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Antibacterial activity and mechanism of action of ε-poly-L-lysine

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ABSTRACT

ε-Poly-L-lysine (ε-PL)[2] is widely used as an antibacterial agent because of its broad antimicrobial spectrum. However, the mechanism of ε-PL against pathogens at the molecular level has not been elucidated. This study investigated the antibacterial activity and mechanism of ε-PL against *Escherichia coli* O157:H7 CMCC44828. Propidium monoazide-PCR test results indicated that the threshold condition of ε-PL for complete membrane lysis of *E. coli* O157:H7 was 10 μg/mL (90% mortality for 5 μg/mL). Further verification of the destructive effect of ε-PL on cell structure was performed by atomic force microscopy and transmission electron microscopy. Results showed a positive correlation between reactive oxygen species (ROS)[3] levels and ε-PL concentration in *E. coli* O157:H7 cells. Moreover, the mortality of *E. coli* O157:H7 was reduced when antioxidant N-acetylcysteine was added. Results from real-time quantitative PCR (RT-qPCR)[4] indicated that the expression levels of oxidative stress genes *sodA* and *oxyR* were up-regulated 4- and 16-fold, respectively, whereas virulence genes *eaeA* and *espA* were down-regulated after ε-PL treatment. Expression of DNA damage response (SOS response)[5] regulon genes *recA* and *lexA* were also affected by ε-PL. In conclusion, the antibacterial mechanism of ε-PL against *E. coli* O157:H7 may be attributed to disturbance on membrane integrity, oxidative stress by ROS, and effects on various gene expressions, such as regulation of oxidative stress, SOS response, and changes in virulence.

1. Introduction

Food safety is an important worldwide public health concern and microbial contamination is recognized as a major source of frequent outbreaks for foodborne illnesses [1]. For example, harmful cucumber contaminated with *Escherichia coli* O104:H4 in Germany and listeriosis caused by *Listeria monocytogenes*-contaminated cantaloupes in the USA resulted in great losses to agricultural production and human health. Although various techniques are used in the food industry to eliminate pathogenic microorganisms, microbial contamination is still difficult to avoid. Foods may also get contaminated with spoilage bacteria during transportation and storage. Preservatives are necessary to inhibit growth of spoilage bacteria and prolong the shelf life of food. An ideal food preservative should possess strong and broad-spectrum antimicrobial activity, and low toxicity without affecting nutritional value and quality of food.

Antimicrobial peptides are a class of natural antimicrobial substances [2] and have received increased attention for food preservation for their excellent antimicrobial activity and established safety. ε-Poly-L-lysine (ε-PL) is widely used to preserve packaged food in certain countries for its broad antimicrobial activity against Gram-negative and Gram-positive bacteria, yeasts, and molds [3,4]. In addition, ε-PL is water soluble, biodegradable, stable [5], and has low toxicity [6]. Natural ε-PL consists of 25–30 L-lysine residues [7] and possesses positively charged amine groups that is responsible for antibacterial activity. This peptide was initially isolated from *Streptomyces albulus* sp. lysinopolymerus strain 346 [8], and later was found in other bacterial or eukaryotic cells [9]. Acute oral toxicity assays showed that ε-PL is non-toxic at high dose levels of 5 g/kg in rats [6]. Absorption, distribution, metabolism, and excretion studies demonstrated that ε-PL has low absorption in rat gastrointestinal tract [6]. Based on its strong antibacterial activity and low toxicity, ε-PL is utilized in Japan as

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[1] Co-first authors.
[2] ε-Poly-L-lysine (ε-PL).
[3] Reactive oxygen species (ROS).
[4] Real-time quantitative PCR (RT-qPCR).
[5] DNA damage response (SOS response).

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food additive for meat or fish sushi (1–5 mg/g), rice, cooked vegetables (0.01–0.5 mg/g), and other food [10]. The US Food and Drug Administration has also authorized it as generally recognized as safe (GRAS) for using ε-PL at levels of up to 50 mg/kg in food (GRAS No. 000135).

The antibacterial activity of ε-PL and its application have been reported in literature [3,4]. However, its antibacterial mechanism at the molecular level has not been elucidated. Only a few publications mentioned that ε-PL can strip the outer membrane and causes abnormal distribution of the cytoplasm [11]. To promote the application of ε-PL, elucidating its antibacterial mechanism is particularly important. In this paper, *E. coli* O157:H7 (CMCC44828), a ε-PL susceptible strain and one of the major Gram-negative foodborne pathogens, was chosen as a model to investigate the mechanism of the effects of ε-PL on this bacterium. Propidium monoazide (PMA)-PCR, transmission electron microscopy (TEM), and atomic force microscopy (AFM) were employed to study the effects of ε-PL on cell morphology and membrane integrity. The concentration of reactive oxygen species (ROS) in ε-PL treated *E. coli* O157:H7 cells were measured by ROS probe. Expression of genes related to oxidative stress, SOS response, and virulence was also investigated by using real-time quantitative PCR (RT-qPCR).

2. Materials and methods

2.1. Microorganisms and ε-PL treatment

The *E. coli* O157:H7 (CMCC44828) was obtained from the National Center for Medical Culture Collections (CMCC) in Beijing, China, and cultured in Luria–Bertani (LB) medium, under aerobic conditions at 37 °C with shaking at 160 rpm. *E. coli* O157:H7 culture (10^6 cfu/mL) was centrifuged at 5000 rpm for 2 min and washed twice with fresh LB. The pellet was resuspended in fresh LB, which was incubated with various concentrations of ε-PL (Bainafo, Zhengzhou, China) and incubated without shaking. Bacterial mortality was calculated after treating the sample with ε-PL and number of *E. coli* O157:H7 cells were counted in comparison with the number of untreated cells.

