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Resistance of early stationary phase *E. coli* to membrane permeabilization by the antimicrobial peptide Cecropin A

Anurag Agrawal, Nambirajan Rangarajan^a, James C. Weisshaar^b

Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI USA 53706

Abstract

Antimicrobial peptides (AMPs) cause bacterial membrane permeabilization and ultimately cell death at low μM concentrations. The membrane permeabilization action of a moth derived AMP Cecropin A on *E. coli* cells in exponential growth (mid-log phase) is well studied. At $1\times\text{MIC}$ concentration, Cecropin A penetrates the lipopolysaccharide (LPS) barrier and causes outer membrane (OM) and cytoplasmic membrane (CM) permeabilization. For non-septating cells, permeabilization of both membranes begins at one pole. For septating cells, OM permeabilization begins at the septal region and CM permeabilization begins at one pole. However, in nature bacteria are frequently found in nutrient-starved conditions. Here we extend our single-cell microscopy assays to the attack of Cecropin A on *E. coli* cells in early stationary phase. Stationary phase *E. coli* is much more resistant to membrane permeabilization by Cecropin A than mid-log phase *E. coli*. A tenfold higher concentration of Cecropin A is required to observe CM permeabilization in the majority of stationary phase cells, and even then permeabilization proceeds more slowly. In addition, the spatial pattern of initial CM permeabilization changes from localized at one pole to global. Studies of lipid mutant strains suggest that a sufficient localized concentration of the anionic phospholipid phosphatidylglycerol (PG) guides the position of initial attack of the cationic AMP Cecropin A on the CM.

Keywords

Antimicrobial peptides; Cecropin A; stationary phase *E. coli*; single-cell time-resolved microscopy

1. Introduction

Antimicrobial peptides (AMPs, also called host-defense peptides) occur in nature as an ancient class of polypeptides, usually <40 aa in length and often cationic [1]. As part of the innate immune system, AMPs exhibit broad spectrum antibacterial activity against both Gram-negative and Gram-positive bacteria. In this era of multi-drug resistance, AMPs may provide templates for the design of new antibacterial agents. Many cationic AMPs are known to permeabilize model lipid bilayers and real bacterial membranes [2, 3]. However, it

^bCorresponding author: weisshaar@chem.wisc.edu.

^aCurrent address: Department of Pharmacology, U. North Carolina at Chapel Hill, Chapel Hill, N.C. 27599, U.S.A.

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is increasingly clear that the massive influx of cationic AMPs after membrane permeabilization [4–6] can impair a wide variety of bacterial mechanisms. Examples include inhibition of transcription and DNA replication, of cytokinesis, of cell wall biosynthesis, and of enzymatic activity and protein synthesis [7]. Most mechanistic studies of the bactericidal effects of AMPs, including the time-honored MIC assay, have focused on bulk, planktonic cultures. We and others have recently used single-cell microscopy to study the mechanism and timing of AMP action on live *E. coli* growing exponentially (mid-log phase cells) [6, 8–16].

In nature bacteria spend much of the time in a nutrient starved condition of minimal growth known as stationary phase. As they approach stationary phase, bacterial populations undergo dramatic changes in morphology, metabolism, transcriptional functions, and translational programs [17, 18]. These changes help protect cells from a variety of stresses including osmotic shock, oxidative stress, heat shock, and acid and base shock [18]. It is well known that stationary phase bacteria are much more resistant than exponentially growing cells to killing by conventional antibiotics such as ampicillin, tetracycline, ciprofloxacin, and streptomycin [19]. Only a handful of studies in bulk cultures have addressed the effectiveness of AMPs in killing stationary phase bacteria. In stationary phase both *E. coli* and *Salmonella* strains showed inhibition of cell lysis by the BPI-derived peptide P2 [20]. Similarly, starved and stationary phase cells of *Salmonella typhimurium* exhibited 200- to 1500-fold greater resistance to polymyxin B compared to cells in exponential growth [21]. A peptide-modified lysin PlyA showed effective antibacterial activity against exponential phase *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, but much reduced activity against the same strains in stationary phase [22]. In contrast, LL-37 at a concentration below 1.0 μ M had higher ability to kill *Staphylococcus aureus* cells in stationary phase than in mid-log phase [23].

