane and binding to nucleic acids (Park et al. 2000; Kobayashi et al. 2000).
1.3. **Histone-derived antimicrobial peptides**

Antimicrobial peptides are usually derived from larger precursors, arriving at the final forms from post-translational modifications or after proteolysis from larger proteins (Kim et al. 2000; Zasloff 2002). One such example of AMPs that are derived from larger proteins after proteolysis is one family of antimicrobial peptides that shares homology with histone subunits. Along with their role in the winding of DNA and the formation of chromatin, different histone protein variants have also shown antimicrobial properties (Norbury and Zhivotovsky 2004; Cho et al. 2002; Parseghian and Luhrs 2006; Lee et al. 2008; Kawasaki and Iwamuro 2008; Konishi et al. 2003; Ruiz-Vela and Korsmeyer 2007). Studies have isolated and identified subunits of histone proteins, termed histone-derived antimicrobial peptides (HDAPs), from fish (Birkemo et al. 2003; Sathyan et al. 2013), amphibians (Cho et al. 2009), and other species (Sathyan et al. 2012; Smith et al. 2010). These peptides have activity against both Gram-positive and Gram-negative bacterial strains (Birkemo et al. 2003; Cho et al. 2009; Sathyan et al. 2012; Smith et al. 2010). Histone H1 from Atlantic salmon, *Salmo salar*, exhibited antimicrobial activity against *E. coli* through a membrane permeabilization mechanism, similar to that of magainin II (Richards et al. 2001). N-terminus of histone H2A contains high numbers of positively charged amino acid residues, allowing histone H2A subunit to serve as a precursor for antimicrobial peptides (Sathyan et al. 2013). Amongst various histone-derived AMPs, those sharing homology with the histone H2A subunit seem to be the most common, including Harriottins, derived from histone H2A subunit of sicklefin chimaera *Neoharriotta pinnata* (Sathyan et al. 2013); Sphistin, derived from crab histone H2A in haemolymphs of *Scylla paramamosain* and exhibited a membrane permeabilization mechanism (Chen et al. 2015); and parasin I, isolated from catfish skin mucus.
and employed membrane permeabilization to kill bacterial cells (Park et al. 1998; Koo et al. 2008). One group of the most extensively studied histone H2A derived AMPs were the buforins, isolated from the stomach tissue of the Asian toad *Bufo bufo gargarizans* (Cho et al. 2009). Buforin I displays stronger *in vitro* antimicrobial activity against a broad range of microbes compared to other AMPs isolated from amphibians, like magainin 2, one of a group of AMPs isolated from the skin of African clawed frog *Xenopus laevis* and utilized membrane permeabilization through pore formation (Ludtke et al. 1996; Cho et al. 2009). Buforin I and its more potent derivative, buforin II, contain a helix-hinge-helix domain structure and kill bacterial cells by crossing the cell membrane without significant permeabilization and binding to nucleic acids (Park et al. 1996; Park et al. 2000; Cho et al. 2009). Studies have found that the Pro hinge within the buforins plays an important role in the translocation of this peptide into bacterial cells; replacement of Pro with Ala leads to decreased translocation and antimicrobial activity and increased membrane permeabilization (Xie et al. 2011; Kobayashi et al. 2000).

The current study focuses on a histone-derived antimicrobial peptide, hipposin, isolated from the skin mucus of Atlantic halibut, *Hippoglossus hippoglossus* L. (Bustillo et al. 2014; Birkemo et al. 2003; Birkemo et al. 2004). The sequence of this relatively large, 51-residue, histone H2A derived antimicrobial peptide can be separated into three regions, two of which are almost identical to two smaller HDAPs with different mechanisms of action against bacteria (Bustillo et al. 2014). The 19 N-terminal residues of hipposin are homologous to parasin I (Table 1; Park et al. 1998; Koo et al. 2008; Bustillo et al. 2014). A 21-amino acid residue section in the middle of the hipposin sequence is identical to buforin II (BF2) (Park et al. 1996; Park et al. 2000; Bustillo et al. 2014; Cho et al. 2009; Pavia et al. 2012; Elmore 2012). The 39-amino acid AMP
parent peptide of BF2, buforin I (BF1), is a fusion of parasin I and BF2 peptide segments (Cho et al. 2009; Bustillo et al. 2014). The C-terminal residues of hipposin, HipC, is a cell-penetrating peptide that can enter the cell without greatly disturbing the cell membrane integrity and has no measurable antimicrobial activity (Bustillo et al. 2014). HipC is found to be the first membrane-crossing histone derived fragment that does not kill bacteria or eukaryotic cells (Bustillo et al. 2014).

Table 1. Amino acid sequences of histone H2A derived antimicrobial peptide, Hipposin, and other AMPs that share homology with segments of Hipposin. Membrane – membrane permeabilization; Translocate – translocation/membrane crossing; Green: presence of antimicrobial activity in antimicrobial activity assay.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hipposin</td>
<td>KGRGKQGGKVRAKWKTRSSRAGLQWPVGRVHRLLRKGNYAHVGAGAPVWL</td>
<td>Membrane</td>
</tr>
<tr>
<td>Buforin I (BF1)</td>
<td>KGRGKQGGKVRAKWKTRSSRAGLQWPVGRVHRLLRKG</td>
<td>Translocate</td>
</tr>
<tr>
<td>Buforin II (BF2)</td>
<td>TRSSRAGLQWPVGRVHRLLRKG</td>
<td>Translocate</td>
</tr>
<tr>
<td>Parasin</td>
<td>KGRGKQGGKVRAKWKTRSS</td>
<td>Membrane</td>
</tr>
<tr>
<td>HipC (HC)</td>
<td>GNYAHRVGAGAPVWL</td>
<td>Translocate</td>
</tr>
</tbody>
</table>

1.4. Applications for translocating, non-antimicrobial peptides

With growing numbers of life-threatening diseases and the development of more effective treatments, increased effort and interest have been placed upon cell-penetrating peptides (CPPs) in utilizing these peptides as delivery systems for highly potent and target specific therapeutic peptides and proteins, whose large size and hydrophilic nature cause difficulty in traversing cellular membranes and other barriers such as the blood brain barrier, gastrointestinal mucosa, and the skin (Kristensen et al. 2016; Dinca et al. 2016; Zhang et al. 2016). The human immunodeficiency virus (HIV) trans-activator of transcription protein, Tat,
was the first CPP discovered (Ruben et al. 1989; Rizzuti et al. 2015; Dinca et al. 2016). pTat, a derivative of Tat protein that harness the ability of Tat protein to cross cellular and tissue barriers, has been conjugated to a variety of molecules, including nanoparticles as a part of nano-carriers for specific delivery of drugs to tumor cells (Torchilin 2007); and used to facilitate the transmembrane transport of small molecules, antibodies, therapeutic peptides and proteins (Rizzuti et al. 2015; Howl et al. 2007). A CPP, p28, with an affinity for cancerous cells, has shown to enhance the tumor suppressor function of p53 by stabilizing p53 and leading to more specific targeting of tumors (Dinca et al. 2016; Yamada et al. 2009). With its translocating but non-antimicrobial characteristics, the HipC fragment of the antimicrobial peptide, hipposin, may have a potential to function as a biological cargo delivery system that can ferry peptides, nucleic acids, small molecules, and other cargos into a cell.

1.5. Characterization of AMP mechanism of action

Characterization of the mechanism of action of antimicrobial peptides of interest, whether naturally-occurring peptides or those that are designed and synthesized, is a crucial step in the understanding of properties that make these peptides effective against bacteria and other microorganisms. Understanding these trends can help to advance the development of novel antimicrobial agents, offering more options for treating antibiotic-resistant illnesses.

