Qualitative and Quantitative Changes to *Escherichia coli* during Treatment with Magainin 2 Observed in Native Conditions by Atomic Force Microscopy

**Kanesha Overton**, Cottey College, Nevada, Missouri

**Helen M. Greer**, Cottey College, Nevada, Missouri

**Megan A. Ferguson**, State University of New York, New Paltz, New York

**Eileen M. Spain**, Occidental College, Los Angeles, California

**Donald E. Elmore**, Wellesley College, Wellesley, Massachusetts

**Megan E. Núñez**, Wellesley College, Wellesley, Massachusetts

**Catherine B. Volle**, Cottey College, Nevada, Missouri

**Abstract**

The bacterial membrane has been suggested as a good target for future antibiotics, so it is important to understand how naturally occurring antibiotics like antimicrobial peptides (AMPs) disrupt those membranes. The interaction of the AMP magainin 2 (MAG2) with the bacterial cell membrane has been well characterized using supported lipid substrates, unilamellar vesicles, and spheroplasts created from bacterial cells. However, to fully understand how MAG2 kills bacteria, we must consider its effect on the outer membrane found in Gram-negative bacteria. Here, we use atomic force microscopy (AFM) to directly investigate MAG2 interaction with the outer membrane of *Escherichia coli* and characterize the biophysical consequences of MAG2 treatment under native conditions. While propidium iodide penetration indicates that MAG2 permeabilizes cells within seconds, a corresponding decrease in cellular turgor pressure is not observed until minutes after MAG2 application, suggesting that cellular homeostasis machinery may be involved.

**Corresponding Author** Catherine B. Volle — Phone: 319-895-4413; cvolle@cornellcollege.edu.

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acslangmuir.9b02726. Supplementary figures showing free MAG2 that does not substantially affect the behavior of the tip and amplitude images of planktonic *E. coli* cells captured by AFM (PDF)

The authors declare no competing financial interest.
responsible for helping the cell maintain turgor pressure despite a loss of membrane integrity. AFM imaging and force measurement modes applied in tandem reveal that the outer membrane becomes pitted, more flexible, and more adhesive after MAG2 treatment. MAG2 appears to have a highly disruptive effect on the outer membrane, extending the known mechanism of MAG2 to the Gram-negative outer membrane.

**Graphical Abstract**

[Image of Magainin 2]

**INTRODUCTION**

Even before penicillin was available as a therapeutic treatment, scientists had already identified the first antibiotic resistant bacterium.\(^1\) The number of antibiotic resistant bacteria has risen dramatically in the past few decades. Antibiotic resistant bacteria represent such an alarming threat that the World Health Organization declared antibiotic resistance one of the three greatest threats to human health,\(^2\) and some clinicians are warning of a coming post-antibiotic era of medical care.\(^1\) Antibiotic usage is so widespread that antibiotics have been detected throughout various ecosystems, and this environmental exposure contributes to the development of antibiotic resistance in bacteria. Today, antibiotic resistant bacteria are being isolated from hospitals, rivers, groundwater, waste water, soil, and animal products. With so many bacteria rapidly gaining resistance to commercially available antibiotics, either through de novo mutations or gene transfer, the scientific community is exploring many different options for the antibiotics of the future.

As we look for new antibiotics, we must also consider how easily bacteria can acquire resistance. Rather than choosing to target an enzyme, to which bacteria can rapidly adapt by mutation, it would be better to choose targets that could delay the appearance of resistant strains. Such a target could be bacterial membranes, given their complex structure composed of proteins, lipids, and carbohydrates. Developing complete resistance to an antibiotic that targets bacterial membranes would likely require multiple mutations in the membrane biosynthesis genes.\(^3\)\(^-\)\(^5\) Antimicrobial peptides (AMPs) are small peptides produced by numerous eukaryotic immune systems, several classes of which kill bacteria by disrupting the membrane. Importantly, because the biochemical characteristics of bacterial and animal cell membranes differ, many AMPs only induce cytotoxicity in bacterial cells. If we understand how AMPs target and disrupt bacterial membranes, we can apply that knowledge to the design of new antibiotic compounds, including non-peptide molecules.
AMPs can be divided into categories based on their net charge and secondary structure.\textsuperscript{6}

One of the best studied AMPs is magainin 2 (MAG2), a cationic, $\alpha$-helical peptide initially isolated in 1987 from the skin of the frog \textit{Xenopus laevis}.\textsuperscript{7} This short peptide is capable of killing a wide array of microbes and shows limited hemolytic activity.\textsuperscript{7-11} MAG2 is initially unstructured in fluid but adopts an $\alpha$-helical conformation upon interaction with lipid bilayers, with the helix axis laying parallel to the bilayer.\textsuperscript{12} This interaction is energetically favorable and induces formation of a hydrophobic face in the $\alpha$-helix, which allows MAG2 to act as a wedge, splitting apart the lipid tails. The initial disruption of the lipid bilayer by MAG2 molecules with a parallel orientation allows new MAG2 molecules, now positioned perpendicular to the lipid bilayer, to further disrupt the membrane. Since MAG2 is cationic, it preferably interacts with the negatively charged lipopolysaccharides (LPS) and other anionic lipids found on the outer surfaces of bacterial cells, which may partially explain its selectivity for microbial cells.\textsuperscript{12-15}

While much of the research on the mechanism of action for MAG2 has focused on its effect on phospholipid membranes, Gram-negative bacteria contain two membranes.\textsuperscript{16} The cell (inner) membrane is composed of negatively charged phospholipids, while the outer membrane is composed of an asymmetric bilayer primarily containing LPS in the outer leaflet and phospholipids in the inner leaflet. Though MAG2 must cross the outer membrane in order to disrupt the cell membrane, less is understood about the biophysical effects of MAG2 interacting with the outer membrane or how MAG2-induced permeabilization affects cellular biophysics over time.

Atomic force microscopy (AFM) has become a powerful tool for examining cellular biophysics.\textsuperscript{17,18} Not only does AFM provide high-resolution images, the tip can be used as a probe to measure the mechanical properties of living cells. Once cells have been identified in an image, the tip can be gently pushed into the cell and retracted, allowing us to measure quantitatively the cell stiffness, flexibility of the outer membrane, and adhesion between the cell surface and the tip. In this work, we describe the qualitative and quantitative changes that occur in \textit{Escherichia coli} (\textit{E. coli}) cells during incubation with MAG2. While others have previously used AFM to investigate the changes in cell morphology after MAG2 treatment, the imaging was carried out in air, which could lead to artifacts as the cells dry.\textsuperscript{19} Furthermore, as the previous work did not image cells over time or collect force data, AFM experiments still have much to tell us about the biophysical consequences of treatment with MAG2. We have developed methodology that allows us to adhere free-swimming \textit{E. coli} to a surface and obtain continuous cellular data in native conditions over the course of MAG2 treatment. We find that, while MAG2 rapidly induces propidium iodide fluorescence, cells do not immediately experience a decrease in turgor pressure. MAG2 interaction with the outer membrane causes a change in elasticity and adhesion as well as increased roughness in the outer surface after treatment. This research provides new insights into the biophysical effects of MAG2 treatment and will hopefully yield valuable information in the search for new antibiotics that target bacterial membranes.
EXPERIMENTAL SECTION

Antimicrobial Peptide Preparation.