Single-cell studies of membrane permeabilization events induced in stationary phase bacteria by AMPs are rare. The susceptibility of *E. coli* to human α -defensin 5 (HD5_{ox}) was shown to be lower in stationary phase compared to mid-log phase cells [10]. The authors suspected a correlation between bacterial susceptibility and altered cellular morphology. Here we employ single-cell microscopy assays to study the timing and spatial localization pattern of cytoplasmic membrane (CM) permeabilization of live *E. coli* cells in stationary phase by the natural AMP Cecropin A. Cecropin A (37-aa, net +6 charge, sequence KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH₂), is derived from the moth *Hyalophora cecropia*. Its positive residues concentrate near the N-terminus of the chain while its hydrophobic residues concentrate near the C-terminus (Figure 1) [24]. Based on studies of both phospholipid vesicles [25, 26] and exponentially growing *E. coli* cells [27], the antibacterial action of Cecropin A is attributed primarily to its ability to rapidly permeabilize both the outer membrane (OM) and the CM.

Here we use the DNA stain Sytox Orange to quantitatively compare the timing and the degree of permeabilization by Cecropin A of the CM of *E. coli* cells in exponential growth and in early stationary phase. This necessitated development of a protocol using spent growth medium to maintain cells in stationary phase during observation under the microscope. The stationary phase cells are much more resistant to Cecropin A action. At a

Cecropin A concentration of 0.5 μM ($1\times\text{MIC}$), all exponentially growing cells were permeabilized quickly. At the same Cecropin A concentration, no CM permeabilization was observed for stationary phase cells over an observation period of 60 min. The cells remain viable; when fresh growth medium without AMP is restored, growth begins immediately. Even at 5 μM ($10\times\text{MIC}$), only 66% of the stationary phase cells exhibited CM permeabilization over 60 min, and these events occurred slowly. We tentatively attribute this greater resistance to alteration of the composition of the cell envelope during stationary phase. In addition, the initial location of CM permeabilization changes from polar to global as cells transition from exponential growth to stationary phase. Studies of mutant strains suggest that a high concentration of the anionic lipid phosphatidylglycerol (PG) at the poles tends to direct the site of initial CM permeabilization to a pole.

2. Materials and Methods

2.1. Bacterial strains, materials, and growth conditions

The wild-type (WT) *E. coli* strain for this study was MG1655 (K-12). The other strains BK12 and BKT29 were a gift from the Weibel lab at the University of Wisconsin-Madison. The strain BKT12 is a triple-CL (cardiolipin) synthase knockout strain from the MG1655 parent strain [28]. These cells cannot make CL and have excess PG, as described in detail below. Strain BKT29 is a mutant of MG1655 that cannot make PG (phosphatidylglycerol) and also lacks CL synthesis associated genes (*clsABC*, *ymdB::Kan^R*) [29].

Bulk cultures were grown in a chemically defined rich medium EZRDM [30], which contains a MOPS-buffered solution supplemented with metal ions (M2130; Teknova), glucose (2 mg/mL), amino acids and vitamins (M2104; Teknova), nitrogenous bases (M2103; Teknova), 1.32 mM K_2HPO_4 , and 76 mM NaCl. Cell cultures were grown overnight at 30°C to stationary phase ($\text{OD} = 0.8\text{--}1.2$ at 600 nm) from glycerol frozen stocks. For the experiments requiring mid-log phase cells, subcultures were grown to exponential phase ($\text{OD} = 0.2\text{--}0.4$ at 600 nm) before plating for imaging.

For the microscopy experiments on early stationary phase cells, a new protocol was developed (Figure S1). The stationary phase cells were obtained from cultures grown overnight (~ 16 h) ($\text{OD} = 0.8\text{--}1.2$ at 600 nm) at 30°C. This early stationary phase cell culture was used directly for experiments. To maintain the cells in stationary phase during the experiments, spent EZRDM was harvested. Cell culture grown overnight was transferred to an Eppendorf tube. The tube was centrifuged so that the cell culture makes a pellet at the bottom of the tube. The supernatant was carefully extracted. This supernatant is defined as spent EZRDM. Spent EZRDM is depleted in nutrients due to bacterial metabolism while the culture evolves from exponential growth to stationary phase. It may also contain toxic metabolic products [17].

Cecropin A (Anaspec, catalog no. 24010, $> 95\%$ purity) was purchased as lyophilized powder and used without further purification. Sterile, ultrapure (18 M Ω) water was used to make peptide stock solutions. The DNA stain Sytox Orange (S11368) was purchased from Thermo-Fisher Scientific. The minimum inhibitory concentration (MIC) for Cecropin A against each strain was determined using a broth microdilution method as previously

described [8]. The 12-hr MIC of Cecropin A for all three strains was 0.5 μ M, reproducible across three trials each.

2.2. Bulk culture growth curve

Growth curves were measured on a Tecan Infinite 200 PRO instrument using a 96 well plate. First, cell cultures were grown overnight at 30°C to stationary phase from a frozen glycerol stock. In 5 wells near the center of the plate, the cell culture was diluted 1:100 in fresh EZRDM to a final volume of 100 μ L. Similarly in 5 other central wells, the cell culture were diluted 1:100 in spent EZRDM to a final volume of 100 μ L. The unused edge wells were filled with water to limit evaporation from the cell culture wells. The plate was maintained at 30°C by the instrument. Optical density at 600 nm wavelength was recorded at intervals of 10 min for 24 hrs. The mean optical density vs time for both fresh and spent EZRDM are plotted in Figure S2.