Conventionally, mechanism of action of an AMP can be determined using various in vitro and in vivo assays, such as propidium iodide uptake assay for probing of membrane permeabilization in vivo in a bacterial system (Bustillo et al. 2014; Steinberg and Lehrer 1997) and artificial model membrane-based assays utilizing spectroscopic measurements for probing of translocation (Park et al. 1998; Xie et al. 2011). However, these techniques can only provide
limited, indirect information about the interaction between peptide and live bacterial cells under physiological conditions.

To this end, in addition to various spectroscopic measurements, antimicrobial activity assays, and molecular dynamic simulations, the mechanisms of action of novel AMPs and their interactions with the bacterial cell membrane can be further characterized and visualized using confocal laser-scanning microscopy. This imaging technique utilizes fluorescently-labeled peptides or fluorophore-containing peptide conjugates to examine the localization of antimicrobial peptides in the presence of bacterial cells, providing insight into a peptide’s mechanism of action. This approach allows more direct, cellular-level visualization of peptide interaction with the bacterial membrane and leans more towards the “single-cell” measurement category compared to the “bulk” measurement of typical biochemical assays. Park and colleagues (2000) examined the localization of buforin II analogs and magainin 2 using biotinylated peptides that can be visualized by streptavidin-FITC (Park et al. 2000). Confocal microscopy images visualized the different localization of the buforin II proline mutant compared to the native BF2, pointing out the importance of the presence of this proline residue in defining the mechanism of action for BF2 (Park et al. 2000). Pavia and colleagues (2012) utilized biotinylated peptides visualized using streptavidin-AlexaFluor 488 fluorophore to visualize the localization of histone derived antimicrobial peptides designed based on properties of BF2, obtaining a qualitative, preliminary understanding of the mechanism of these novel peptides that leads to more comprehensive studies via additional methods (Pavia et al. 2012; Cutrona et al. 2015).
While confocal microscopy imaging does allow the visualization and characterization of peptide-membrane interaction in live bacterial cells, the technique is limited by the small size and the shape of the bacterial cells, for example, the rod shape of *Escherichia coli*. The physical dimensions of the bacteria make it difficult to acquire clear and conclusive images necessary for the consistent and reliable determination of the localization of the peptide in the bacterium. In confocal microscopy imaging, consecutive, independent focal planes, or “slices”, are produced when the point of laser light travels through the thickness axis of the sample, creating a stack of images for the sample, a bacterium, for example. Due to the small size of a bacterium, the focal planes can overlap and fluorescence signals from different focal planes can contaminate each other, producing blurry and inconsistent images (Scheme 2). For example, for a membrane permeabilizing peptide, its membrane localizing fluorescence can “contaminate” the supposedly independent focal plane for the interior of a bacterial cell, giving the false appearance that the peptide fluorescence is also inside the bacterium, leading to ambiguities in determination of peptide localization and mechanism characterization (Figure 1b and 1c).

Giant unilamellar vesicles (GUVs) have been used instead of bacterial cells for the characterization of the mechanism of action of antimicrobial peptides and cell-penetrating peptides through visualization via confocal microscopy imaging. Ambroggio and colleagues (2005) visualized the membrane permeabilization of Maculatin 1.1, Citropin 1.1, and Aurein 1.2 in GUVs and differentiated the lytic mechanisms of these peptides between the carpet model and the pore-forming model (Ambroggio et al. 2005). Islam and colleagues (2014) visualized the entry and membrane permeabilization of cell-penetrating peptide, Transportan 10, into single GUV through the decrease in vesicle interior fluorescence due to the leakage of fluorophores
through pores formed by Transportan 10 over time (Islam et al. 2014). Single-cell, time-resolved microscopy studies have also been utilized to explore the sequence of events that occur via the addition of AMPs in bacterial cells (Choi et al. 2016). Antimicrobial peptide alamethicin was found to kill *Bacillus subtilis* through a series of events starting from cell growth halting, membrane permeabilization, and leakage of cell content (Barns et al. 2016).

While the use of GUVs bypasses the resolution issues that the imaging of bacterial cells faces, the utilization of artificial model membranes take away the more physiological relevant aspect of the interaction between AMP and live cell. In order to overcome the resolution limitations posed by imaging of bacterial cells and to characterize mechanism of action of AMPs in a model that is as biologically relevant as possible, *Escherichia coli* spheroplasts show promise in fulfilling the above mentioned condition. *E. coli* spheroplasts in this study are prepared by exposing normal *E. coli* bacteria to cephalexin, which prevents cell division and leads to the formation of “snake-like” bacterial filaments (Martinac et al. 1987). These “snake” filaments are then treated with a mixture of reagents: EDTA and Tris to disrupt cell wall integrity through the removal of metal ions; lysozyme to digest the peptidoglycan layer; and DNase I to lower the amount of clumping in the mixture (Martinac et al. 1987; Goldschmidt and Wyss 1967). *E. coli* spheroplasts have been commonly used for studies of the relationship between protein localization and membrane curvature (Renner and Weibel 2011), ion channels (Shaikh et al. 2014), and mechanical properties of various membranes (Sun et al. 2014). The present project examines the potential of using *E. coli* spheroplasts combined with confocal microscopy imaging in the characterization of the mechanism of action of AMPs.
1.6. **Overview**

The first portion of this study aims to improve resolution from three aspects: 1) enlarge the size of the bacteria; 2) change the shape of the bacteria; and 3) label the membrane with a membrane dye to create more contrast for the peptide fluorescence. These three aspects are achieved through the adaptation of *Escherichia coli* spheroplasts as a model system for the characterization of an antimicrobial peptide’s mechanism of action. *E. coli* spheroplasts are spherical in shape and are at least three to four times larger (2 – 5μm) than the width axis (approximately 1μm) of normal bacterial cells. The larger size of the spheroplasts increases the ease of focusing on the target and allows for clearer images with increased resolution and more convincing evidence of peptide localization. The spherical shape of the spheroplasts allows for images to be taken from any angle of the sample with equal clarity. The successful adaptation of *E. coli* spheroplasts with confocal microscopy will allow the procedure to be used for the further characterization of a wide variety of molecules, including AMPs and other cell-penetrating peptides without antimicrobial activity.

The second portion of the current study utilizes the optimized method to further investigate the mechanism of action of HipC, a peptide segment derived from hipposin that has been found to be cell-penetrating but with no significant antimicrobial activity. With its translocating and non-antimicrobial property, HipC has the potential to serve as a transporting vehicle that can “pull” a “cargo” molecule across the bacterial membrane. The characterization and confirmation of the mechanism of action of HipC serves as a critical checkpoint in the continuation of the investigation into chimeric peptide constructs containing the HipC segment.
Scheme 1. Two general categories of AMP mechanism of action, membrane permeabilization (left) and translocation (right). Green: lipid bilayer; Purple and Blue: peptide. Figure modeled after Maria LaBouyer’s 2014 Ruhlman slides.
2. **Materials and Methods**

2.1. *Peptide Design and Synthesis*

The peptide sequences used in this study are shown in Table 1. Peptides were synthesized both with and without an N-terminally linked biotin or FITC at >95% purity by NeoBioSci (Cambridge, MA). All peptide stocks were quantitated from tryptophan absorbance using the average of at least three $A_{280nm}$ measurements.

