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Extracytoplasmic Stress Responses Induced by Antimicrobial Cationic Polyethylenimines

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Abstract

The ability of an antimicrobial, cationic polyethylenimine (PEI⁺) to induce the three known extracytoplasmic stress responses of *Escherichia coli* was quantified. Exposure of *E. coli* to PEI⁺ in solution revealed specific, concentration-dependent induction of the Cpx extracytoplasmic cellular stress response, ~2.0–2.5 fold at 320 µg/mL after 1.5 hours without significant induction of the σ^E or Bae stress responses. In comparison, exposure of *E. coli* to a non-antimicrobial polymer, polyethylene oxide (PEO), resulted in no induction of the three stress responses. The antimicrobial small molecule vanillin, a known membrane pore-forming compound, was observed to cause specific, concentration-dependent induction of the σ^E stress response, ~6-fold at 640 µg/mL after 1.5 hours, without significant induction of the Cpx or Bae stress responses. The different stress response induction profiles of PEI⁺ and vanillin suggest that although both are antimicrobial compounds, they interact with the bacterial membrane and extracytoplasmic area by unique mechanisms. EPR studies of liposomes containing spin-labeled lipids exposed to PEI⁺, vanillin, and PEO reveal that PEI⁺ and PEO increased membrane stability whereas vanillin was found to have no effect.

Keywords

antimicrobial polymer; cationic polyethylenimine; extracytoplasmic stress response; poly(ethylene oxide); vanillin

Introduction

Pathogenic bacteria pose a significant threat to human health, both in situations involving inadvertent exposure (contamination of the food supply[1], hospital-borne infections[13]) and where bacteria are leveraged as biological weapons[16]. Surface coatings derived from cationic, alkylated polyethylenimines (PEI⁺, Fig. 1A) have demonstrated antimicrobial activity against a wide variety of bacteria[5,9,12]. A suggested mechanism by which these polymers kill bacteria involves membrane disruption. This hypothesis has been supported by experiments where antimicrobial activity was measured as polymer structure was varied. The biocidal effects of PEI⁺ are influenced by the cationic charge of PEI⁺[4], the size of the polymer, and length of the alkyl side chain.[12] Further evidence for this mechanism has

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been obtained through the Live/Dead two-color fluorescent method that reveal ruptured bacterial membranes in the presence of PEI+[12].

We have taken a unique approach to studying the mechanism of action of PEI+ by examining the cellular response of bacteria induced by exposure to the polymer. Gram-negative bacteria such as *Escherichia coli* possess extracytoplasmic stress responses that allow them to both sense and respond to perturbations of their cellular envelope, including potentially lethal environmental stresses. Each of the three characterized responses (σ^E , CpxRA and BaeSR) have both unique inducing signals as well as those that overlap with the other two responses. The σ^E stress response detects stresses that perturb the outer membrane such as heat and ethanol[14], the Cpx stress response detects changes in pH and certain misfolded periplasmic proteins[2,6], and the Bae stress response is sensitive to both sodium tungstate and some flavonoids[10]. However, certain signals such as the presence of the misfolded PapE and PapG pilin subunits derived from uropathogenic *E. coli* (UPEC) can either activate both the Cpx and Bae responses (PapE) or all three responses simultaneously (PapG) [7,14]. The existence of these three known stress responses and their correlation with specific types of inducing signals allows us to employ stress response activation as an assay to assess potential mechanisms by which PEI+ causes bacterial cell death. For comparison, stress response activation was also studied using two other compounds as inducers, a non-antimicrobial polymer polyethylene oxide (PEO, Fig. 1B) and a small molecule with demonstrated antimicrobial activity, vanillin (Fig. 1C). PEO was chosen as a comparison for PEI+ in order to elucidate if any observable stress response induction by PEI+ was due solely to its polymeric structure and therefore unrelated to its biocidal properties. Vanillin provided contrast as a small-molecule antimicrobial with an established mechanism of action involving membrane disruption[3].

Materials and Methods

Polymer synthesis

Chemical reagents were purchased from Sigma-Aldrich or Fisher Scientific. Branched polyethylenimine (MW 750 kDa) was purchased from Aldrich as an aqueous solution and lyophilized before use. PEI+ was synthesized using literature procedures using dodecyl bromide and methyl iodide as alkylating agents[5,12]. PEO (MW 900 kDa) was purchased from Acros Organics.