The antimicrobial peptide magainin II (GIGKWLHASAKFGKAFVGEIMNS, MAG2), containing an F5W mutation for easier quantitation, was synthesized by Genscript with >95% purity. Previous studies have shown that this substitution of tryptophan for phenylalanine does not affect the behavior of the peptide. Stocks of the peptide were prepared by rehydrating a small amount of the lyophilized peptide in distilled water and determining the concentration using the absorbance at 280 nm. These stock solutions were then diluted to the appropriate concentration for further use.

Minimum Inhibitory Concentration of MAG2 for Planktonic E. coli Cells.

The minimum inhibitory concentration (MIC), defined as the lowest concentration of MAG2 that inhibits visible growth, was determined using slight alterations to a previously published protocol. Luria Broth (LB) was inoculated with an overnight culture of E. coli ZK1056 (from M.O. Martin and R. Kolter) and allowed to shake at 37 °C until the optical density at 600 nm (OD$_{600}$) was above 0.2 AU. The measured OD$_{600}$ was then used to determine the cells per milliliter in the culture (1 AU = 8 × 10$^8$ cells/mL), and the cell suspension was diluted with LB to 1 × 10$^6$ cells/mL.

Next, using a sterile 96-well plate, 50 μL of bacteria was transferred to each of the 12 wells in a row. MAG2 stock was diluted in LB and was serially diluted (1:1). In separate wells, 50 μL of the bacteria alone and LB alone served as positive and negative growth controls, respectively.

After addition of the MAG2, the 96-well plate was incubated overnight at 37 °C, 220 rpm. The well with no bacterial growth and the lowest concentration of MAG2 was identified as the MIC. The MIC determination was performed twice in triplicate.

Propidium Iodide Uptake Assay.

The ability of MAG2 to permeabilize cells was determined using a propidium iodide (PI) uptake assay. An overnight E. coli culture was used to inoculate fresh LB, which was grown at 37 °C until the OD$_{600}$ was over 0.4 AU, approximately 3–4 h. The culture was then centrifuged at 880g for 10 min. The supernatant was discarded, and each pellet was resuspended in 10 mL of 10 mM sodium phosphate buffer at pH 7.4 (PB). The resulting bacterial suspensions were further cleaned by centrifugation and resuspension in 10 mL of PB as described above before measuring the OD$_{600}$ and adjusted to 0.5 ± 0.01 AU by either dilution with PB or re-centrifugation and suspension in a smaller volume.

Once OD$_{600}$ values were normalized, 1 mL of the suspension was placed in each of two cuvettes. Ten microliters of 2 mg/mL PI solution was added to each and gently mixed. The cuvettes were then transferred to a Varian Cary Eclipse fluorescence spectrophotometer. Fluorescence readings ($\lambda_{ex} = 535$ nm, $\lambda_{em} = 617$ nm) were taken every 15 s. Readings were started, and the samples were allowed to equilibrate for 5 min before addition of MAG2.
Since PI can diffuse across the outer membrane, this equilibration allows PI to move into the periplasmic space, where it is retained until the cell membrane is permeabilized.

For the fluorescence spectrometry experiments, the MAG2 stock was diluted to the appropriate concentration in PB. After the system finished equilibrating, the readings were paused, MAG2 was added to one of the cuvettes and quickly mixed by pipetting, and the readings were resumed. Both the treated and untreated samples were read for at least 15 min after addition of MAG2. The PI uptake was measured with fresh bacterial preparations four times for each MAG2 concentration.

In order to normalize the data between different experiments, the emission value of the control sample was subtracted from the emission value of the matched treated sample. The time point immediately preceding MAG2 addition was then set to zero, and the subsequent data points were adjusted by subtracting the same value. The normalized values from each separate experiment were then averaged, and the standard error was determined for each 15 s time point.

**Preparation of Planktonic E. coli Cells for Imaging.**

To obtain fluorescence and atomic force microscopy (AFM) images of cells grown in liquid culture, the cells must first be attached to a surface. This was accomplished using Corning Cell-Tak cell and tissue adhesive, deposited onto a clean glass coverslip. Cell-Tak was diluted 1:5 with 5% acetic acid. Glass coverslips were plasma-cleaned for 3 min using a Harrick Plasma FDC-32G plasma cleaner set to low. The clean coverslips were placed in a Petri dish, and 15 μL of diluted Cell-Tak was pipetted in the center of each coverslip. Coverslips were left to dry overnight at room temperature before washing with copious amounts of PB. Bacteria (1 × 10⁷) were pipetted onto the dried circle of Cell-Tak and incubated for approximately 2 h at room temperature. The coverslips were then gently rinsed with PB and were then used in either live/dead fluorescent staining or AFM experiments.

**Live/Dead Cell Staining.**

Cells were stained using the Invitrogen LIVE/DEAD BacLight bacterial viability kit following the manufacturer’s instructions. After cells were affixed to the coverslip with Cell-Tak, they were treated with MAG2 or PB for 30 min. The samples were then rinsed with PB, and 50 μL of stain was applied. The samples were allowed to incubate in the dark for 15 min at room temperature before imaging with a Nikon Ti microscope using a Nikon PlanApo Lambda 40X objective, with a numerical aperture of 0.9. Images of cells stained with SYTO 9 were captured using a Nikon ET-GFP filter, while cells stained with PI were imaged with a Nikon ET-mCherry filter. Images were then imported to ImageJ, where they were adjusted for brightness and overlaid, and a scale bar was added.

**AFM.**

Images of bacteria in PB were obtained in intermittent contact mode using an MFP-3D BIO AFM (Asylum Research) as previously described. Briefly, silicon nitride tips with a nominal spring constant of 0.32 N/m were used to obtain both images and cellular force data. In order to obtain data both before and after MAG2 exposure, coverslips containing the
bacteria of interest were attached to the bottom of a 50 mm diameter Petri dish modified for injection with a syringe and kept submerged in PB throughout the experiment. After acquiring an initial image, a group of cells were selected for investigation and centered in the field of a 3–5 μm scan area. Initial force measurements, designated as time 0, were acquired on the center of each cell. Force data was obtained on 12 untreated cells and 31 treated cells.

After initial images and force measurements were obtained, a concentrated solution of MAG2 diluted in PB was injected by a syringe without the need to move the AFM head, allowing continuous collection of force data on individual cells. Force curves were obtained every 5 min, with five force curves acquired per location. Images were obtained between force measurements to ensure that cells remained in the same location and were still intact. Final images of the system were obtained after completion of the force measurements acquired at 30 min, and other areas of the sample likewise imaged between 30 and 60 min post-injection. These images were analyzed as a group to determine the root-mean-square average roughness (Rq) for the treated samples. The Rq was determined by exporting amplitude images to ImageJ and analyzing at least three random squares drawn along the longitudinal axis of the bacterium using the “roughness_calculation” plugin. The Rq values were compiled by time and treatment, and the average and standard errors were calculated.