2.2. *Radial Diffusion Assays (RDA)*

The radial diffusion assay was performed using *E. coli* (ATCC #25922), *E. coli* Top10, *Staphylococcus epidermidis* (Carolina #155556), *Bacillus thuringiensis* (Carolina #154926), and *Serratia marcescens* (Carolina #155450) in a manner similar to previous studies (Steinberg and Lehrer 1997; Bustillo et al. 2014). A single colony picked from a TSB plate was incubated overnight at 37°C (*E. coli* and *S. epidermidis*), 30°C (*B. thuringiensis*), and 28°C (*S. marcescens*) in 2 mL tryptic soy broth (TSB). The overnight culture is then diluted 1:100 in fresh TSB and grown for 2.5 hours for *E. coli* strains and for 3.5 hours for other strains until OD$_{600}$ of 0.5 – 0.7.

Bacteria were pelleted via centrifugation at 880xg for 10 minutes at 4°C, washed once with 10mM phosphate buffer (pH 7.4), then pelleted again and re-suspended in 10mM phosphate buffer (pH 7.4). 4x10$^6$ CFU of bacteria are mixed with 10mL of molten agarose gel (10mM phosphate, 1% TSB v/v, 1% agarose w/v, pH 7.4) and allowed to solidify on a petri dish. Wells were formed in the solid media using a Pasteur pipette attached to a bleach trap. One microliter ($\mu$L) of 10$^{-4}$M peptide solution was added to each well, and plates were incubated at respective temperatures for 3 hours. Ten milliliter (mL) of overlay gel (30% w/v TSB, 1% w/v
agarose) was poured over the underlay gel and incubated for 12 – 18 hours at appropriate temperatures for each bacterial strain. The diameter of bacterial clearing around each well was measured at 7x magnification. The diameter of clearance around a well that contains only deionized water (negative control) was 1mm. Data were collected from at least three independent plates.

2.3. Preparation of E. coli Spheroplasts

2.3.1. Multi-cell 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. (1987). 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 (25μg/mL final concentration) and allowed to grow to OD$_{600}$ of 0.5 – 0.7. 3mL of this culture was diluted 1:10 into ampicillin-containing TSB media and cephalexin was added to reach a final concentration of 60μg/mL. The culture was then shaken at 37°C for 2 – 3 hours until single-cell filaments reached sufficient length (about 50 – 150μm) observable under light microscope at 1000x oil immersion magnification.

Filaments were harvested by centrifugation at 1500 x g for 4 minutes, and the pellet was rinsed without resuspension by gentle addition of 1mL of 0.8 M sucrose with 1 min incubation at room temperature and then re-suspended in 3mL of 0.8M sucrose after supernatant has been removed via pipetting. The following reagents were added in order: 150μL of 1M Tris Cl (pH 7.8); 120μL of lysozyme (5mg/ml); 30μL of DNase I (5mg/ml); and 120μL of 0.125M sodium
EDTA (pH 8.0). This mixture was incubated at room temperature for 6 - 10 minutes to hydrolyze the peptidoglycan layer, and appearance of spheroplasts during spheroplast formation was followed under microscope at 1000x. 1 mL of Solution A (20mM MgCl$_2$, 0.7M sucrose, 10mM Tris Cl at pH 7.8) was gradually added over 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 (10mM MgCl$_2$, 0.8M sucrose, 10mM 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. Prepared spheroplasts were either used immediately or stored at -80˚C to be used within a one-week period.

2.3.2 Single-cell Spheroplasts

Single-cell spheroplasts were prepared following the same procedure as the preparation of multi-cell spheroplasts described previously, except with the omission of the second incubation with cephalixin. Following the addition of four reagents for hydrolysis of the peptidoglycan layer, the appearance of single-cell spheroplasts as cluster of circular cells during spheroplast formation was followed under microscope at 1000x. Prepared single-cell spheroplasts were either used immediately or stored in -80˚C freezer to be used within one-week period.
2.4.  Confocal Microscopy Imaging

2.4.1.  Bacteria

Peptide localization in *E. coli* (Top10 containing a pET45b plasmid for ampicillin resistance) were done in a manner similar to that described in Bustillo et al. 2014. An overnight culture grown (37˚C in shaking incubator, approximately 180 rpm) from one plate-picked colony was diluted 1:100 in TSB liquid media in the presence of ampicillin (25μg/ml final concentration) and allowed to grow to mid-logarithmic phase, an OD₆₀₀ of 0.5 – 0.7. Bacteria were then pelleted by centrifugation at 880 x g, washed, and re-suspended in sterile 10mM sodium phosphate buffer (pH 7.4). Bacterial cells at a concentration of 10⁷ CFU/mL were incubated with 4μg/mL of either biotinylated peptide or FITC-labeled peptide (peptide stock concentration range between 1.1E-4M and 6.2E-4M) for 30 minutes at 37˚C. Cell/peptide solutions were then placed on a poly-L-lysine coated glass slide. Biotinylated peptides were rendered fluorescent by the addition of streptavidin conjugated with AlexaFluor 488 (Invitrogen) at a final concentration of 5μg/mL. 1μL of Di-8-ANEPPS (Biotium, Hayward, CA) membrane dye (30μM in DMSO) was also added to membrane labeled samples. Cells were visualized with a Leica TCS SP5 laser scanning confocal microscope with excitation at 488 nm by an argon laser at 20% power output, 20% transmission, and emission ranges of 499 - 532 nm (FITC), 500 - 535 nm (AlexaFluor 488) and 670 - 745 nm (membrane dye 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 of 0.04 – 0.08 μm thickness were evaluated visually for localization of peptide fluorescence within the spheroplast.
2.4.2. **Spheroplasts**

Single-cell or multi-cell spheroplasts were either prepared immediately before or thawed from frozen stock (frozen for no more than one week) at -80°C and diluted 1:2 (multi-cell spheroplasts) and at least 1:5 (single-cell spheroplasts) in 0.8M sucrose, respectively. Diluted spheroplasts were then placed on a poly-L-lysine coated glass slide and incubated with equal volume of FITC-labeled peptide or biotinylated peptides (peptide stock concentration range between 1.1E-4M and 6.2E-4M) for 1 minute (or 10 - 20 minutes for FITC HipC only) for FITC-peptide only samples or biotinylated peptide-only samples. Streptavidin-conjugated AlexaFluor 488 (Invitrogen) was added to biotinylated peptide-spheroplast mixture to create a 1:1:1 peptide-fluorophore-spheroplast mixture for biotinylated peptide samples. 1μL of Di-8-ANEPPS (Biotium, Hayward, CA) membrane dye (30μM in DMSO) was also added to membrane labeled samples. Spheroplasts were visualized with a Leica TCS SP5 laser scanning confocal microscope following the same procedure described above for bacterial cells.

2.5. **Propidium Iodide Uptake Assay (PI) for Membrane Permeabilization**

2.5.1. **Bacteria**

An *E. coli* single bacterial colony picked from a plate was grown overnight in TSB media (30% w/v TSB) at 37°C. The overnight culture was diluted 1:100 in fresh TSB and allowed to grow to 2.5 hours to mid-logarithmic phase with OD<sub>600</sub> of 0.5 – 0.7. Bacteria were pelleted via centrifugation at 880xg for 10 minutes at 4°C, washed once with 10mM phosphate buffer (pH 7.4), pelleted again, and re-suspended to an OD<sub>600</sub> of 0.5. 3 mL of this bacterial solution was exposed to propidium iodide (final concentration of 20μg/mL from 2mg/mL stock) and allowed
to equilibrate. PI/DNA complexation was measured at an excitation of 535nm and emission of 617nm on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA). Following 5-minute equilibration to ensure the thorough mixture of PI with bacterial cells and the stabilization of fluorescence signal baseline, peptide solution was added to a concentration of 2μM and fluorescence intensity was monitored for a 40-minute period. Increase in fluorescence due to the peptide’s presence was quantified by comparing the averaged fluorescence 5 minutes after peptide addition to the averaged fluorescence intensity during the one minute before peptide addition.