2.3. Microfluidics chamber for time-lapse imaging

Single-cell imaging experiments were performed in a microfluidics chamber described earlier [31]. The chamber consists of a single rectilinear channel of uniform height of 50 μ m, width of 6 mm, and length of 11 mm. The total chamber volume is $\sim$ 10 μ L. The glass coverslip was cleaned first in acetone and then through plasma oxidation. A polydimethylsiloxane (PDMS) slab containing the chamber pattern was then bonded to the coverslip. After chamber assembly, 0.01% poly-*L*-lysine (molecular weight >150,000 Da) was injected through the chamber for 30 min, followed by thorough rinsing with Millipore water. During imaging, the chamber was maintained at 30°C by a TC-344B dual channel temperature controller and CC-28 heating cables attached to RH-2 heater blocks (Warner Instruments). Injection of solutions into the flow chamber was performed with a syringe pump using 1 mL NORMJECT injection syringes. The flow of fresh, aerated medium or of spent EZRDM is continuous throughout the observation. This maintains a stable external bulk Cecropin A concentration around the plated cells.

2.4. Microscopy

Single-cell imaging experiments were performed on a Nikon Eclipse Ti inverted microscope with a 100 $\times$, 1.45 N.A. phase contrast objective (Nikon). Images were further magnified 1.5 $\times$ using the built-in magnification lens of the microscope. The pixel size corresponds to 106 ± 10 nm at the sample. Sytox Orange was imaged using 561 nm excitation (Coherent Sapphire laser). The laser was expanded to illuminate the field of view uniformly. Emission filter HQ605/75 (Chroma) was used for the red channel (Sytox Orange). Fluorescence images were detected by an EMCCD camera Andor iXon 897.

The one-color imaging experiments used red fluorescence from Sytox Orange to observe CM permeabilization. Intervening phase contrast images were acquired to monitor cell length vs time. Time-lapse movies of 30–60 min duration were obtained, with red channel fluorescence and phase contrast images interleaved (3 s per complete cycle) and an exposure time of 50 ms/frame. μ Manager software was used for data acquisition and switching of filters between frames using a LB10-NW filter wheel (Sutter). At $t = 0$, EZRDM with Cecropin A and 5 nM Sytox Orange is injected into the flow chamber. For cells in

exponential growth, the solution of Cecropin A and Sytox Orange was made in fresh EZRDM. To maintain cells in stationary phase during imaging, spent EZRDM was used. Cytoplasmic membrane permeabilization events caused by Cecropin A are monitored using the change in intensity and spatial distribution of Sytox Orange fluorescence over time. When both OM and CM have been permeabilized to Sytox Orange, the dye gains access to the cytoplasm, binds to the chromosomal DNA, and becomes fluorescent.

Faster, one-color imaging movies (fluorescence only) were obtained to identify the spatial pattern of CM permeabilization to Sytox Orange [9]. Time-lapse movies of red channel fluorescence from Sytox Orange were acquired at 0.5 s per cycle with an exposure time of 50 ms/frame. μ Manager software was used for data acquisition. These experiments were performed at 5 μ M Cecropin A to ensure that permeabilization occurs during a shorter period. Time-lapse movies of 30–60 min duration were obtained.

2.5. Data analysis

Images were acquired using the software μ Manager (version 1.4) [32]. Image J (version 1.49m) and Microcal Origin (version 9.0) were used for the data analysis. The intensity analysis was performed using a custom macro code written in ImageJ. For measuring the total fluorescence intensity in a single cell, a region of interest (ROI) enclosing the cell was drawn and the background was subtracted by duplicating the same ROI on an area that did not contain a cell. For the Sytox Orange signal, the time point at which the background corrected Sytox Orange signal begins to rise is defined as t_{CM} for that cell. The rate of Sytox leakage through the CM is characterized by the risetime t_{Sytox} , which is defined as the interval over which the Sytox Orange intensity increased from 10% to 90% of its maximum value. The measurement process was repeated for each well plated, well isolated cell in the field of view.