Force data consists of both an extension and retraction curve. As previously described, the force extension curve is composed of three components: the linear approach, the nonlinear compression, and the linear compression.\textsuperscript{26,27} The slope of the linear compression was used in eq 1 to calculate the cellular spring constant ($k_{cell}$).

$$\frac{1}{k_{\text{effective}}} = \frac{1}{k_{cell}} + \frac{1}{k_{\text{cantilever}}} \quad (1)$$

While the slopes of the linear compressions were calculated using the MFP-3D software in Igor Pro, the change in force and the change in distance of the nonlinear region were measured as described previously.\textsuperscript{26,27} The retraction data was analyzed for the presence or absence of an adhesion event.

**RESULTS AND DISCUSSION**

**E. coli Grown in Liquid Are Sensitive to MAG2.**

*E. coli* cells grown in liquid were exposed to decreasing concentrations of magainin 2 (MAG2) in a 96-well microtiter plate assay to determine the minimum inhibitory concentration (MIC).\textsuperscript{22} The well with the lowest concentration of MAG2 that displayed no visual bacterial growth was designated the MIC. We determined that the MIC of MAG2 for planktonic cells of this strain was 16.5 μM. Though MIC determinations can vary depending on the volume, growth media, and number of cells per well, the sensitivity of the *E. coli* in this study appears to be similar to reported MICs of MAG2 for other *E. coli* strains, values of which range from 2 to 55 μM.\textsuperscript{7,10,20,28-30}
Fluorescence Microscopy Indicates that the Majority of *E. coli* Cells Adhered to a Surface Are Permeabilized by MAG2.

While the bacterial cells in the MIC experiments were in liquid culture, we must adhere the cells to a surface for investigation by atomic force microscopy (AFM). To ensure that the process of adhering the cells to a surface for our AFM experiments did not affect the cells’ susceptibility to MAG2, we used fluorescence microscopy to observe the cell viability of treated and untreated cells after they had been fixed to a surface with Cell-Tak (Figure 1). After incubation on a Cell-Tak coated surface, the cells were treated with 16.5 μM MAG2 diluted in 10 mM sodium phosphate buffer, pH 7.4 (PB), or an equal volume of PB for 30 min before staining with the Invitrogen’s BacLight bacterial viability kit. The cellular dye SYTO 9 can readily cross the bacterial membranes and stains all cells green. Propidium iodide (PI) has increased fluorescence upon binding to DNA but cannot cross the intact bacterial cell membrane. Only cells whose cell membrane is degraded, either by MAG2 or normal cell death, will take up PI and fluoresce red. The untreated cells are predominately green, with few, if any, cells exhibiting red fluorescence (Figure 1A), but cells are treated with 16.5 μM MAG2 for 30 min are mostly dead (Figure 1B). Thus, we are confident that the vast majority of adhered cells in the AFM experiments will be affected by MAG2 treatment within our experimental time-frame.

MAG2 Permeabilizes the Cell within Seconds.

While live/dead staining indicates that the cells in our AFM experiments will be permeabilized by MAG2 after 30 min of treatment, we also wanted to investigate how quickly MAG2 could permeabilize the cell. We used PI, the same molecule we used in our fluorescence microscopy, to monitor the ability of MAG2 to permeabilize the membranes of cells suspended in PB using fluorescence spectrometry. We measured PI fluorescence in the presence of MAG2 at the MIC, 16.5 μM, and at concentrations just above (24.8 μM) and just below (12.4 μM). At each concentration tested, the PI fluorescence rose immediately and continued to increase over the first 120 to 240 s, after which the fluorescence plateaued as the system reached equilibrium (Figure 2). It is important to note that, in order to observe PI fluorescence, we must use more cells than were used in the MIC experiments. Thus, while 16.5 μM is given as the MIC, we would not expect that this concentration of MAG2 is high enough to kill all the cells in the cuvette. As the maximum fluorescence increases with increasing MAG2 concentration, it is possible that, at higher concentrations of MAG2, more cells are acquiring pores, leading to higher fluorescence. Conversely, it is also possible that higher concentrations of MAG2 causes larger pores to form, letting more PI enter the cells and causing higher fluorescence. Regardless of the concentration of MAG2 used, PI does not require MAG2-induced disruption to move across the outer membrane, so PI access to DNA is reliant on MAG2-induced disruption of the inner cell membrane in these experiments. However, MAG2 cannot permeabilize the cell membrane without first crossing the outer membrane. We observe such a fast increase in PI fluorescence that MAG2 must disrupt the outer membrane seconds after addition in order to permeabilize the cell membrane before the first reading, which was obtained only 15 s after adding MAG2 to the system.
Previous studies have shown that the structure of the outer membrane is an important factor in the susceptibility of bacteria to AMPs, which indicates that the interaction between the AMP and the outer membrane is likely important. The outer membrane of Gram-negative bacteria such as E. coli is composed of an asymmetric lipid bilayer. The outer leaflet contains lipopolysaccharides (LPS) that consist of a lipid molecule (lipid A), an inner and outer polysaccharide core, and a repeating polysaccharide O-antigen. Some strains of bacteria, called rough strains, make LPS with some or all of the polysaccharides missing. Some of the earliest work characterizing bacterial susceptibility to AMPs indicated that the LPS length, or the amount of polysaccharide present, had a direct effect on the MIC for a given bacteria. Rana et al. showed that Salmonella typhimurium (S. typhimurium) with shorter LPS were more sensitive to MAG2. E. coli ZK1056 is a rough strain, containing only the inner core and lipid A moieties, and is similar to the most susceptible of the S. typhimurium mutants. This shortened LPS structure could explain why MAG2 permeabilizes the outer membrane so quickly in our PI uptake assay. Though decreased LPS length often is related to increased AMP susceptibility, several other characteristics of LPS also correlate with enhanced AMP effectiveness, including the size and structure of the polysaccharides, their location and net charge, and the LPS packing density.

To better understand the effect of MAG2 on the cell and its interaction with the outer membrane, we must directly investigate its effect on living bacteria.

### Cell Stiffness Decreases after Treatment with MAG2.

AFM force measurements, consisting of extension and retraction curves, provide vital information about the biophysical properties of the cell. The extension curve consists of three distinct regimes (Figure 3A). Initially, as the tip approaches the cellular surface, there is a change in distance but no change in force; the tip encounters minimal resistance on its path though the buffer to the cell surface (Figure 3Ai). Next, the tip encounters the bacterial surface, which produces a nonlinear change in distance and force, reflecting the initial interaction between the tip and the bacterial cell wall (Figure 3Aii). Finally, the tip encounters resistance from the cellular turgor pressure, and there is a linear change in both distance and force (Figure 3Aiii). Representative extension curves from untreated cells (Figure 3B) and MAG2-treated cells (Figure 3C) show each of these regimes. Control curves taken on the underlying glass substrate over the course of the experiment demonstrate that the tip does not retain cell surface biopolymers or significantly accumulate free MAG2 (Supplementary Figure 1). While MAG2 is likely in the imaging solution and attracted to the negatively charged silicon nitride tip, the interaction between the two is weak compared to the interaction of the MAG2 with the cell. Indeed, for experiments in which an AMP must remain attached to the tip, it must be covalently linked to prevent AMP dissociation as the tip moves through the fluid.