2.5.2. **Spheroplasts**

Multi-cell spheroplasts either prepared immediately before or thawed from frozen stock (frozen for no more than one week) at -80°C were diluted in 0.8M sucrose to an OD$_{600}$ of 0.010 – 0.012. 3 mL of this spheroplast solution was exposed to propidium iodide (final concentration of 20μg/mL from 2mg/mL stock) and allowed to equilibrate. PI/DNA complexation was measured at an excitation of 535nm and emission of 617nm on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA). Following 5-minute equilibration to ensure the thorough mixture of PI with spheroplasts and the stabilization of fluorescence signal baseline, peptide solution was added to a concentration of 2μM and fluorescence intensity was monitored for a 40-minute period. Increase in fluorescence due to the peptide’s presence was quantified by comparing the averaged fluorescence 5 minutes after peptide addition to the averaged fluorescence intensity during the one minute before peptide addition.
3. RESULTS AND DISCUSSION

3.1. Determination of peptide mechanism of action via confocal microscopy imaging of bacterial cells is restricted by resolution

To further investigate the mechanism of action of our peptides of interest, confocal microscopy imaging was utilized to examine the localization of fluorophore-conjugated peptide in a bacterium. Representative image stacks of peptide exhibiting membrane permeabilization mechanism, DP, and translocation mechanism, Des1, are shown in Figure 1a (Figure 1a; Table 2). However, the accurate characterization of peptide mechanism based on localization inside bacterial cells is limited by the small size and rod shape of the bacterium and the resolution limit of the instrument. This leads to ambiguity in the determination of localization of peptide when signals from the membranes of a bacterial cell “bleed-through” into the interior “slice” of the cell, as observed in image stacks visualized with fluorophore-streptavidin biotinylated peptide conjugates and membrane dye di-8-ANEPPS (Figure 1b and 1c).

More specifically, a typical E. coli bacterium has a thickness between 0.5μm and 1.0μm and a length around 2.0μm while the thinnest “slice” that our instrument was able to resolve is 0.773μm (Scheme 2). The small size of a bacterium pushes the resolution limit and more than likely, if not always, the supposedly individual focal planes (“slices”) taken inside the cell would contain an average of signals from one or both membranes and the center of a bacterial cell, leading to uncertainty in distinguishing the source of the fluorescence signal, whether from inside the cell or on the bacterial cell membrane. Additionally, the orientation of a bacterium at the time of imaging is also uncontrollable. The rod shape of bacterial cells affects the signal “contamination” between focal planes or “slices” and therefore makes it difficult to specify the location of a particular peptide in a bacterial cell. In order to determine the localization of our
peptides and their respective mechanism of action in a more reliable and consistent manner, we needed to develop a method that can bypass the resolution limitation posed by our instrument and the small size of bacterial cells.

Figure 1. Representative confocal images of *E. coli* bacterial cells exposed to fluorophore-peptide conjugates reveals resolution limitations. a) Representative images of membrane permeabilizing peptide, DP, and translocating peptide, Des1. Distance between top and middle slices: 1.3μm (DP) and 0.6μm (Des1); Distance between middle and bottom slices: 0.5μm (DP) and 0.2μm (Des1). b) Representative image stack showing “contamination” in the center focal plane or “slice”. Figure taken from Maria LaBouyer 2015 Ruhlman slides. c) Representative image stack using the membrane dye di-8-ANEPPS showing “contamination” in the interior “slice”. Distance between top and middle slices, 0.5μm, and between middle and bottom slices, 1.0μm. Images from top, middle, and bottom of a z-stack of each bacterial cell were chosen. Green: Streptavidin-conjugated Alexa Fluor 488 and biotinylated peptide; Red: membrane dye, di-8-ANEPPS.
Table 2. Sequences of the 10 peptides used in this study, including generally accepted mechanism of action and modifications on these peptides. Membrane = membrane permeabilization/membrane localization. All 10 peptides have their un-modified versions and those marked with X have both un-modified and modified forms. X indicates a peptide N-terminally labeled with either biotin or FITC.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mechanism</th>
<th>Amino Acid Sequence</th>
<th>Biotinylated</th>
<th>FITC</th>
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<tr>
<td>MscL TM1 (TM)</td>
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<tr>
<td>TM + HipC (TH)</td>
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<td>HipC + TM (HT)</td>
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<tr>
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<td>Translocation</td>
<td>ARDNKKTRIWPRLQAVRN</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Des1 + Parasin (DP)</td>
<td>Membrane</td>
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<td>X</td>
<td></td>
</tr>
<tr>
<td>Parasin + Des1 (PD)</td>
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</tr>
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<td>Translocation</td>
<td>GNYAHRVGAGAPVWL</td>
<td>X</td>
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Scheme 2. Overlay of a typical *E. coli* bacterial cell dimensions with the highest resolution of our microscope. Green: bacterial cell; Purple box: thinnest focal plane with a thickness of 0.773μm, the resolution limit of the instrument. Figure modeled after Maria LaBouyer 2015 Ruhlman slide.
3.2. Confocal microscopy imaging of Escherichia coli spheroplasts presents a feasible method for bypassing resolution limitation

In order to overcome these resolution limitations, we have utilized *Escherichia coli* spheroplasts in confocal microscopy imaging of fluorophore-peptide conjugates or directly fluorescently-labeled peptides. Spheroplasts are produced by growing Gram-negative bacteria in the presence of antibiotics, such as cephalexin, that prevent bacteria from dividing into individual cells but still allow bacteria to grow. The resulting elongated bacterial “snakes” are then exposed to a mixture of EDTA, Tris chloride, lysozyme, DNase I, and magnesium chloride, which disrupts the outer cell wall, hydrolyzes the peptidoglycan layer, and leads to the formation of spherical spheroplasts that are usually between 2 - 5μm in diameter. The larger size of these spheroplasts increases the ease of focusing on the target and allows clearer images with increased resolution and more convincing evidence of peptide localization. The spherical shape of the spheroplasts allows for images to be taken from any angle of the sample with equal penetration, regardless of the orientation of the spheroplast at the time of imaging.

3.2.1. Presence of divalent cation, Mg\(^{2+}\), affects the translocation of fluorophore-streptavidin biotinylated peptide conjugates into *E. coli* spheroplasts