The cytoplasmic membrane permeabilization pattern was classified as either local or global from movies obtained at 0.5 s per camera cycle. This classification was performed using the time dependence of the axial intensity profiles for each cell. For each cell, total Sytox Orange intensity was projected along the x -axis (long axis) of the cell to provide the axial spatial distribution of Sytox Orange within the cytoplasm at several time points for each cell. In the cells that exhibit localized CM permeabilization, the distribution of Sytox Orange intensity initially increases near one endcap. The pattern gradually changes to a uniformly stained nucleoid distribution over time. In the cells that exhibit global CM permeabilization, the intensity increases simultaneously across the entire nucleoid length, even at early times after the onset of flow of the AMP. The two cases can be distinguished quantitatively by comparing the full width at half maximum (FWHM) of the axial intensity curves at different time points for each cell. If the FWHM increases vs time, the permeabilization is local. If the FWHM remains essentially constant vs time, the permeabilization is global.

3. Results

3.1. Spent EZRDM medium maintains cells in stationary phase

A bulk growth curve comparison was performed for *E. coli* cells in fresh EZRDM and spent EZRDM to determine if cells can be maintained in a state of minimal growth by the spent medium. The optical density at 600 nm was measured at 10 min intervals for a period of 24 hr. In fresh EZRDM, the cells follow a typical bacterial growth curve (Figure S2). The doubling time was 57 ± 1 min. In spent EZRDM, we observed negligible optical density increase (Figure S2).

3.2. Single-cell growth of stationary phase cells in fresh and spent EZRDM

Single-cell growth controls were performed using the microfluidic chamber as sample cell for microscopy experiments. Phase contrast images of *E. coli* cells enable observation of cell morphology and measurement of cell length vs time. The average length of *E. coli* cells in the mid-log phase of growth in fresh EZRDM was $5.5 \pm 1.2 \mu\text{m}$ (± 1 standard deviation). The mid-log phase cells keep growing and dividing in the microfluidic chamber when a flow of fresh EZRDM is maintained. These cells show an average increase of ~25% in cell length over 30 min (Figure S3). The stationary phase cells are significantly shorter than the exponential phase cells when observed through the microscope (Figure S4). The average length of *E. coli* cells in the stationary phase was $2.0 \pm 0.4 \mu\text{m}$. These stationary phase cells are much shorter than even the non-septating cells in mid-log phase of growth, whose average length was $4.5 \pm 0.6 \mu\text{m}$. The cell diameter is similar in both cases. On average, the stationary phase cells show <5% increase in cell length when spent medium is flowed through the chamber for 1 hr (Figure 2). However, when fresh EZRDM is flowed across newly plated stationary phase cells they begin to grow in a few minutes (Figure 2). The stationary phase cells show an average increase of ~60% in cell length over 1 hr of flowing fresh EZRDM. Use of spent EZRDM greatly inhibits growth of stationary phase cells.

We also checked that exposing plated stationary phase cells to spent EZRDM did not affect cell viability. Following a 30-min flow of spent EZRDM, the plated stationary phase cells immediately began to grow when the medium was switched to fresh EZRDM (Figure S5). Once the medium was switched, the cells increase in length by an average of ~25% over the next 30 min (Figure S5).

3.3. Cecropin A action on stationary phase cells

Next we performed live-cell imaging experiments to measure the time course of Cecropin A action on single *E. coli* cells growing exponentially or in stationary phase. These experiments were performed using the imaging assays described in our previous work [14]. Briefly, for *E. coli* cells in exponential growth phase, a solution of Cecropin A and 5 nM Sytox Orange in fresh EZRDM flowed through the microfluidic chamber containing plated mid-log cells (Methods). For cells in stationary phase, the Cecropin A and Sytox Orange solution was prepared with spent EZRDM. For both cases, interleaved images of phase contrast and Sytox Orange were obtained with a 3 s cycle time. The onset of Cecropin A flow is defined as $t = 0$. The time when the Sytox Orange signal starts to rise is defined as

t_{CM} , the time of the onset of cytoplasmic permeabilization to the dye and, by inference, to Cecropin A itself.

For *E. coli* cells maintained in mid-log phase, the experiments were performed at 0.5 μ M of Cecropin A, 1 $\times$ the 12 hr MIC for *E. coli* cells in fresh EZRDM. All the cells in mid-log phase exhibited CM permeabilization to Sytox Orange within 10 min of the onset of Cecropin A flow (Table 1 and Figure 3). The mean time to the onset of cytoplasmic membrane permeabilization was $\langle t_{CM} \rangle = 300 \pm 51$ s for the 49 cells analyzed. The mean Sytox rise time for the cells was $t_{Sytox} = 3.0 \pm 0.6$ min. Cells that were permeabilized to Sytox Orange never recovered growth during the remainder of the 30 min observation period.