We calculated the cell stiffness from the linear compression region. The slope of this regime provides the $k_{\text{effective}}$, a combination of the cellular spring constant ($k_{\text{cell}}$) and the spring constant of the cantilever ($k_{\text{cantilever}}$). The $k_{\text{cantilever}}$ is measured at the beginning of each experiment, allowing us to determine the $k_{\text{cell}}$, or cellular spring constant, from the linear slope of the extension curve. The cellular spring constant is directly related to the turgor pressure generated by the cytoplasm pushing against the cell wall.
To establish our measurement parameters, we examined planktonic *E. coli* cells affixed to a glass coverslip, submerged in PB, over 30 min, taking force measurements at 5 min intervals. We acquired images in between force measurements to ensure that the cells remained in place. Cells in buffer have a remarkably stable spring constant over 30 min in which we collected data (Figure 4, blue squares). However, when we treated cells with 16.5 μM MAG2, the spring constant decreased dramatically over 30 min, with some cells losing over half their initial turgor pressure (Figure 4, orange diamonds). It is important to note that this loss of turgor pressure is not instantaneous; some cells appear to maintain a regular turgor pressure at least 5 min after addition of MAG2. Thus, while our PI fluorescence indicates that MAG2 permeabilizes the cell within seconds (Figure 2), there is not always an immediate corresponding decrease in the turgor of the compromised cell. While the turgor pressure of every cell observed in the AFM experiments decreased within 10 min after the start of treatment, there is some variation in cellular response time, likely reflecting the natural heterogeneity of cells within the population. Other experiments where groups of cells are monitored during treatment with AMPs also reveal a range of response times. High-speed AFM imaging has previously revealed that, while some *E. coli* cells respond to treatment with the AMP CM15, a cecropin A and melittin hybrid, within seconds, others have no response until 12 min after the start of treatment.46

Using AFM, others have also observed a decrease in cell stiffness after treatment with membrane-disrupting AMPs. A capsule-deficient *Klebsiella pneumoniae* strain susceptible to the AMP melittin, a cationic AMP originally isolated from bee venom, showed an almost 90% decrease in cell stiffness after exposure to melittin for 120 min.47 While they observe a decrease in spring constant at their first time point, force measurements were not obtained until after 40 min, indicating that the maintenance of turgor pressure could be limited to the first minutes of peptide treatment. Gram-positive *Bacillus subtilis* also showed a 50% decrease in turgor pressure after a 120 min treatment with the linear AMP trichokonin VI, which also disrupts membranes.48 Their first measurements at 30 min did not show a significant decrease in cell stiffness, but this could reflect either the differences in AMP effectiveness or the difference between Gram-negative and Gram-positive cell wall structure.

The slower time frame associated with the drop in cellular turgor pressure stands in contrast to the fast permeabilization of the outer and cell membranes observed in our fluorescence spectroscopy experiments. However, Gram-negative bacteria are complex organisms with two membranes and molecular machinery dedicated to maintaining membrane stability. While small molecules can pass through the MAG2-permeabilized membranes, there are likely other mechanisms responsible for performing the ultimately futile task of preventing a loss of cellular turgor pressure. Using fluorescence microscopy, Gee et al. showed that pore formation by melittin is reduced in living cells compared to model membranes and suggest that cellular intervention is responsible for the reduction.49 We may be observing a similar phenomenon whereby cellular homeostatic mechanisms are preventing a complete loss of turgor pressure, even though the cell membranes are compromised. Alternatively, recent experiments by Yang et al., using a GFP that accumulates in the periplasm, suggest that the *E. coli* outer and cellular membranes are able to reseal after initial pore formation by melittin, which could prevent an immediate loss of turgor pressure.50 They propose that membrane rescaling is driven by a time-dependent buildup of melittin in the membrane, the
stress of which eventually causes catastrophic membrane permeabilization. It is interesting to consider whether a similar phenomenon would be observed for MAG2, though further empirical data is needed to reach such a conclusion.

**The Outer Membrane of E. coli Cells Becomes More Elastic after MAG2 Treatment.**

We can investigate the effect of MAG2 on the outer membrane of the *E. coli* cells by analyzing the nonlinear regime of the extension curve. This region corresponds to the initial contact between the tip and the outer membrane and contains both Δ force and Δ distance components. Extension curves previously measured on these cells have distance components roughly corresponding to the size of the outer membrane (10–20 nm) and force measurements between 0.5 and 1 nN, and the nonlinear regions of the untreated control cells measured here agreed with these parameters (Figure 5). However, when the *E. coli* cells are treated with 16.5 μM MAG2, both the distance and force components of the nonlinear region increase (Figure 5, purple circles), indicating that the surface appears thicker and more easily deformed to the approaching tip. Representative nonlinear regions are shown in Figure 5B-E.

Through its interaction with the outer membrane, MAG2 could be directly (through insertion into the membrane) or indirectly (by activating membrane remodeling mechanisms) changing the alignment and packing of the molecules in the membrane, which is consistent with previously demonstrated membrane disruption by MAG2. MAG2 adopts a helical structure upon contact with lipid membranes, forming a hydrophobic face that disrupts the membrane, allowing additional MAG2 molecules to insert into the membrane, a process previously captured by AFM. AFM imaging has revealed that treatment of supported phospholipids bilayers composed of dimyristoylphosphatidylcholine (DMPC) with MAG2 induced remodeling of the bilayer, with small parts released into the surrounding aqueous environment. AFM imaging has also revealed that a more hydrophobic analogue, magainin H2, caused the formation of disordered lipid protrusions extending from a model membrane composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). Both DMPC and POPC are zwitterions while LPS is anionic, so LPS should have stronger interactions with the cationic MAG2. In whole cells, treatment of *E. coli* with MAG2 caused an increased susceptibility to detergent-mediated disruption of the outer membrane, indirectly supporting the proposed MAG2-mediated remodeling of the outer membrane.

Alternatively, our observed increase in the force and distance components of the nonlinear region could reflect an increase in repulsive forces between the tip and outer membrane, rather than a purely structural change. The nonlinear regime is a “repulsive” regime caused by repulsive electrostatic and van der Waals forces between the cellular surface and the AFM tip. The addition of MAG2 to the cell surface could represent an electrostatic change in the bacterial surface as MAG2 has an overall positive charge compared to the negative charge of the Gram-negative LPS, and pore formation allows cations to flow from the bacterial cytoplasm to the environment. The increase in positive charges on the bacterial surface could be at least partially responsible for the observed changes in the nonlinear regime after MAG2 treatment; however, the increase in adhesion between the tip and the cell surface observed in the retraction curves (vide infra) suggest that any change in repulsive
forces between the tip and the surface is minimal. Most likely, the change in the nonlinear region reflects a combination of outer membrane structural perturbation and changes in electrostatic forces.

**Treatment with MAG2 Increases the Surface Roughness of the *E. coli* Outer Membrane.**

To directly assess how MAG2 exposure alters the morphology of the outer membrane, we obtained detailed images of cells incubated 30 to 60 min with 16.5 μM MAG2. Because these images were taken after force acquisition, we were able to investigate the outer membrane structure of cells not included in the force analysis but exposed to the same conditions. In the absence of MAG2, images taken at the beginning and end of the time course appear similar (Figure 6A,B). The bacterial surface appears smooth, and there is a lack of pili or other surface structures, consistent with other AFM images of bacteria in liquid. However, after incubation in 16.5 μM MAG2, there is a noticeable change in the roughness of the cells’ surface (Figure 6C,D) as the surface of the treated cells becomes more textured and small pits are observed in the outer membrane. While this is a qualitative observation, the surface of every treated cell imaged had the same roughened appearance.