Minimum Inhibitory Concentrations (MICs) of *n*-butanol and Growth Kinetics of *n*-butanol-treated strains

A MIC for *n*-butanol (*n*BuOH) was determined in liquid culture by assessing growth of the MC4100 gene-fusion-bearing strains in the presence of a broad range of *n*BuOH concentrations. A *n*BuOH concentration at which bacterial growth is clearly not compromised was determined for subsequent solubilization of the PEI+ polymer and its use in the experiments outlined below. Since *n*BuOH has previously been shown to cause a mild induction of the Cpx stress response,[15] all quantitative PEI+ induction data was normalized to appropriately-matched *n*BuOH-treated controls. All strains were subcultured to OD₆₀₀ $\approx$ 0.05 in 5 mL of LB and grown in the presence of varying concentrations of *n*BuOH. OD₆₀₀ measurements were taken at 15 minute intervals over a 3 hour time course.

Western Blot Analysis

Overnight cultures of wild-type *Escherichia coli* containing either *cpxP'*-lacZ⁺, *rpoHP3'*-lacZ⁺, or *spy'* lacZ⁺ gene fusions were subcultured to OD₆₀₀ $\approx$ 0.05 in 5 mL of LB. For PEI⁺, cultures were started with either specific concentrations of the PEI⁺ polymer dissolved in *n*BuOH or appropriately matched *n*BuOH controls. For vanillin, cultures were started with

either specific concentrations of vanillin dissolved in ethanol or appropriately matched ethanol controls. For PEO, cultures were started with either specific concentrations of the PEO polymer dissolved in LB or left untreated. All cultures were grown at 37 °C for 3 h. Samples were collected, normalized for the number of cells per milliliter, harvested by centrifugation, then resuspended in 100 µL of SDS/PAGE loading buffer and lysed by boiling for 5 min. Twenty milliliters of each sample were electrophoresed on a SDS-10% polyacrylamide gel, transferred to nitrocellulose, and subjected to Western blot analysis. Detection was performed using the Vectastain-ABC alkaline phosphatase kit (Vector Labs). Quantitative analysis of LacZ levels was performed using ImageJ software (NIH) (W. Rasband, ImageJ 1.41o. <http://rsb.info.nih.gov/ij>. Java 1.6.0_13). Reported stress response induction ratios were obtained from experiments performed in triplicate or greater.

EPR study

Spin-labeled liposomes were prepared by drying down lipids from a polar extract of *E.coli* and the nitroxide-based spin-label 5-doxyl-phosphatidylcholine in a molar ratio of 99:1. The combined lipids, originally dissolved in CHCl₃, were dried under a stream of nitrogen gas then placed in a vacuum desiccator overnight at room temperature. The resulting lipid film was then suspended in 50 mM MOPS, pH 7.0 buffer to a concentration of 10 mM. Unilamellar vesicles were formed by subjecting the lipid suspension to 5 freeze-thaw cycles followed by extrusion through a 200 nm polycarbonate membrane using a mini-extruder apparatus (Avanti Polar Lipids). Reagents were added to the liposomes at final concentrations of 320 µg/mL (PEI+ and PEO) or 640 µg/mL (vanillin) and allowed to incubate overnight at 4 °C. Volume-matched controls containing the solvents *n*BuOH, MOPS buffer, or ethanol were prepared for the PEI+, PEO, and vanillin samples, respectively. The lipid concentration was held constant at 7.5 mM for all samples and controls. Samples were drawn into 50 µL silica glass capillaries, sealed with wax then inserted into a standard-diameter EPR tube.

Continuous-wave (CW) EPR spectra were collected at 25 °C on a Bruker (Billerica, MA) EMX X-band spectrometer equipped with the standard resonance cavity and temperature controller. The spectra were recorded at a microwave power of 10 mW, using a 100 kHz modulation frequency, a 1.0 G modulation amplitude, and a 100 G field width centered at 3350 G. The motional parameter $2T_{||}$ (spectral breadth) which reflects mobility of the nitroxide spin label, was determined by measuring the distance in G between the outermost hyperfine extrema.