For cells maintained in stationary phase, we found no evidence of CM permeabilization at the same 0.5 μ M Cecropin A concentration. None of the stationary phase cells (0 of 126 cells) exhibited CM permeabilization during an observation period of 60 min (Table 1 and Figure 3). No cell shrinkage was observed. Stationary phase cells all recovered growth immediately when fresh EZRDM was flowed following a 30-min Cecropin A treatment (Figure S6). Evidently 30-min exposure to 0.5 μ M Cecropin A, the 12-hr MIC for exponentially growing cells, did not affect the viability of the stationary phase cells.

We also tested the effects of a tenfold larger concentration of Cecropin A on stationary phase cells. Even at 10 $\times$ MIC (5 μ M of Cecropin A), only 66% of stationary phase cells exhibited CM permeabilization within the observation period of 60 min (Table 1 and Figure 3). The mean time to the onset of CM permeabilization was $\langle t_{CM} \rangle = 1421 \pm 105$ s for the cells that were permeabilized (Table 1). The stationary phase cells are much more resistant to the antibacterial activity of Cecropin A compared with the exponentially growing cells. The mean Sytox rise time for the stationary phase cells that were permeabilized by 5 μ M of Cecropin A was $t_{Sytox} = 14.6 \pm 1.3$ min, five times slower than the Sytox risetime for exponential growing cells attacked by only 0.5 μ M Cecropin A. These data indicate that cytoplasmic membrane permeabilization requires much higher bulk Cecropin A concentration in stationary phase cells compared to exponentially growing cells. In addition, the resulting combined permeability of the cell envelope (OM and CM) to Sytox Orange is much lower.

As a final control, we tested the possibility that some unknown components present in spent EZRDM might interfere with the activity of Cecropin A. Exponential phase cells were plated in the microfluidic device as described earlier. Fresh EZRDM flowed initially to wash away unbound cells. The flow of Cecropin A plus Sytox Orange in spent EZRDM began at $t = 0$. At the Cecropin A concentration of 0.5 μ M, all 28 exponential phase cells exhibited the same polar CM permeabilization pattern previously observed in fresh EZRDM. The mean time to the onset of CM permeabilization was $\langle t_{CM} \rangle = 287 \pm 21$ s, quite similar to the result $\langle t_{CM} \rangle = 300 \pm 51$ s for Cecropin A in fresh EZRDM. Evidently Cecropin A is comparably active in both fresh and spent EZRDM.

3.4. Spatial pattern of CM permeabilization in WT cells

In the earlier study [9], localized initial permeabilization of both the outer and cytoplasmic membranes of *E. coli* by Cecropin A was observed. OM permeabilization was detected using an *E. coli* strain that exports GFP to the periplasm. CM permeabilization was detected using Sytox Orange. The initial disruption of both membranes occurred preferentially either at the septum or at the cell poles, i.e., in regions of strong membrane curvature. For the present study of stationary phase cells, we had difficulty finding an induction protocol yielding sufficient periplasmic GFP to enable imaging studies. Nevertheless, we are able to use Sytox Orange to report on the spatial pattern of cytoplasmic membrane permeabilization.

As before, we flowed a solution of Cecropin A (5 μ M) and Sytox Orange (0.5 nM) in spent medium across plated stationary phase cells beginning at $t = 0$. Images were obtained at the faster rate of 0.5 sec per camera frame. In all 30 cells analyzed, Sytox Orange enters the cytoplasm essentially simultaneously from the entire periphery of the cell. This is shown most clearly in the axial projections, in which the Sytox fluorescence increases initially over the entire length of the cell (Figure 5C, D). We refer to this behavior as global permeabilization of the cytoplasmic membrane. In contrast, all 40 of the non-septating, mid-log phase cells show initial CM permeabilization that is localized at one cell pole (Figure 5A, B). These results are summarized qualitatively in Table 2.

3.5. Phosphatidylglycerol may guide initial site of CM permeabilization

In exponentially growing cells, the anionic phospholipids cardiolipin (CL) and phosphatidylglycerol (PG) (Figure 1) concentrate in the polar and septal regions of *E. coli* [28], the same regions of the CM preferentially targeted by the cationic peptide Cecropin A. PG makes up 15–20% of total *E. coli* lipids in exponential growth [28], but only ~5% in stationary phase conditions [33]. CL makes up only ~5% of lipids in exponential growth [28], but 15–20% in stationary phase [33]. In exponential growth at 27°C and at 37°C, a study found no significant difference in the %CL between CM lipids and total lipids (CM plus OM) [34]. The change in lipid composition on transition to stationary phase led us to the hypothesis that a sufficient concentration of the anionic lipid PG in the polar or septal regions might guide the location of the initial attack of the cationic peptide Cecropin A on the CM, perhaps due to electrostatic interactions.