In order to validate the feasibility of using *E. coli* spheroplasts to determine peptide localization, we examined the cellular localization of a number of previously characterized peptides (Table 2). We exposed *E. coli* spheroplasts to peptides N-terminally labeled with a biotin molecule and then added streptavidin-conjugated Alexa Fluor 488 fluorophore for imaging and visualization (Scheme 3). Figure 2 shows that the permeabilizing peptides, DP and PD, are localized on the spheroplasm membrane as expected, but, there is no localization of the translocating peptides, BF2 and Des1 (Figure 2; Table 2). This indicates that the process of
turning normal bacterial cells into spheroplasts may have altered the membrane property of
the bacteria (Figure 2). A further search within the literature on the roles of reagents used in
the spheroplast formation process suggested that the stabilizing agent, Mg$^{2+}$, may have a role in
changing the permeability of the membrane (Goldschmidt and Wyss 1967; Martinac et al. 1987).
The divalent positive charge on a magnesium ion can interact favorably through electrostatics
with the negatively charged phosphate head group on a lipid molecule. Within a lipid
membrane, Mg$^{2+}$ can potentially “pull” its neighboring lipid molecules closer together, leading
to a less permeable lipid membrane overall. In fact, translocation of biotinylated peptides
visualized by AlexaFluor 488-conjugated streptavidin into normal bacterial cells was reduced in
bacterial cells incubated with Mg$^{2+}$ as suggested by the decreased fluorescence intensity inside
those cells (Figure 3). Preliminary propidium iodide (PI) uptake studies on the permeabilization
of the bacterial cell membrane by DP showed a reduction of membrane permeabilization in the
presence of Mg$^{2+}$ compared to its absence (Figure 4a and 4b). Within the less permeable
membrane, the larger size of the fluorophore-streptavidin conjugate used to visualize
biotinylated peptides makes it more difficult to cross the membrane, leading to decreased
translocation of peptides into the cell. It is not conclusive from these images, however, whether
Mg$^{2+}$ led to membrane localization of the peptides or caused a decrease in overall translocation
and thus, the fluorescence signal into the cell, which made it seem that the localization of the
peptide had altered.
Figure 2. Confocal images of representative spheroplasts incubated with permeabilizing peptides, DP and PD, and translocating peptides, BF2 and Des1. Distance between top and middle slices: 2.8µm (DP), 0.8µm (PD), 1.8µm (BF2), and 0.8µm (Des1); Distance between middle and bottom slices: 1.0µm (DP), 0.4µm (PD), 1.3µm (BF2), and 1.1µm (Des1). Images from top, middle, and, bottom of a z-stack of each bacterial cell were chosen. Green: Streptavidin-conjugated AlexaFluor 488 and biotinylated peptide.
Figure 3. Confocal images of the translocation of Des1 into bacterial cells exposed to 5mM (left) and 0mM of MgCl₂ (right). Distance between top and middle slices: 0.8μm (5mM) and 0.7μm (0mM); Distance between middle and bottom slices: 0.3μm (5mM) and 1.3μm (0mM). Images from top, middle, and bottom of a z-stack of each multi-cell spheroplast were chosen. Green: N-terminally biotinylated peptide conjugated to streptavidin-conjugated Alexa Fluor 488.

Figure 4. Representative fluorescence intensity traces and average fluorescence intensity increase for propidium iodide entry into bacterial cells exposed to permeabilizing DP, in the presence and absence of 60mM (stock concentration) MgCl₂ (a and b). DP: un-labeled DP; Error bar = standard deviation.
3.2.2. Changing fluorophore-peptide conjugate allows translocating peptides to enter spheroplasts

Magnesium ions play an important role in the stabilization of spheroplasts, which are osmotically very fragile due to the removal of the outer bacterial cell wall. Replacement of Mg$^{2+}$ with other divalent and monovalent cations, such as Ca$^{2+}$ and Na$^+$, in either equivalent ionic strength or ionic concentrations either leads to no spheroplast formation or conversion of spheroplast back into their bacterial cell form. Reduction or complete removal of Mg$^{2+}$ in reagents led to less stable spheroplasts but did not resolve the inability of translocating peptides to gain entry across spheroplast membrane.

Turning attention to other aspects of the visualization process, we focused on the system of fluorophore and peptides used in imaging. This system of biotinylated peptides and streptavidin-conjugated fluorophore has been used in the imaging of bacterial cells in past studies (Park et al. 2000; Koo et al. 2008; Bustillo et al. 2014; Scheme 3). However, the fairly large size of the final, fluorescent conjugate has posed a concern and question in how does a large construct cross the bacterial cell membrane. The inability of this fluorescent conjugate to cross spheroplast membrane, and possibly even the bacterial membrane under some conditions, raised additional uncertainty on the reliability of past images in characterizing the mechanism of action of peptides using localization of peptides in bacterial cells. In order to answer these questions, we replaced our previous system of fluorophore-peptide with peptides directly labeled with FITC on the N-terminal. This current fluorophore-peptide system is smaller in size, with a 1:1 fluorophore-peptide ratio, and allows for more direct and free interaction between peptide and lipid membrane. Figure 5 shows that the membrane permeabilization control, BF2 P11A, and the translocation control, BF2, localized to the membrane and
spheroplast interior, respectively (Figure 5). The remaining optimization process and further data collection were conducted using FITC-labeled peptides.

Scheme 3. Fluorophore-peptide systems used for confocal imaging of bacterial cells and spheroplasts. Figure adapted from Maria LaBouyer 2015 Ruhlman slides. Left: N-terminally biotinylated peptide conjugated to streptavidin-conjugated Alexa Fluor 488. Right: N-terminally FITC-labeled peptide.
Figure 5. Representative confocal images of spheroplasts exposed to translocating peptide, BF2 (left) and permeabilizing peptide BF2 P11A (right). Distance between top and middle slices: 0.5μm (BF2) and 1.4μm (BF2 P11A); distance between middle and bottom slices: 1.7μm (BF2) and 0.7μm (BF2 P11A). Images from top, middle, and bottom of a z-stack of each multi-cell spheroplast were chosen. Green: FITC-labeled peptide.

3.3. Image resolution of spheroplast improved upon optimization via addition of membrane dye, increase in size, and uniformization of shape

To further confirm the viability of using spheroplasts as a method for characterizing peptide mechanism of action via confocal imaging, we utilized well-characterized BF2 (Park et al. 2000) as translocation positive control, and BF2 P11A (Xie et al. 2011) and Magainin 2 (Ludtke et al. 1996) as negative (permeabilizing) controls (Table 2). We examined the localization of these peptides in normal bacterial cells, single-cell spheroplasts, and multi-cell spheroplasts. Full composite images of FITC BF2, FITC BF2 P11A, and Magainin 2 in bacterial cells, single-cell spheroplasts, and multi-cell spheroplasts are shown in the Appendix section (Appendix Figures 1 – 3). For the following section on optimization, images for translocation and permeabilization controls are of FITC BF2 and FITC BF2 P11A, respectively, unless indicated otherwise.
Labeling spheroplast membranes with di-8-ANEPPS creates contrast with the fluorescence signal from the interior of the spheroplast for translocating peptides and allows for more reliable qualification of the localization of the peptide of interest visually (Figure 6, right). For membrane permeabilizing peptides, the co-localization of membrane dye and FITC-labeled peptide on the spheroplast membrane pinpoints the location of the peptide (Figure 6, left). The decrease in fluorescence intensity of membrane dye observed in permeabilization peptide images can be attributed to the disruption of lipid molecule arrangement by the permeabilizing peptides and therefore, the resulting reduction of membrane dye-lipid molecule interactions.