When we determined the relative root mean square (Rq) for the roughness of the cell surfaces, untreated cells generally increased in Rq over the course of the experiment, but treated cells had a significantly greater increase in roughness (Table 1, P < 0.001). These Rq values are similar to previously reported Rq values of *E. coli* cells treated with the AMP CM15. Because the time requirement between force acquisitions necessitates that we obtain less detailed images during the experiment, we are currently unable to determine when exactly the roughness of the cell surface changed, though it does appear to occur only after we observe a decrease in turgor pressure, sometime between 15 and 30 min after MAG2 is introduced (Supplementary Figure 2).

We can determine that the change in roughness we observe in the treated bacteria represents a localized change in the outer membrane rather than a change in whole cell morphology because we image the cells in fluid. A previous study indicated qualitatively that MAG2-treated *E. coli* imaged with AFM had increased surface roughness. However, the images were obtained in air rather than in fluid, and drying can affect the cell morphology as observed by AFM, potentially either causing the rough surface directly or amplifying the effect of MAG2 treatment. AFM experiments with other AMPs have also demonstrated an increased surface roughness, either qualitatively or quantitatively, but the majority of these studies relied on a drying step to fix bacteria to the surface and are imaged in air rather than in fluid. Not only can drying affect the surface morphology, but it can also make it difficult to distinguish between changes in surface roughness and more dramatic cellular remodeling, such as cellular collapse due to dehydration. We have previously reported significant changes in the cellular morphology of healthy cells imaged in air, and many of the same changes appear in images of AMP-treated cells imaged in air. However, our experiments here maintained native conditions throughout, indicating that the increased roughness is a feature of treatment with pore-forming AMPs.

Others have imaged in fluid and also observed an increased surface roughness after treatment with pore-forming AMPs. When Fantner et al. investigated the effect of the AMP
CM15 on *E. coli* cells in fluid using high-speed AFM, they observed a rapid increase in surface roughness, with some cells showing an increased roughness in under a minute.\(^{46}\) While the Gram-positive bacterium *B. subtilis* has a different outer surface than the Gram-negative *E. coli*, Su et al. also observed an increase in surface roughness of *B. subtilis* cells after a 30 min treatment with the AMP trichokonin VI.\(^{48}\) They observed this increase in surface roughness without a decrease in turgor pressure, which suggests that the roughness of the outer membrane is caused by the interaction of the AMP with the outer surface rather than a decrease in turgor pressure. We have also observed that *E. coli* ZK1056 cells with decreased turgor pressure remain smooth in fluid (Volle et al., unpublished data),\(^{26}\) suggesting that the rough surface we observe after MAG2 treatment is caused by MAG2 remodeling the outer membrane rather than the result of the decrease in turgor pressure. However, given that we observe changes in the cell surface only after the cell has lost turgor pressure, the change in roughness could represent a final failing of the cellular mechanisms responsible for maintaining membrane integrity.

**Cells Treated with MAG2 Display Higher Affinity for the AFM Tip than Their Untreated Counterparts.**

Not only does the nonlinear region of the extension curve change upon MAG2 treatment, we also observe much stronger adhesion between the AFM tip and the bacterial surface (Figure 7). The force component indicates the strength of the adhesion; a larger force is the result of a stronger adhesion. At the beginning of each experiment, there was no noticeable adhesion between the tip and the bacterial surface (Figure 7A,C). ZK1056 cells grown in a biofilm do display a significant adhesion to the AFM tip, but those cells produce a matrix of exopolymERIC substances (EPS) that aid in adhesion to the surface.\(^{26,27}\) Planktonic cells, such as those used in these experiments, would have no need for EPS production as they live suspended in an aqueous environment. We observed small adhesion events after 30 min without MAG2 treatment (Figure 7B), which could indicate that, because the cells are alive and attached to a surface, they are in the earliest stages of biofilm development and have started excreting EPS.

Notably, the adhesion events observed in the absence of MAG2 are small compared to the adhesion between the tip and the cells treated with MAG2 for 30 min (Figure 7D), indicating that the surface of cells treated with MAG2 are sticking to the tip more strongly. These adhesion events could be due to increased membrane flexibility, which is consistent with the observed increase in both the force and distance of the nonlinear region. The purpose of the cell wall is to maintain cellular integrity, even during osmotic stress,\(^{16}\) so, in an untreated cell, the rigidity and interconnectedness of the cell wall components would quickly break any contacts between the bacterial surface and the tip. However, as MAG2 inserts into the outer and cell membranes, the connections between the outer membrane and the rest of the cell wall may become disrupted, allowing the outer membrane to remain attached to the tip for a longer distance during the retraction and requiring a larger force to break the connection between the tip and surface. These adhesion events were likely not due to increased secretion of EPS since these cells had all been attacked by MAG2 and were in the process of dying.
We have previously observed similar increases in adhesion forces when *E. coli* cells were invaded by the predatory bacterium *Bdellovibrio bacteriovorus.*\(^{26}\) As part of its attack, *Bdellovibrio* secretes enzymes to degrade the cell wall of its prey, disrupting the membrane and degrading the peptidoglycan. We ascribed the changes in adhesion after *Bdellovibrio* invasion to increased contact between the tip and the degraded membrane, and increased adhesion after MAG2 treatment is likely due to the same phenomenon. Treatment of *B. subtilis* with the AMP trichokonin VI also produced increased adhesion forces between the bacterial surface and the tip as well.\(^{48}\) Notably, increased adhesion was only observed after incubating the cells with trichokonin VI for 2 h, which may suggest that the adhesion we observe after only 30 min of MAG2 treatment is specific to AMP interaction with the Gram-negative outer membrane, which is absent in Gram-positive bacteria.

**CONCLUSIONS**

In this work, we describe the biophysical effects of MAG2 treatment on *E. coli* cells in native conditions. While PI uptake indicated that MAG2 permeabilized the cell membrane within seconds, the cellular turgor pressure generally remained stable on the order of minutes before decreasing. After the turgor pressure decreased, we observed that MAG2 caused noticeable changes to the outer membrane structure as treated cells appeared pitted, had more flexible outer membranes, and adhered more strongly to the AFM tip. The roughness in the outer membrane was not a transient change as the membrane structure was stable enough to capture in our AFM images. Thus, the remodeling of the outer membrane and gradual failing of the cell’s homeostatic machinery leading to a loss of turgor pressure are likely important in the bactericidal activity of MAG2.

These changes in the cell lead to the conclusion that, for MAG2-susceptible bacteria, interaction of the peptide with the outer membrane is a highly disruptive process. However, not all Gram-negative bacteria are as susceptible to MAG2 as this strain of *E. coli* and the outer membrane, particularly the LPS structure, play an important role in MAG2 effectiveness. Smooth LPS, which contains repeating polysaccharides called the O-antigen, has previously been shown to moderate the bactericidal activity of pore-forming AMPS.\(^{38,63,64}\) Thus, in order to fully comprehend how some bacteria may evade AMP-mediated permeabilization, we must understand how MAG2 interacts with different forms of the Gram-negative outer membrane. Here, we used a rough *E. coli* strain, but future work must also investigate the biophysical consequences of MAG2 on smooth strains. Furthermore, these experiments must be conducted in a cellular context as model membranes are not always the best predictor of a cellular mechanism. Targeting the bacterial membrane is a prudent choice for new antibiotics as the rate of resistance is low, and understanding how AMPs permeabilize different bacterial membranes could bring us closer to that goal.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
ACKNOWLEDGMENTS

The authors wish to thank Louise Darling for her help with the fluorescence microscopy and Asriel Walker for her help with the cell adhesion. This work was supported by a National Science Foundation Major Research Instrumentation grant (number DBI1528288).