To test this hypothesis we conducted our fast imaging Sytox Orange experiments on mutant strains that lack either the CL synthesis machinery (strain BKT12, which we call CL-) or both the PG and CL synthesis machinery (strain BKT29, which we call CL-PG-). In WT cells, CL biosynthesis proceeds by condensation of two molecules of PG, catalyzed by cardiolipin synthase A [35]. In exponential growth, the fractional abundance of PG is greater in CL- cells compared to WT cells; evidently the enhanced concentration of PG compensates for the loss of CL in this mutant [28]. In addition, the enhancement of PG in the polar regions vs the cylindrical region is more pronounced in CL- mutant cells (4.4-fold) than in WT cells (2.7-fold) [28].

Fast Sytox Orange imaging experiments (0.5 s/frame) at 5 μ M Cecropin A were conducted for both mid-log phase and stationary phase cells of the BKT12 (CL-) and BKT29 (CL-PG-) strains. The results are summarized in Table 2. For the BKT 12 cells (lacking CL, but having extra PG), all 38 of the mid-log phase, non-septating cells analyzed exhibit the localized membrane permeabilization pattern in which Sytox Orange initially enters from one pole of the cell (Figure S7 and Table 2). This is the same behavior observed in exponentially growing WT cells. However, the majority of the stationary phase cells (85% of 41 cells) now also exhibit initial CM permeabilization localized at one endcap (Figure S7 and Table 2), in contrast to the behavior of WT stationary phase cells.

For the BKT29 strain (lacking both CL and PG), a majority of the cells in mid-log phase (58% of 78 cells) exhibit the global pattern (Figure 6 and Table 2). Sytox Orange enters the cytoplasm essentially simultaneously from the entire periphery of these cells. This is different from the behavior of WT exponentially growing cells. For the stationary phase cells, the majority behavior (64% of 14 cells) is again global membrane permeabilization (Figure 6 and Table 2), similar to the behavior of WT stationary phase cells. These data are consistent with the hypothesis that in both mid-log phase and stationary phase cells, a sufficiently high concentration of PG localized at the cell poles may guide localized attack of the CM by Cecropin A at one pole.

4. Discussion

Single-cell imaging studies of AMPs including LL-37 [8, 13, 16], Cecropin A [9, 14], CM15 [11], and melittin [15] have been reported by our group previously. These studies were performed on *E. coli* cells in exponential growth phase, typical for biophysical assays. The observed mechanisms of action include localized or global OM and CM permeabilization [8, 9, 14], permanent or transient OM and CM permeabilization to GFP [15], induction of oxidative stress [11, 13], and rigidification of the cytoplasmic medium [16]. In those cases studied with good time resolution (LL-37, Cecropin A, and melittin), OM and CM permeabilization initiate locally, at the septal region or one endcap. A periplasmic HaloTag assay revealed a sequence of OM and CM permeabilization and re-sealing events to GFP common to LL-37, CM15, and melittin [36].

In our experience, once the bulk concentration of AMP exceeds the threshold necessary to permeabilize the OM to GFP, the CM is invariably subsequently permeabilized to Sytox, and the permeabilized cells never recover growth. We recently suggested a two-step model of OM permeabilization of *E. coli* cells in exponential growth by Cecropin A [14]. First a threshold concentration of Cecropin A must penetrate the kinetic barrier of the anionic lipopolysaccharide (LPS) layer to build a sufficient local concentration at the membrane bilayer. The subsequent OM disruption is localized in space and stable in time. For non-septating cells in exponential growth, both the OM and the CM were permeabilized locally at one pole of the cell.

Here we extend our study of Cecropin A to *E. coli* cells in early stationary phase, an important growth phase of bacteria in nature. To maintain cells in stationary phase during imaging, we substituted spent EZRDM for the usual flow of fresh aerated EZRDM. This

prevents growth during imaging (Figure 2) but does not affect viability when the medium is switched to fresh EZRDM (Figure S5). The 12-hr MIC of Cecropin A for *E. coli* cells in exponential phase (0.5 μ M) is sufficient to permeabilize all the cells under observation within 10 min (Table 1 and Figure 3). However, stationary phase cells are impervious to this concentration of Cecropin A. Over an observation period of 60 min under the flow of 0.5 μ M Cecropin A, none of the stationary phase cells had its CM permeabilized to Sytox Orange. In addition, all stationary phase cells treated with 0.5 μ M Cecropin A for 30 min recovered growth when fresh EZRDM was supplied (Table 1, Figure 3, and Figure S6). Even at a concentration of 10 $\times$ the MIC for Cecropin A (5 μ M), only 66% of stationary phase cells had their CM permeabilized within the 60 min observation period. The CM permeabilization that did occur at 5 μ M Cecropin A proceeds slowly compared to the exponential phase cells at 0.5 μ M. Finally, the mean Sytox rise time for stationary cells at 5 μ M Cecropin A was $\sim$ 5 times longer than for exponential phase cells at 0.5 μ M Cecropin A, indicating a smaller combined degree of permeabilization of the OM/CM envelope.