**Figure 6.** Labeling spheroplast membrane with di-8-ANEPPS improves contrast of confocal images. Left: permeabilizing peptide, BF2 P11A. Right: translocating peptide, BF2. Distance between top and middle slices: 1.5μm (BF2 P11A) and 1.0μm (BF2); distance between middle and bottom slices: 1.1μm (BF2 P11A) and 1.5μm (BF2). Images from top, middle, and bottom of a z-stack of a multi-cell spheroplast were chosen. Green: FITC-labeled peptide; Red: membrane dye, di-8-ANEPPS.
In order to test whether the improved resolution for spheroplasts was only due to the change in shape, we compared images taken with “single-cell” and “multi-cell” spheroplasts. Increasing the size of spheroplasts from single-cell spheroplasts, typically 1μm in diameter, to multi-cell spheroplasts, usually between 2 - 5μm in diameter, allows more “slices” to be taken for the interior of the spheroplasts (Figure 7). This also insures that the selected center “slice” of a particular image z-stack has minimal “contamination” by signals from the membranes of the spheroplast. The lower fluorescence of translocating peptides inside the larger, multi-cell spheroplasts is most likely due to the increase in volume and space that a particular translocating peptide needed to fill (Figure 7). Single-cell spheroplasts tend to cluster together, increasing the difficulty of focusing on one single spheroplast during imaging (Figure 7, left).
Figure 7. Changing size from single-cell spheroplast to multi-cell spheroplast improves resolution. Top full panel: translocating peptide, BF2. Bottom full panel: permeabilizing peptide, BF2 P11A. Distance between top and middle slices: BF2 – 1.0μm (single-cell) and 1.0μm (multi-cell); BF2 P11A – 0.5μm (single-cell) and 1.5μm (multi-cell). Distance between middle and bottom slices: BF2 – 1.0μm (single-cell) and 1.5μm (multi-cell); BF2 P11A – 0.4μm (single-cell) and 1.1μm (multi-cell). Images from top, middle, and bottom of a z-stack of each single-cell spheroplast (left) or multi-cell spheroplast (right) were chosen. Green: FITC-labeled peptide; Red: membrane dye, di-8-ANEPPS.
Furthermore, we examined the effect of shape change from rod shaped bacteria to spherical spheroplasts, separately from the change in size, by comparing normal *E. coli* bacterial cells to single-cell spheroplasts. The shape change from rod-shaped bacterial cells to spherical spheroplasts alone leads to observable resolution improvement (Figure 8). In bacterial cells, both translocating peptide (top panel) and permeabilizing peptide (bottom panel) display fluorescence signal inside the cell, suggesting a translocation mechanism (Figure 8). In comparison, the shape change without enlargement in size in single-cell spheroplast allowed for a distinction in mechanism as presented by the absence of fluorescence inside single-cell spheroplast for membrane permeabilizing peptide, FITC BF2 P11A and the filled spheroplast outlined by membrane dye (in red) for translocating peptide, FITC BF2 (Figure 8). However, this does not necessarily mean that normal bacterial cells are not suitable at all for such imaging work. As shown in the bottom full panel of Figure 8, the membrane dye does seem to localize to the bacterial cell membrane and does not show “contamination” in the center focal plane of the bacterial cell (Figure 8, bottom panel). The observations of both cases, where fluorescence signals from the membrane “contaminate” the cell interior focal planes and where fluorescence signals from the membrane do not “contaminate” the cell interior focal planes, point out that imaging with bacterial cells may be more prone to these occurrences of artefact but this does not indicate that imaging in normal bacterial cells is an infeasible approach. The observation of both occurrence simply emphasizes that determination of the peptide localization based on images of bacterial cells alone should be done cautiously, especially if membrane-localizing fluorescence is robust.
Figure 8. Changing shape from bacterial cell to single-cell spheroplast improves resolution. Top full panel: translocating peptide, BF2. Bottom full panel: permeabilizing peptide, BF2 P11A. Distance between top and middle slices: BF2 – 0.5μm (bacteria) and 1.0μm (single-cell); BF2 P11A – 0.5μm (bacteria) and 0.5μm (single-cell). Distance between middle and bottom slices: BF2 – 1.0μm (bacteria) and 1.0μm (single-cell); BF2 P11A – 0.4μm (bacteria) and 0.4μm (single-cell). Images from top, middle, and bottom of a z-stack of each bacterial cell (left) or single-cell spheroplast (right) were chosen. Green: FITC-labeled peptide; Red: membrane dye, di-8-ANEPPS.
Together, the three aspects of optimization, addition of the membrane dye, uniformization of the shape, and enlargement of the spheroplast size, using *E. coli* spheroplast improve the resolution limitations caused by the small size and rod shape of the bacterial cells and the resolution limit of the instrument. Out of three cell types, multi-cell spheroplasts exhibit the largest qualitative resolution enhancement, bypassing the concern of signal contamination between individual focal planes, or “slices”, within a z-stack and giving the clearest insights into a peptide’s mechanism of action.

Conventionally, use of confocal imaging in bacterial cells has primarily served as a qualitative visualization of a particular peptide’s mechanism of action in bacteria. With spheroplasts, in addition to providing higher quality confocal images compared to that of bacterial cells, we can quantify the percentage of images showing peptide translocation or membrane permeabilization (Table 3). It is much more time-consuming to acquire good images of bacterial cells compared to that of spheroplasts. The larger counts of quality images collected of spheroplasts would allow us to be more quantitative in comparing mechanisms between peptides. This may also allow us to be less biased since spheroplasts are easier to find so that we are not selecting cells that have certain features, enabling us to obtain a more representative collection of peptide localization from a particular sample. Again, consistent with previous trends observed for translocation (Table 3), BF2 shows entrance into larger percentage of single-cell spheroplast (n = 113; 85%) and multi-cell spheroplasts (n = 67; 62%) compared to BF2 P11A (n = 126; 69%, n = 101; 25%) and magainin (n = 23; 8.7%, n = 61; 18%).

None of the peptides exhibit exclusively the translocation or membrane permeabilization behavior. This occurrence may be related to the inherent differences between
spheroplasts and bacterial cells or the exact timing of imaging. For example, it is possible that a membrane permeabilizing peptide may localize on the membrane at the beginning of exposure period but then begin the entering spheroplasts with increased permeabilization of the membrane, contributing to signals inside the spheroplasts. A further possibility may be that peptides’ mechanisms of action may not belong to a solely binary but rather, behave on a more continuum of mechanisms ranging from mostly translocation to mostly membrane permeabilization. Confocal imaging with spheroplasts gives us a means to explore these trends more closely and in a more quantitative manner.

Table 3. Percentage of cells/spheroplasts showing translocation or membrane permeabilization for FITC BF2, FITC BF2 P11A, FITC Magainin, and FITC HipC in FITC only samples (green) and FITC and membrane dye labeled (Di-8-ANEPPS) samples (purple). a) Normal bacterial cells; b) Single-cell spheroplasts; c) Multi-cell spheroplasts. White column = sum of FITC only and membrane dye labeled samples.

<table>
<thead>
<tr>
<th></th>
<th>All Cells</th>
<th>FITC only</th>
<th>With Di-8</th>
<th>%</th>
<th>FITC only</th>
<th>With Di-8</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FITC BF2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translocation</td>
<td>59</td>
<td>24</td>
<td>35</td>
<td>95%</td>
<td>100%</td>
<td>92%</td>
<td></td>
</tr>
<tr>
<td>Membrane</td>
<td>3</td>
<td>3</td>
<td></td>
<td>5%</td>
<td>0%</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>62</td>
<td>24</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FITC BF2 P11A</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translocation</td>
<td>47</td>
<td>24</td>
<td>23</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Membrane</td>
<td></td>
<td></td>
<td></td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>47</td>
<td>24</td>
<td>23</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>FITC Magainin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>17</td>
<td>100%</td>
<td>100%</td>
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<td></td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>26</td>
<td>9</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FITC HipC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translocation</td>
<td>23</td>
<td>22</td>
<td>1</td>
<td>100%</td>
<td>100%</td>
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<td></td>
<td></td>
<td></td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>23</td>
<td>22</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### b) Single-cell Spheroplast