Results and Discussion

Antimicrobial, cationic polyethylenimine (PEI+) was synthesized from a commercially-available, branched polyethylenimine using literature procedures[5,12] by alkylating with dodecyl bromide and methyl iodide. Although methods for preparing antimicrobial surface coatings from PEI+ exist, studies of *E. coli* stress response induction required formulating new protocols for allowing PEI+ to exert its antimicrobial effect in solution since adhesion to surfaces is known to induce one of the three extracytoplasmic stress responses in *E. coli*[11]. PEI+ was found to be minimally-soluble in water and LB growth media but is soluble in *n*-butanol (*n*BuOH). However, we observed that *n*BuOH alone can affect bacterial growth. Therefore, we determined through MIC studies that up to 0.4% v/v *n*BuOH could be added to cultures of *E. coli* before growth was significantly affected (Online Resource Fig. S1A).

With this information, we prepared solutions of PEI+ in *n*BuOH that were added to cultures of *E. coli* such that the amount of *n*BuOH was less than 0.4% v/v. The effect of PEI+ on these cultures as a function of concentration is shown in (Online Resource Fig. S1B).

Growth inhibition after 3 hours was observed starting at 80 $\mu\text{g/mL}$ and bacterial density was reduced by approximately 50% at 320 $\mu\text{g/mL}$.

In contrast to PEI+, PEO was substantially more soluble in growth media and demonstrated no growth inhibition at 80-320 $\mu\text{g/mL}$ of polymer (Online Resource Fig. S2A). The small-molecule antimicrobial vanillin was highly soluble in ethanol and allowed introduction of vanillin to growth cultures using volumes of ethanol that did not affect bacterial densities. The effect of vanillin on the growth of *E. coli* cultures is shown in Online Resource Fig. S2B. Concentrations of vanillin between 320-640 $\mu\text{g/mL}$ produced a comparable reduction in bacterial densities as 80-320 $\mu\text{g/mL}$ PEI+ and were used in the stress response induction studies presented below.

Three different strains bearing reporter gene fusions were used as readouts of stress response induction. Each gene fusion couples the expression of stress response proteins, Cpx, σ^E , or Bae, to the production of a reporter protein, β -galactosidase (LacZ). The induction of each stress response was assessed by measuring the amount of β -galactosidase produced by the bacteria after exposure to PEI+, PEO, or vanillin.

Quantification of the stress response induction by PEI+, PEO, and vanillin was obtained by introducing 80-320 $\mu\text{g/mL}$ of each polymer or 320-640 $\mu\text{g/mL}$ of vanillin, dissolved in the appropriate alcohol if needed, to bacterial cell cultures. After 1.5 hours, an aliquot of the culture was removed and the cells lysed. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on each lysate. The separated proteins were transferred to nitrocellulose paper and Western blotting was conducted utilizing an anti- β -galactosidase antibody. The resulting blots were scanned and ImageJ software was used to quantify the intensity of each β -galactosidase band after normalizing to a cross-reactive band to control for small differences in the amount of sample loaded in each lane.

Since PEI+ and vanillin required solubilization in small volumes of *n*-butanol and ethanol, respectively, and alcohols are known stress response inducers,[15] the stress response induction by identical volumes of both alcohols was also determined. Therefore, we defined the quantified stress responses of PEI+ and vanillin as *the ratio of the stress response induction of the antimicrobial compound versus the stress response induction of the corresponding volume of alcohol used to solubilize the compound (induction ratio)*. This method of analysis ensured that the magnitude of the stress response induction of each compound is independent of any effect due to the alcohol alone. For experiments involving PEO, a water-soluble polymer, the induction ratio is derived from comparing the quantified stress responses of PEO to that of a sample where an equivalent volume of water was added.

Figure 2 presents the quantified stress response induction of Cpx, σ^E , and Bae by PEI+, PEO, and vanillin after 1.5 hours. A sample Western blot for determining the induction of Cpx by PEI+ is shown in Online Resource Fig. S3. Similar results are seen at 3.0 hours. PEI+ at a concentration of 320 $\mu\text{g/mL}$ induces the Cpx stress response 2.0-2.5-fold but does not induce σ^E or Bae (Fig. 2A); induction of the Cpx stress response by PEI+ is dose-dependent (Fig. 2B and Online Resource Fig. S3). The magnitude of Cpx induction is less than that caused by alkaline pH (12- to 15-fold)[2] or misfolded periplasmic proteins PapE and PapG (~6-fold)[7] but comparable to that of the misfolded maltose-binding protein variant MaIE31 (1.5-2.5-fold).[6] Therefore PEI+ can be considered alongside previously characterized Cpx inducing signals.