REFERENCES

(1). Davies J; Davies D Origins and Evolution of Antibiotic Resistance. Microbiol. Mol. Biol. Rev 2010, 74, 417–433. [PubMed: 20805405]

(2). Infectious Disease Society of America. The 10 x '20 Initiative: Pursuing a Global Commitment to Develop 10 New Antibacterial Drugs by 2020. Clinical Infectious Diseases 2010, 50, 1081–1083. [PubMed: 20214473]

(3). Peschel A; Sahl H-G The Co-Evolution of Host Cationic Antimicrobial Peptides and Microbial Resistance. Nat. Rev. Microbiol 2006, 4, 529–536. [PubMed: 16778838]

(4). Lai Y; Gallo RL AMPed up Immunity: How Antimicrobial Peptides Have Multiple Roles in Immune Defense. Trends Immunol. 2009, 30, 131–141. [PubMed: 19217824]

(5). Fjell CD; His JA; Hancock REW; Schneider G Designing Antimicrobial Peptides: Form Follows Function. Nat. Rev. Drug Discovery 2012, 11, 37–51.

(6). Shagaghi N; Palombo EA; Clayton AHA; Bhave M Antimicrobial Peptides: Biochemical Determinants of Activity and Biophysical Techniques of Elucidating Their Functionality. World J. Microbiol. Biotechnol 2018, 34, 62. [PubMed: 29651655]

(7). Zasloff M Magainins, a Class of Antimicrobial Peptides from Xenopus Skin: Isolation, Characterization of Two Active Forms, and Partial cDNA Sequence of a Precursor. Proc. Natl. Acad. Sci 1987, 84, 5449–5453. [PubMed: 3299384]

(8). Soravia E; Martini G; Zasloff M Antimicrobial Properties of Peptides from Xenopus Granular Gland Secretions. FEBS Lett. 1988, 228, 337–340. [PubMed: 3125066]

(9). Westerhoff HV; Juretić D; Hendler RW; Zasloff M Magainins and the Disruption of Membrane-Linked Free-Energy Transduction. Proc. Natl. Acad. Sci 1989, 86, 6597–6601. [PubMed: 2671997]

(10). Zasloff M; Martin B; Chen HC Antimicrobial Activity of Synthetic Magainin Peptides and Several Analogues. Proc. Natl. Acad. Sci 1988, 85, 910–913. [PubMed: 3277183]

(11). Cruciani RA; Barker JL; Durell SR; Raghunathan G; Guy HR; Zasloff M; Stanley EF Magainin 2, a Natural Antibiotic from Frog Skin, Forms Ion Channels in Lipid Bilayer Membranes. Eur. J. Pharmacol 1992, 226, 287–296. [PubMed: 1383011]

(12). Bechinger B The SMART Model: Soft Membranes Adapt and Respond, Also Transiently, in the Presence of Antimicrobial Peptides. J. Pept. Sci 2015, 21, 346–355. [PubMed: 25522713]

(13). Wenk MR; Seelig J Magainin 2 Amide Interaction with Lipid Membranes: Calorimetric Detection of Peptide Binding and Pore Formation. Biochemistry 1998, 37, 3909–3916. [PubMed: 9521712]

(14). Wieprecht T; Beyermann M; Seelig J Binding of Antibacterial Magainin Peptides to Electrically Neutral Membranes: Thermodynamics and Structure. Biochemistry 1999, 38, 10377–10387. [PubMed: 10441132]

(15). Matsuzaki K; Harada M; Funakoshi S; Fuji J; Miyajima K Physicochemical Determinants for the Interactions of Magainins 1 and 2 with Acidic Lipid Bilayers. Biochim. Biophys. Acta, Biomembr 1991, 1063, 162–170.

(16). Silhavy TJ; Kahne D; Walker S The Bacterial Cell Envelope. Cold Spring Harbor Perspect. Biol 2010, 2, a000414.

(17). Dufrêne YF Atomic Force Microscopy, a Powerful Tool in Microbiology. J. Bacteriol 2002, 184, 5205–5213. [PubMed: 12218005]

(18). Formosa-Dague C; Duval RE; Dague E Cell Biology of Microbes and Pharmacology of Antimicrobial Drugs Explored by Atomic Force Microscopy. Semin. Cell Dev. Biol 2018, 73, 165–176. [PubMed: 28668355]
(19). Meincken M; Holroyd DL; Rautenbach M Atomic Force Microscopy Study of the Effect of Antimicrobial Peptides on the Cell Envelope of *Escherichia coli*. Antimicrob. Agents Chemother 2005, 49, 4085–4092. [PubMed: 16189084]

(20). Matsuzaki K; Murase O; Tokuda H; Funakoshi S; Fujii N; Miyajima K Orientational and Aggregational States of Magainin 2 in Phospholipid Bilayers. Biochemistry 2002, 33, 3342–3349.

(21). Wade HM; Darling LEO; Elmore DE Hybrids Made from Antimicrobial Peptides with Different Mechanisms of Action Show Enhanced Membrane Permeabilization. Biochim. Biophys. Acta, Biomembr 2019, 1861, 182980. [PubMed: 31067436]

(22). Wiegand I; Hilpert K; Hancock REW Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. Nat. Protoc 2008, 3, 163–175. [PubMed: 18274517]

(23). Xie Y; Fleming E; Chen JL; Elmore DE Effect of Proline Position on the Antimicrobial Mechanism of Buforin II. Peptides 2011, 32, 677–682. [PubMed: 21277926]

(24). Pavia KE; Spinella SA; Elmore DE Novel Histone-Derived Antimicrobial Peptides Use Different Antimicrobial Mechanisms. Biochim. Biophys. Acta, Biomembr 2012, 1818, 869–876.

(25). Sharifian Gh M; Wilhelm MJ; Dai H-L Label-Free Optical Method for Quantifying Molecular Transport Across Cellular Membranes *In Vitro*. J. Phys. Chem. Lett 2016, 7, 3406–3411. [PubMed: 27518496]

(26). Volle CB; Ferguson MA; Aidala KE; Spain EM; Núñez ME Quantitative Changes in the Elasticity and Adhesive Properties of *Escherichia coli* ZK1056 Prey Cells during Predation by *Bdellovibrio bacteriovorus* 109J. Langmuir 2008, 24, 8102–8110. [PubMed: 18572929]

(27). Volle CB; Ferguson MA; Aidala KE; Spain EM; Núñez ME Spring Constants and Adhesive Properties of Native Bacterial Biofilm Cells Measured by Atomic Force Microscopy. Colloids Surf., B 2008, 67, 32–40.