Stationary phase cells are much more resistant to the antibacterial action of Cecropin A than exponentially growing cells. Our standard assay for OM permeabilization to periplasmic GFP was unsuccessful for stationary phase cells, so we remain uncertain whether or not Cecropin A at 0.5 μ M was able to penetrate the OM and reach the periplasmic space. We suspect that it did not, primarily because in exponentially growing cells CM permeabilization always occurs whenever OM permeabilization occurs. In addition, no cell shrinkage was observed.

As *E. coli* cells transition from exponential growth to stationary phase, they undergo a wide variety of morphological, metabolic, transcriptional, and translational changes [17, 18]. It seems natural to attribute the greater resistance to membrane permeabilization by Cecropin A to changes in the cell envelope, a significant feature of the adaptation strategy by stationary phase cells against external stress. In the outer membrane, the overall amount of lipopolysaccharide (LPS) increases [18]. This enhancement of the outermost protective barrier may explain the diminished susceptibility of stationary phase cells to the AMP. Accordingly, in earlier work we showed that removal of phosphate charges and/or sugar layers from the core oligosaccharide layer of K-12 *E. coli* enabled faster penetration of the OM by Cecropin A [14]. In stationary phase, the peptidoglycan layer composition is altered and its thickness increases [37]. Crosslinking of OM proteins to the peptidoglycan layer is enhanced [17]. The cytoplasmic membrane undergoes multiple modifications that make it more ordered with reduced fluidity [38]. One important change in the CM is the alteration of phospholipid head group composition [18, 33]. The percentage of cardiolipin (CL) increases and the percentage of phosphatidylglycerol (PG) decreases in stationary phase. Any of these changes to the cell envelope might contribute to the relative inability of Cecropin A to penetrate the OM and subsequently permeabilize the CM of *E. coli* in early stationary phase.

A striking result from the present work is the different spatial pattern of CM permeabilization to Sytox Orange for WT cells in exponential growth vs stationary phase, summarized in Table 2. Initial CM permeabilization is localized at one pole in non-septating, exponential phase *E. coli* and global in stationary phase cells. Minicell studies have shown that the anionic phospholipids cardiolipin (CL) and phosphatidylglycerol (PG) concentrate

in the polar regions of exponentially growing *E. coli* [28], the same regions preferentially attacked by the cationic peptide Cecropin A [9]. PG makes up 15–20% of *E. coli* lipids in exponential growth [28], but only ~5% in stationary phase conditions [33]. Conversely, CL makes up only ~5% of lipids in exponential growth, but 15–20% in stationary phase. This suggests the hypothesis that a sufficiently large concentration of PG sequestered to the poles may guide the initial attack of Cecropin A at the poles of the *E. coli* cytoplasmic membrane. For WT cells, such a localized concentration of PG is present in exponential growth, but may well be absent in stationary phase.

To test this hypothesis, we extended our studies of the spatial pattern of CM permeabilization to mutant strains lacking CL (CL- strain BKT 12) or lacking both CL and PG (CL-PG- strain BKT 29). In exponentially growing CL- cells, CM permeabilization primarily initiates locally, much as in the WT cells. Remarkably, most of the CL- cells in stationary phase are also locally permeabilized, in contrast to the global permeabilization of WT stationary phase cells. This is consistent with our hypothesis under the assumption that the enhancement of PG and its greater sequestration at the cell poles, as previously observed for exponentially growing CL- cells, carries over to stationary phase CL- cells. For the CL-PG- mutant strain, a majority of the cells exhibit the global CM permeabilization pattern in both exponential and stationary phase cells (Table 2). The correlation is imperfect; roughly 40% of the CL-PG- cells continue to exhibit localized CM permeabilization in both exponential growth and stationary phase. However, the marked change from WT behavior lends additional credence to the hypothesis that a threshold amount of PG concentrated at the poles exerts a guiding influence on the initial location of CM permeabilization by Cecropin A.