<table>
<thead>
<tr>
<th>All Cells</th>
<th>FITC only</th>
<th>With Di-8</th>
<th>FITC only %</th>
<th>With Di-8 %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FITC BF2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translocation</td>
<td>155</td>
<td>59</td>
<td>96</td>
<td>84%</td>
</tr>
<tr>
<td>Membrane</td>
<td>30</td>
<td>13</td>
<td>17</td>
<td>16%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>185</td>
<td>72</td>
<td>113</td>
<td></td>
</tr>
</tbody>
</table>

| **FITC BF2 P11A** | | | | |
| Translocation | 121 | 33 | 88 | 66% | 57% | 70% |
| Membrane | 63 | 25 | 38 | 34% | 43% | 30% |
| **Total** | 184 | 58 | 126 | | |

| **FITC Magainin** | | | | |
| Translocation | 5 | 3 | 2 | 7% | 7% | 9% |
| Membrane | 62 | 41 | 21 | 93% | 93% | 91% |
| **Total** | 67 | 44 | 23 | | |

| **FITC HipC** | | | | |
| Translocation | 23 | 2 | 21 | 100% | 100% | 100% |
| Membrane | 62 | 41 | 21 | 93% | 93% | 91% |
| **Total** | 23 | 2 | 21 | | |

### c) Multi-cell Spheroplast

<table>
<thead>
<tr>
<th>All Cells</th>
<th>FITC only</th>
<th>With Di-8</th>
<th>FITC only %</th>
<th>With Di-8 %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FITC BF2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translocation</td>
<td>71</td>
<td>29</td>
<td>42</td>
<td>49%</td>
</tr>
<tr>
<td>Membrane</td>
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<td><strong>Total</strong></td>
<td>146</td>
<td>79</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

| **FITC BF2 P11A** | | | | |
| Translocation | 33 | 7 | 26 | 19% | 9% | 26% |
| Membrane | 143 | 68 | 75 | 81% | 91% | 74% |
| **Total** | 176 | 75 | 101 | | |

| **FITC Magainin** | | | | |
| Translocation | 19 | 8 | 11 | 18% | 18% | 18% |
| Membrane | 86 | 36 | 50 | 82% | 82% | 82% |
| **Total** | 105 | 44 | 61 | | |

| **FITC HipC** | | | | |
| Translocation | 68 | 3 | 65 | 96% | 75% | 97% |
| Membrane | 3 | 1 | 2 | 4% | 25% | 3% |
| **Total** | 71 | 4 | 67 | | |
3.4.  **FITC-labeled peptides exhibit similar levels of antimicrobial activity against Gram-negative and Gram-positive bacteria but have lower activity compared to unlabeled peptides**

FITC-labeled peptides exhibited similar levels of antimicrobial activity against Gram-negative *E. coli* and Gram-positive *S. epidermidis* (Figure 9a). However, the FITC-labeled peptides have lower activity compared to the unlabeled peptides in *E. coli* (Top10 strain), indicating that the addition of the fluorescent label does affect the function of the peptides to a certain degree (Figure 9b). Amongst BF2, BF2 P11A, Magainin, and HipC (Table 2), Magainin showed greatest reduction in activity in FITC-labeled version compared to the unlabeled version. This observed reduction in antimicrobial activity of FITC-labeled Magainin may contribute to a decrease in the percentage of permeabilization observed in confocal imaging and FITC-labeled Magainin may be less effective in localizing on the membrane than its unlabeled counterpart (Table 3). HipC, both FITC-labeled and unlabeled forms, showed no activity against all three bacterial strains examined, slightly inconsistent with previous observation of unlabeled HipC with a slight activity against *E. coli* (1.49 ± 0.14 mm) and against *S. epidermidis* (2.54 ± 0.15 mm) but consistent with a past study (Figure 9; Table 4; Bustillo et al. 2014). As the primary positive and negative controls, BF2 (translocation) and BF2 P11A (permeabilization) still retained a portion of their respective antimicrobial activity with the FITC-label, suggesting that they should still follow a similar set of mechanism of interaction with the lipid membrane during confocal imaging (Table 2).
Figure 9. Average diameter of clearance: a) FITC-labeled peptides in *E. coli* (ATCC #25922), *E. coli* Top10, and *S. epidermidis*. b) FITC-labeled and unlabeled peptides in *E. coli* Top10. Well diameter = 1mm; Negative control: deionized water (dH$_2$O); Error Bars: Standard Error of Mean, calculated over multiple wells from at least three independent replicates.
Table 4. HC shows slight antimicrobial activity against three bacteria strains while TM, HT, and TH show no activity against all four bacteria. HC=HipC; TM=MscL TM1; HT=HipC+TM1; TH=TM1+HipC; M = magainin. Well diameter = 1mm; Negative control: deionized water (dH₂O); Positive control: Magainin (M); Diameter of clearance: Mean±SE, calculated over multiple wells.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>HC</th>
<th>TM</th>
<th>HT</th>
<th>TH</th>
<th>M</th>
<th>dH₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>1.49±0.14</td>
<td>1.05±0.01</td>
<td>1.05±0.01</td>
<td>1.05±0.01</td>
<td>6.84±0.40</td>
<td>1.04±0.02</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>1.06±0.02</td>
<td>1.05±0.02</td>
<td>1.08±0.02</td>
<td>1.09±0.02</td>
<td>3.01±0.13</td>
<td>1.03±0.02</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>1.54±0.08</td>
<td>1.00±0.00</td>
<td>1.13±0.06</td>
<td>1.01±0.01</td>
<td>6.78±0.47</td>
<td>1.00±0.00</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>2.54±0.15</td>
<td>1.00±0.00</td>
<td>1.17±0.09</td>
<td>1.00±0.00</td>
<td>6.27±0.45</td>
<td>1.01±0.01</td>
</tr>
</tbody>
</table>

3.5. **Determination of mechanism of action of histone-derived peptide segments using the current adapted approach**

This optimized approach of confocal microscopy imaging of spheroplasts can be used to determine the mechanism of action of hybrid peptides, consist of peptide segments that each has respective mode of interaction with the membrane and modularly designed with the aim of exhibiting a desired mechanism or set of mechanisms. A set of peptides are the previously discussed, translocating hipposin-derived peptide, HipC (HC), the first transmembrane domain of the mechano-sensitive channel of large conductance protein, MscL TM1 (TM), and their two chimeric fusions, HC+ TM (HT) and TM+HC (TH) (Table 2). These four peptides were tested for antimicrobial activity. These experiments, using a radial diffusion assay (RDA), were performed on two gram-negative bacteria, *Escherichia coli* and *Serratia marcescens*, and two gram-positive bacteria, *Bacillus thuringiensis* and *Staphylococcus epidermidis*. TM and the two chimeric peptide constructs, HT and TH, did not show antimicrobial activity against all four strains of bacteria. Each peptide caused an average diameter of clearance of around 1mm for each strain, consistent with the diameter of the well and that of the negative control of deionized water (Table 4). On the other hand, HipC (HC), were found to show slight antimicrobial activity, with
average diameter of clearance values of 1.49±0.14 mm, 1.54±0.08 mm, and 2.54±0.15 mm against *E. coli*, *S. marcesens*, and *S. epidermidis*, respectively (Table 4). These larger values of diameter of clearance are dramatically lower compared to that of a peptide with significant activity, magainin 2. It is important to note that the weak antimicrobial activity of HipC disappeared once another peptide was attached to HipC, either at the N-terminal or the C-terminal. This observation of the absence of HipC antimicrobial activity in the chimeric peptides initiated the interest in HipC’s potential as a “vehicle” that can potentially transport another “cargo” peptide into the bacterial cell, leading to subsequent studies of HipC mechanism using confocal microscopy imaging and other assays such as propidium iodide uptake assay.