(28). Wieprecht T; Dathe M; Beyermann M; Krause E; Maloy WL; MacDonald DL; Bienert M Peptide Hydrophobicity Controls the Activity and Selectivity of Magainin 2 Amide in Interaction with Membranes. Biochemistry 1997, 36, 6124–6132. [PubMed: 9166783]

(29). Bessalle R; Kapitkovsky A; Gorea A; Shalit I; Fridkin M All-D-Magainin: Chirality, Antimicrobial Activity and Proteolytic Resistance. FEBS Lett. 1990, 274, 151–155. [PubMed: 2253768]

(30). Bessalle R; Haas H; Goria A; Shalit I; Fridkin M Augmentation of the Antibacterial Activity of Magainin by Positive-Charge Chain Extension. Antimicrob. Agents Chemother 1992, 36, 313–317. [PubMed: 1605597]

(31). Stocks SM Mechanism and Use of the Commercially Available Viability Stain, BacLight. Cytometry, Part A 2004, 61A, 189–195.

(32). Yeh C-JG; Hsi B-L; Faulk WP Propidium Iodide as a Nuclear Marker in Immunofluorescence. II. Use with Cellular Identification and Viability Studies. J. Immunol. Methods 1981, 43, 269–275. [PubMed: 7019344]

(33). Rosenfeld Y; Shai Y Lipopolysaccharide (Endotoxin)-Host Defense Antibacterial Peptides Interactions: Role in Bacterial Resistance and Prevention of Sepsis. Biochim. Biophys. Acta, (BBA) Biomembr 2006, 1758, 1513–1522.

(34). Hancock REW; Diamond G The Role of Cationic Antimicrobial Peptides in Innate Host Defences. Trends Microbiol. 2000, 8, 402–410. [PubMed: 10989307]

(35). Nikaido H Outer Membrane Barrier as a Mechanism of Antimicrobial Resistance. Antimicrob. Agents Chemother 1989, 33, 1831–1836. [PubMed: 2692513]

(36). Raetz CRH; Whitfield C Lipopolysaccharide Endotoxins. Annu. Rev. Biochem 2002, 71, 635–700. [PubMed: 12045108]

(37). Weiss J; Beckerdlite-Quagliata S; Elsbach P Resistance of Gram-Negative Bacteria to Purified Bactericidal Leukocyte Proteins. J. Clin. Invest 1980, 65, 619–628. [PubMed: 6986410]

(38). Rana FR; Macias EA; Sultany CM; Modzrakowski MC; Blazyk J Interactions between Magainin and *Salmonella typhimurium* Outer Membranes: Effect of Lipopolysaccharide Structure. Biochemistry 1999, 30, 5858–5866. [PubMed: 2043628]
(39). Xu H; Murdacha AE; Chen W; Aidala KE; Ferguson MA; Spain EM; Núñez ME Characterizing Pilus-Mediated Adhesion of Biofilm-Forming E. coli to Chemically Diverse Surfaces Using Atomic Force Microscopy. Langmuir 2013, 29, 3000–3011. [PubMed: 23421314]

(40). André J; Koch MJH; Bartels R; Brandenburg K Biophysical Characterization of Endotoxin Inactivation by NK-2, an Antimicrobial Peptide Derived from Mammalian NK-Lysin. Antimicrob. Agents Chemother 2004, 48, 1593–1599. [PubMed: 15105110]

(41). Gutfmann T; Hagge SO; David A; Roes S; Böhling A; Hammer MU; Seydel U Lipid-Mediated Resistance of Gram-Negative Bacteria against Various Pore-Forming Antimicrobial Peptides. J. Endotoxin Res 2005, 11, 167–173. [PubMed: 15949145]

(42). Strauss J; Kadilak A; Cronin C; Mello CM; Camesano TA Binding, Inactivation, and Adhesion Forces between Antimicrobial Peptide Cecropin P1 and Pathogenic E. coli. Colloids Surf., B 2010, 75, 156–164.

(43). Lozeau LD; Alexander TE; Camesano TA Proposed Mechanisms of Tethered Antimicrobial Peptide Chrysopsin-I as a Function of Tether Length Using QCM-D. J. Phys. Chem. B 2015, 119, 13142–13151. [PubMed: 26388176]

(44). Arnoldi M; Fritz M; Bäuerlein E; Redmacher M; Sackmann E; Boullitch A Bacterial Turgor Pressure Can Be Measured by Atomic Force Microscopy. Phys. Rev. E 2000, 62, 1034–1044.

(45). Yao X; Walter J; Burke S; Stewart S; Jericho MH; Pink D; Hunter R; Beveridge TJ Atomic Force Microscopy and Theoretical Considerations of Surface Properties and Turgor Pressures of Bacteria. Colloids Surf., B 2002, 23, 213–230.

(46). Fantner GE; Barbero RJ; Gray DS; Belcher AM Kinetics of Antimicrobial Peptide Activity Measured on Individual Bacterial Cells Using High-Speed Atomic Force Microscopy. Nat. Nanotechnol 2010, 5, 280–285. [PubMed: 20228787]

(47). Mularski A; Wilksch JJ; Wang H; Hossain MA; Wade JD; Separovic F; Strugnell RA; Gee ML Atomic Force Microscopy Reveals the Mechanobiology of Lytic Peptide Action on Bacteria. Langmuir 2015, 31, 6164–6171. [PubMed: 25978768]

(48). Su H-N; Chen Z-H; Song X-Y; Chen X-L; Shi M; Zhou B-C; Zhao X; Zhang Y-Z Antimicrobial Peptide Trichokinin VI-Induced Alterations in the Morphological and Nanomechanical Properties of Bacillus subtilis. PLoS One 2012, 7, e45818. [PubMed: 23049870]

(49). Gee ML; Burton M; Grevis-James A; Hossain MA; McArthur S; Palombo EA; Wade JD; Clayton AHA Imaging the Action of Antimicrobial Peptides on Living Bacterial Cells. Sci. Rep 2013, 3, 1557. [PubMed: 23532056]

(50). Yang Z; Choi H; Weisshaar JC Melittin-Induced Permeabilization, Re-Sealing, and Re-Permeabilization of E. coli Membranes. Biophys. J. 2018, 114, 368–379. [PubMed: 29401434]

(51). Camesano TA; Logan BE Probing Bacterial Electrosteric Interactions Using Atomic Force Microscopy. Environ. Sci. Technol 2000, 34, 3354–3362.

(52). Vadillo-Rodríguez V; Busscher HJ; Nordé W; de Vries J; Dijkstra RJB; Stokroos J.; van der Mei, H. C. Comparison of Atomic Force Microscopy Interaction Forces between Bacteria and Silicon Nitride Substrata for Three Commonly Used Immobilization Methods. Appl. Environ. Microbiol 2004, 70, 5441–5446. [PubMed: 15345431]

(53). Hall K; Lee T-H; Mechler AI; Swann MJ; Aguilar M-I Real-Time Measurement of Membrane Conformational States Induced by Antimicrobial Peptides: Balance between Recovery and Lysis. Sci. Rep 2014, 4, 5479. [PubMed: 24969959]

(54). Marin-Medina N; Mescola A; Alessandri A Effects of the Peptide Magainin H2 on Supported Lipid Bilayers Studied by Different Biophysical Techniques. Biochim. Biophys. Acta, Biomembr 2018, 1860, 2635–2643. [PubMed: 30292399]

(55). Matsuzaki K; Sugishita K; Harada M; Fujii N; Miyajima K Interactions of an Antimicrobial Peptide, Magainin 2, with Outer and Inner Membranes of Gram-Negative Bacteria. Biochim. Biophys. Acta (BBA) Biomembr 1997, 1327, 119–130.