Why would localized, polar permeabilization of the CM be guided by polar concentration of the anionic lipid PG (–1 charge on head group, two tails), but not by polar concentration of the anionic lipid CL (–2 charge on head group, four tails)? In Figure 1 we show a ribbon diagram of the α -helical structure of Cecropin A with sidechains drawn explicitly. The positively charged residues are concentrated towards the N-terminal portion of the peptide. On the same scale, we show the chemical structure of PG and CL. We speculate that at the same total anionic lipid charge density (i.e., for PG concentration twice that of CL), PG can distribute its anionic charges more flexibly and thus better accommodate binding to the cationic side chains of Cecropin A. Thus concentrated PG at the poles may induce stronger preferential binding of the AMP at the poles than concentrated CL, whose two charges are more spatially constrained and whose tails are bulkier. The resulting localized buildup of a high Cecropin A concentration may then lead to localized, polar CM permeabilization. In this picture, global permeabilization of the CM occurs as the default result when a sufficient localized polar concentration of PG is absent. Accordingly, when the global permeabilization mechanism is observed it occurs more slowly than the localized permeabilization mechanism. Our data do not speak to the long-standing mechanistic issue of detergent-like permeabilization vs pore formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AMP	antimicrobial peptide
OM	outer membrane
CM	cytoplasmic membrane
MIC	minimum inhibitory concentration
CL	cardiolipin
PG	phosphatidylglycerol

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Figure 1.

Ribbon diagram of Cecropin A and chemical structures of major anionic phospholipids of *E. coli*, phosphatidylglycerol (PG) and cardiolipin (CL). Cecropin A residues are color-coded as follows: gray, hydrophobic; blue, positive; red, negative; green, neutral. Positive and negative charges on all three molecules are labelled. The Cecropin A structure is adapted from the Figure 1 of Ref. [39]. The chemical structures of PG and CL are adapted from the Figure 1 of Ref. [28].

Figure 2. Relative cell length ($L_t/L_{t=0}$) vs time for plated stationary phase cells of *E. coli* in a flow of fresh or spent EZRDM medium as indicated. Four individual cells in each case.

Figure 3.

Phase contrast and Sytox Orange images for single-cell experiments. A) Phase contrast and B) Sytox Orange images of WT mid-log phase cells after 60 min of treatment with 0.5 μ M Cecropin A. C) Phase contrast and D) Sytox Orange images of WT stationary phase cells after 60 min of treatment with 0.5 μ M Cecropin A. E) Phase contrast and F) Sytox Orange images of WT stationary phase cells after 60 min of treatment with 5 μ M Cecropin A.

Figure 4.

Two-channel imaging (phase contrast plus Sytox Orange fluorescence) with 3 s cycle time for a typical WT cell in exponential growth (panels A-C) and in stationary phase (panels D-F). Constant flow of a fixed concentration of Cecropin A plus 5 nM Sytox Orange begins at $t = 0$. Cecropin A concentration is 0.5 μ M in exponential growth (A) and 5 μ M for stationary phase (D). Phase contrast snapshots at times indicated. (B and E) Sytox Orange snapshots. (C and F) Sytox Orange total fluorescence intensity and cell length vs. time.

WT mid-log phase, non-septating cell

WT stationary phase cell

Figure 5.

(A) Typical pattern of Sytox Orange entry into the cytoplasm for a non-septating mid-log phase cell of the WT strain. (B) Axial intensity projections vs time (min). (C) Typical pattern of Sytox Orange entry into the cytoplasm for a stationary phase cell of the WT strain. (D) Axial intensity projection vs time (min).

BKT29 (-CL and -PG) mid-log phase non-septating cell

BKT29 (-CL and -PG) stationary phase cell

Figure 6.

(A) Typical pattern of Sytox Orange entry into the cytoplasm for a non-septating mid-log phase cell of the strain BKT29 (-CL-PG). (B) Axial intensity projections vs time (min). (C) Typical pattern of Sytox Orange entry into the cytoplasm for a stationary phase cell of the BKT29 strain (-CL-PG). (D) Axial intensity projections vs time (min).

Table 1.

Timing of cytoplasmic membrane permeabilization to Sytox Orange.

Strain	Cecropin A concentration (μ M)	Growth Phase	% CM permeabilization	$\langle t_{CM} \rangle, s^a$
MG1655 (WT)	0.5	mid-log	100% (49/49 cells)	300 ± 51
MG1655	0.5	stationary	0% (0/126 cells)	—
MG1655	5	stationary	66% (46/70 cells)	1421 ± 105

^aRange is ± 1 SEM.

Table 2.

Dominant spatial pattern of CM permeabilization by 5 μ M Cecropin A for different strains.

Strain	Growth Phase	Permeabilization pattern ^a
MG1655 (WT)	mid-log	local (100%) (40/40 cells)
MG1655 (WT)	stationary	global (100%) (30/30 cells)
BKT 12 (-CL)	mid-log	local (100%) (38/38 cells)
BKT 12 (-CL)	stationary	local (85%) (35/41 cells)
BKT 29 (-CL and -PG)	mid-log	global (58%) (45/78 cells)
BKT 29 (-CL and -PG)	stationary	global (64%) (9/14 cells)

^aAt 5 μ M of Cecropin A, all cells of all strains were permeabilized during the observation period. The percentages indicate what fraction of the permeabilized cells exhibited the stated pattern.