The polymeric character of PEI+ appears to not be solely responsible for its ability to induce the Cpx stress response. The non-antimicrobial polymer PEO failed to induce any of the 3 extracytoplasmic stress responses at concentrations identical to those of PEI+ sufficient to

induce Cpx (Fig. 2C). This result suggests that the stress response induction by PEI+ is not simply attributable to its polymeric nature since a polymer of similarly high molecular weight but with no antimicrobial activity did not produce the same effect.

In contrast to PEI+ and PEO, the antimicrobial small molecule vanillin induces only the σ^E stress response, approximately 12-14-fold in the presence of 640 $\mu\text{g/mL}$ vanillin (Fig. 2D). Induction of σ^E is associated with perturbations that specifically disrupt outer membrane protein-folding[14]. The reported mechanism of antimicrobial activity of vanillin, primarily as an outer membrane pore-forming compound[3] appears entirely consistent with the induction of σ^E in our strain.

Although the proposed mechanism of antimicrobial activity of PEI+ also involves membrane disruption, PEI+ does not induce the σ^E stress response. Instead we observe that PEI+ is an inducer of the Cpx stress response, a response most typically induced by accumulation of misfolded and/or mislocalized protein at the periplasmic face of the inner membrane. While these results do not preclude PEI+ acting as a membrane disruptor, the distinct inducing profiles of PEI+ and vanillin suggest that the two compounds likely interact with bacterial membranes in different ways.

Further evidence for different interactions of PEI+ and vanillin with the bacterial membrane was seen in EPR studies of spin-labeled liposomes, an established method for observing changes in membrane stability.[8] The spin-labeled liposomes were exposed to PEI+ (320 $\mu\text{g/mL}$), PEO (320 $\mu\text{g/mL}$) and vanillin (640 $\mu\text{g/mL}$). The effects of PEI+ on the EPR spectral parameters were compared to those of the corresponding solvent controls (*n*BuOH for PEI+, buffer for PEO, and ethanol for vanillin). Fig. 4 shows the change in spectral breadth, $2T_{||}$, a measure of the mobility of the spin label within the liposome. Both PEI+ and PEO are seen to decrease the mobility of the spin label, consistent with the notion that these agents exert a stabilizing effect on the membrane. Conversely, vanillin has a negligible effect on spin label mobility. While these results do not produce a definitive molecular-level picture of how PEI+, PEO, and vanillin interact with bacterial membranes, they provide an independent experimental method for corroborating the observation from the stress response induction data that PEI+ and vanillin have different interactions with membranes.

In conclusion, we have determined that an antimicrobial, cationic polyethylenimine is a specific inducer of the Cpx stress response in *E. coli*. In contrast, the small molecule antimicrobial vanillin, known to disrupt membranes, was observed to specifically induce the σ^E stress response. These results suggest that extracytoplasmic stress response induction can provide an intriguing window into the differences in bactericidal action of antimicrobial compounds. In future, we will examine stress response induction by other antimicrobial polymers and other types of biocides such as antimicrobial peptides[17].

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Abbreviations

PEI+	cationic alkylated polyethylenimine
PEO	polyethyleneoxide
<i>n</i>BuOH	<i>n</i> -butanol

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a) cationic polyethylenimine (PEI+)
R = methyl or dodecyl

b) poly(ethylene oxide)
PEO

c) vanillin

Fig. 1.
Chemical structure of a) PEI+, b) PEO and c) vanillin.

Fig. 2.

(a) *E. coli* stress response induction by PEI+ (320 micrograms/mL) after 1.5 hours (b) increasing stress response induction by PEI+ at concentrations of 80 -320 micrograms/mL (c) *E. coli* stress response induction by PEO (320 micrograms/mL) after 1.5 hours with comparison to 320 micrograms/mL PEI+ (d) *E. coli* stress response induction by vanillin (640 micrograms/mL) after 1.5 hours with comparison to 320 micrograms/mL PEI+. Error bars shown represent standard deviation of the data

Fig. 3.
Effect of PEI+, PEO, and vanillin on $2T_{\parallel}$ parameter of EPR spectrum of liposomes containing a spin-label on the 5-position of the phosphoacyl chain.