(56). da Silva A Jr.; Tesche O Effects of the Antimicrobial Peptide PGLa on Live Escherichia coli. Biochim. Biophys. Acta 2003, 1643, 95–103. [PubMed: 14654232]

(57). Oh YJ; Plochberger B; Rechberger M; Hinterdorfer P Characterizing the Effect of Polymyxin B Antibiotics to Lipopolysaccharide on Escherichia coli Surface Using Atomic Force Microscopy. J. Mol. Recognit. Journal of Molecular Recognition 2017, 30, 1–7.

Langmuir. Author manuscript; available in PMC 2020 August 17.
(58). Zdybicka-Barabas A; Januszani B; Mak P; Cytryńska M An Atomic Force Microscopy Study of Galleria Mellonella Apolipophorin III Effect on Bacteria. Biochim. Biophys. Acta, Biomembr 2011, 1808, 1896–1906.

(59). Torcato IM; Huang Y-H; Franquelim HG; Gaspar D; Craik DJ; Castanho MARB; Henriques ST Design and Characterization of Novel Antimicrobial Peptides, R-BP100 and RW-BP100, with Activity against Gram-Negative and Gram-Positive Bacteria. Biochimica et Biophysica Acta (BBA) - Biomembranes 2013, 1828, 944–955. [PubMed: 23246973]

(60). Soon RL; Nation RL; Hartley PG; Larson I; Li J Atomic Force Microscopy Investigation of the Morphology and Topography of Colistin-Heteroresistant Acinetobacter baumannii Strains as a Function of Growth Phase and in Response to Colistin Treatment. Antimicrob. Agents Chemother 2009, 53, 4979–4986. [PubMed: 19786595]

(61). Alves CS; Melo MN; Franquelim HG; Ferre R; Planas M; Feliu L; Bardaji E; Kowalczyk W; Andreu D; Santos NC; Fernandes MX; Castanho MARB Escherichia coli Cell Surface Perturbation and Disruption Induced by Antimicrobial Peptides BP100 and PepR. J. Biol. Chem 2010, 285, 27536–27544. [PubMed: 20566635]

(62). Li A; Lee PY; Ho B; Ding JL; Lim CT Atomic Force Microscopy Study of the Antimicrobial Action of Sushi Peptides on Gram Negative Bacteria. Biochim. Biophys. Acta (BBA) Biomembr 2007, 1768, 411–418.

(63). Groisman EA; Parra-Lopez C; Salcedo M; Lipps CJ; Heffron F Resistance to Host Antimicrobial Peptides Is Necessary for Salmonella Virulence. Proc. Nat. Acad. Sci 1992, 89, 11939–11943. [PubMed: 1465423]

(64). Banemann A; Deprisch H; Gross R The Lipopolysaccharide of Bordetella Bronchiseptica Acts as a Protective Shield against Antimicrobial Peptides. Infect. Immun 1998, 66, 5607–5612. [PubMed: 9826332]
Figure 1.
Representative images from live/dead staining of planktonic *E. coli* cells after 30 min in the (A) absence or (B) presence of 16.5 μM magainin II. Cells stained with SYTO 9, a membrane-permeable cellular dye that enters all cells, appear green. However, cells stained with membrane-impermeable propidium iodide have lost membrane integrity and appear red.
Figure 2.
Propidium iodide (PI) fluorescence increases after addition of magainin II (MAG2) to planktonic *E. coli* cells. The green diamonds show the average PI fluorescence after addition of 12.4 μM MAG2, the orange squares show the average PI fluorescence after addition of 16.5 μM magainin II, and the blue circles show the average PI fluorescence after addition of 24.8 μM magainin II. The error bars indicate the standard error from four individual experiments.
Figure 3.
Extension curve of the force cycle is composed of three regimes, shown in panel (A): (i) the linear approach as the tip moves toward the surface, (ii) the nonlinear compression as the tip makes initial contact with the cell surface, and (iii) the linear compression as the tip pushes into the cell and encounters resistance from the cell’s turgor pressure. (B) Two representative force curves from an untreated cell at time 0 are shown in blue, while representative force curves from the same cell after a 30 min incubation in PB are shown in green. (C) Two representative force curves from a treated cell at time 0 are shown in orange, while two force curves from the same cell after 30 min of MAG2 treatment are shown in purple.
Figure 4.
MAG2 causes a decrease in cellular turgor pressure after several minutes of treatment. Cell stiffness measurements were taken on treated (orange diamonds) and untreated (blue squares) cells over a 30 min period. The $k_{cell}$ was calculated as described in eq 1. Each data set represents a single cell, and each point represents the average $k_{cell}$ determined from five force acquisitions. The standard deviation is presented, but the value is too small to be discernable from the marker. To account for differences in the starting stiffness between cells, each was normalized to $k_{cell}$ at time 0. Images were captured after each force acquisition to ensure that cells remained in place. These data are representative of the cells observed (untreated $n = 12$, treated $n = 31$).
Figure 5.
Nonlinear regions of untreated cells cluster together regardless of time, but the nonlinear region of *E. coli* cells treated with MAG2 for 30 min has larger changes in both force and distance. As seen in panel (A), the nonlinear regions of untreated cells at time 0 (blue diamonds) and after 30 min (green triangles) display a high degree of overlap. However, when comparing the nonlinear regions of treated cells at time 0 (orange squares) and those of treated cells after 30 min exposure to MAG2 (purple circles), the cells at time 0 cluster with the other untreated cells, but the treated cells have significantly larger changes in force and distance (Student’s *t*-test, *P* < 0.001 for both force and distance). Representative examples of the nonlinear region for (B) untreated cells at time 0, (C) untreated cells at 30 min, (D) treated cells at time 0, and (E) treated cells at 30 min are shown below, with the nonlinear region highlighted.
Figure 6.
Amplitude images of planktonic *E. coli* cells captured by AFM. Images were obtained in (A, B) the absence or (C, D) the presence of 16.5 μM magainin II. The images in panels (A) and (C) were obtained at 0 min. The image in panel (B) was obtained at 60 min, the image in panel (D) was obtained at 50 min. The data presented here are from different experiments but are representative of the cells observed (untreated *n* = 20, treated *n* = 56).
Figure 7.
MAG2 treatment increases the adhesive forces between the AFM tip and the bacterial surface. The adhesion between the tip and untreated cells was analyzed (A) at the beginning and (B) at the end of the 30 min experiment. Likewise, the adhesion between the tip and the treated cells was also analyzed (C) before MAG2 injection and (D) at the end of the 30 min experiment. Each panel shows four representative retraction curves, each from a different cell.
Table 1.
MAG2 Treatment Increases the Surface Roughness of *E. coli* Cells

| cells  | Rq at time 0 (nm) | Rq at time 30+ (nm) |
|--------|------------------|---------------------|
| untreated | 118.3 ± 8.2      | 168.7 ± 3.8         |
| treated  | 118.2 ± 2.1      | 195.9 ± 7.2         |