3.5.1. Validation of the translocation mechanism of action of the histone-derived peptide segment, HipC

HipC (HC), the C-terminal domain of histone-derived antimicrobial peptide, hipposin, was found to be translocating but does not exhibit significant antimicrobial activity (Bustillo et al. 2014). The mechanism of action of HipC was examined using the AlexaFluor 488-conjugated streptavidin with biotinylated peptide system in normal bacterial cells by Bustillo and colleagues (Bustillo et al. 2014). Data on HipC translocation into lipid vesicles were also collected but were not overly conclusive so it is valuable to investigate HipC’s mechanism of action further using the present system of confocal imaging in *E. coli* spheroplasts. Confocal images of FITC-labeled HipC in bacterial cells, single-cell spheroplasts, and multi-cell spheroplasts labeled with Di-8-ANEPPS membrane dye, reveal peptide localization inside the cell/spheroplast, also consistent with percentage of translocation of 100% (n = 21) into single-cell spheroplasts and 97% (n = 67) into multi-cell spheroplasts (Figure 10; Table 3). However, compared to the other translocating peptide, BF2, HipC has significantly lower translocation
efficiency into spheroplasts, requiring longer incubation of peptide with spheroplasts, between 10 and 20 minutes compared to the 1-minute incubation time for all other peptides, and a much lower count of internalization per sample per imaging session. Spheroplast samples with FITC HipC often have a much higher background fluorescence noise compared to other peptide samples, possibly implying a slower rate of initial association with spheroplasts membrane. While HipC does exhibit strong translocation behavior once it associates with the spheroplast membrane, its slow and limiting rate of initial association with spheroplast membranes will likely not allow HipC to serve as a potential, effective shuttle for delivery of molecules into bacterial cells.

Figure 10. Representative confocal images of bacterial cells (left), single-cell spheroplasts (middle), and multi-cell spheroplasts (right) exposed to HipC shows translocation. Distance between top and middle slices: 0.8μm (bacteria), 0.3μm (single-cell), and 1.1μm (multi-cell); distance between middle and bottom slices: 0.9μm (bacteria), 0.3μm (single-cell), and 1.1μm (multi-cell). Images from top, middle, and bottom of a z-stack of each cell type were chosen. Green: FITC-labeled peptide; Red: membrane dye, di-8-ANEPPS. Yellow/orange: co-localization of FITC-peptide and membrane dye.
3.6. **Preliminary permeabilization assay in spheroplasts further supports the feasibility of using E. coli spheroplasts as a viable model system**

Preliminary PI uptake studies of translocating peptide, Des1, and permeabilizing peptide, DP, in spheroplasts showed permeabilizing trends consistent with their respective mechanisms of action (Figure 11a and 11b). Fluorescence intensity trace of Des1 (translocating peptide) remains fairly consistent throughout the time course, indicating that Des1 translocation into spheroplasts did not lead to significant disruption of spheroplast membrane, which would have led to propidium iodide entry and an increase in fluorescence intensity (Figure 11a). In comparison, fluorescence intensity trace of DP (membrane permeabilizing peptide) in spheroplasts reveals characteristic increase and then plateauing of fluorescence after the addition of propidium iodide, indicating the successful permeabilization of spheroplast membrane by DP and the resulting entrance of PI and increase in fluorescence intensity. These initial data shows promising results that spheroplasts likely show similar trends in membrane permeabilization studies with PI. Examination of these trends with additional replicates and more diverse set of peptides is ongoing work to further support the use of spheroplasts as a suitable model for peptide-bacterial membrane interaction.

![Figure 11. Representative fluorescence intensity traces (a) and average fluorescence intensity increase (b) for propidium iodide entry into spheroplasts exposed to translocating peptide, Des1 (blue), and permeabilizing peptide, DP (red). Error bar = standard deviation.](image-url)
4. Summary

Examination of FITC-labeled peptide localization in *E. coli* spheroplasts via confocal microscopy imaging presents a viable method for characterizing the mechanism of action of a particular peptide of interest. This method provides higher-quality images by bypassing resolution limitations encountered during bacterial cell imaging and allows for preliminary calculation of percentage of images showing translocation and membrane permeabilization behaviors, adding a more quantitative perspective to a previously qualitative approach. This approach, when combined with antimicrobial activity assays and membrane permeabilization assays such as propidium iodide uptake assay, offer a more reliable way to gain insights into antimicrobial peptides and/or other cell-penetrating/membrane-active peptides. Additionally, spheroplasts have been noted previously to be able to revert back into individual bacterial cells, potentially implying that spheroplasts may retain their viability to some degree (Liu et al. 2006 and Sun et al. 2014). Sun and colleagues (2014) have also found that spheroplasts metabolically maintain a membrane reservoir in order to adjust cytoplasmic volume and to equalize osmolality on both sides of the membrane, suggesting that spheroplasts are viable and can serve as a reasonable model for bacterial system (Sun et al. 2014).

Through the use of primary positive and negative controls, BF2 and BF2 P11A, and two other previously characterized peptides, magainin 2 (permeabilizing) and HipC (translocation) (Table 2), we were able to obtain image data consistent with previously characterized mechanisms of action for these four peptides (Park et al. 2000; Xie et al. 2011; Ludtke et al. 1996). Data from this further quantification step raised an observation that a peptide may not exclusively exhibit a translocation or permeabilizing mode of interaction, but rather, employs a
combination of both mechanisms. This observation can also be attributed to the inherent
difference between bacterial cells and spheroplasts. Further studies, with more sample
collection on more diverse set of antimicrobial peptides and cell-penetrating peptides without
antimicrobial activity, need to be conducted in order to explore this observation in more depth
and to further validate the feasibility of this approach.
5. References


Appendix Figure 1. Representative confocal images of bacterial cells (top left), single-cell spheroplasts (top right), and multi-cell spheroplasts (bottom middle) exposed to BF2 shows translocation. Distance between top and middle slices: 0.5μm (bacteria), 1.0μm (single-cell), and 1.0μm (multi-cell); distance between middle and bottom slices: 1.0μm (bacteria), 1.0μm (single-cell), and 1.5μm (multi-cell). Images from top, middle, and bottom of a z-stack of each cell type were chosen. Green: FITC-labeled peptide; Red: membrane dye, di-8-ANEPPS. Yellow/orange: co-localization of FITC-peptide and membrane dye.
Appendix Figure 2. Representative confocal images of bacterial cells (top left), single-cell spheroplasts (top right), and multi-cell spheroplasts (bottom middle) exposed to BF2 P11A shows membrane localization. Distance between top and middle slices: 0.5μm (bacteria), 0.5μm (single-cell), and 1.5μm (multi-cell); distance between middle and bottom slices: 0.4μm (bacteria), 0.4μm (single-cell), and 1.1μm (multi-cell). Images from top, middle, and bottom of a z-stack of each cell type were chosen. Green: FITC-labeled peptide; Red: membrane dye, di-8-ANEPPS. Yellow/orange: co-localization of FITC-peptide and membrane dye.
Appendix Figure 3. Representative confocal images of bacterial cells (top left), single-cell spheroplasts (top right), and multi-cell spheroplasts (bottom middle) exposed to Magainin shows membrane localization. Distance between top and middle slices: 0.5μm (bacteria), 0.5μm (single-cell), and 0.8μm (multi-cell); distance between middle and bottom slices: 0.1μm (bacteria), 0.5μm (single-cell), and 1.8μm (multi-cell). Images from top, middle, and bottom of a z-stack of each cell type were chosen. Green: FITC-labeled peptide; Red: membrane dye, di-8-ANEPPS. Yellow/orange: co-localization of FITC-peptide and membrane dye.