All of the tests were performed at least in triplicate. Data were reported as means ± SD and analyzed using SigmaPlot 11.0 (Systat Software, San Jose, CA, USA).

2.2. PMA-PCR assay

Aliquots of 10^6 cfu/mL *E. coli* O157:H7 cells were resuspended in PBS (phosphate buffer solution, pH 7.4) and treated for 15 h with 0–500 μg/mL of ε-PL. Then cells were treated with 5 μg/mL of PMA (Biotium, USA) and extracted the genomic DNA of *E. coli* O157:H7 by Bacteria Gen DNA Kit (ComWin Biotech, Beijing, China) used as the templates in the PCR reactions as in Yanez et al. (2011) [12]. The primers for the unique gene of *E. coli* O157:H7,*frbE* [13] was used in PCR (Table 1).

2.3. Examination of cell morphology by AFM and TEM

Samples of *E. coli* O157:H7 (10^6 cfu/mL) washed with PBS were transferred to a mica piece and then air dried. *E. coli* O157:H7 nanostructures were imaged by AJ-III AFM (Shanghai AJ Nano-Science Development Co., Ltd., Shanghai, China) in the tapping mode (cantilever length of 100 μm) with Nanosytems ACT Si probes at a scan rate of 1.6 Hz. Scan size was 4.18045 μm. The height and phase images were collected and further analyzed using iNano SPM-ELeChem software (Shanghai AJ Nano-Science Development Co., Ltd., Shanghai, China) to display the cell surface.

The same *E. coli* O157:H7 suspension samples (10^6 cfu/mL) were dropped on a carbon-coated copper grid and observed under a HITACHI H600 TEM (Hitachi Instrument, Tokyo, Japan) operated at 75 kV. Bacteria micrographs were recorded at a magnification of 150,000 times.

2.4. Detection of ROS

About 10^8 cfu/mL of *E. coli* O157:H7 were harvested by centrifugation and then washed three times with fresh LB medium. Bacteria cells were re-suspended in a medium containing the non-fluorescence ROS probe 2′,7′-dichlorfluorescein-diacetate (DCFH-DA; Beyotime, Jiangsu, China) (1:2000, v/v). The mixture was shaken at 37 °C for 30 min in order for the probe to enter the cells. Cells were collected by centrifugation at 5000 rpm for 2 min and then washed twice with fresh medium to remove the free probe. The cells were then treated with ε-PL under aerobic conditions at 37 °C for 15 h. The fluorescent signal intensity of the oxidized product of DCFH-DA by ROS, namely, DCF, was de-

Table 1
Genes and primers selected for PCR.

Gene function protein encoded	Gene	Sequence (5′–3′)	Length (bp)	Reference
Unique gene of E. coli O157:H7				
Perosamine synthetase	*rfbE*	TTATACGGACATCCATGTGA TAATTCCACGCCAACCA	625	This study
Oxidative stress response genes				
Superoxide dismutase	*sodA*	CCGATTATGGGCCTGGAT CAAAACGTGCCGCTGC	122	[14]
Transcriptional regulator	*oxyR*	CGCGATCAGGCAATGG CAGCGCTGGCAGTAAAGTG	129	[14]
SOS response genes				
DNA strand exchange and recombination protein	*recA*	CATCTCTACCGGTTCGCTTTC GCGTGTTCAGCATCGATAAAC	176	[15]
DNA-binding transcriptional repressor of SOS regulon	*LexA*	CGGTCAGGTCGTTGTCG CTGCTGACGAAGGTCAACG	130	This study
Virulence genes				
Intimin	*eaA*	TGACCAAAAGCAACATGACC CCAGCGATACCAAGAGCG	133	[16]
Higher expression of LEE pathogenicity island gene	*EspA*	GCAAACAGTGAAGGCGG CACATCAGAACGTGCACTCG	127	[15]
Housekeeping genes				
Gyrase subunit A	*gyrA*	GTTCCATCAGCCCTTCAATG TTGATAACTATGACGGCACGG	195	[17]

tected at an emission wavelength of 535 nm (Ex. 488 nm) by a fluorescence spectrophotometer (F-4500, HITACHI Instrument, Tokyo, Japan). ROS level of ε-PL-treated with fluorescence was calculated by comparing the fluorescence intensity of treated bacteria (F) with that of untreated bacteria (Fc).

2.5. RT-qPCR analyses of gene expression

To quantify gene expression in bacterial cells by RT-qPCR, 1 mL of the *E. coli* O157:H7 culture in the late log phase of growth was treated with 0 or 5 μg/mL of ε-PL for 1 h and then used for total RNA extraction with a TransZol RNA extraction kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized from the RNA sample using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Kyoto, Japan). qPCR was conducted using SYBR green PCR master Mix (Applied Biosystems) and Primers used in the RT-qPCR are listed in Table 1. A 7900HT FAST real-time PCR system (Applied Biosystems, Foster City, CA) was used for qPCR. Data analysis was carried out using the $2^{-\Delta\Delta CT}$ method [18] The gyrase gene *gyrA*, a housekeeping gene [17], was used as the reference for data normalization.

3. Results

3.1. Effects of ε-PL concentration, treatment temperature, pH, and treatment length on the antibacterial activity of ε-PL against E. coli O157:H7

To optimize the antibacterial action of ε-PL, several factors were studied with *E. coli* O157:H7 culture, including ε-PL concentration, treatment temperature, pH, and length of treatment. The mortality of*E. coli* O157:H7 was dependent on concentration (Supplementary Fig. S1A). At 37 and 20 °C, the susceptible range of ε-PL for *E. coli* O157:H7 was between 1 and 5 μg/mL. The antibacterial effect reached a plateau (90% mortality) when the concentration of ε-PL was increased to 10 μg/mL. When the temperature was maintained at 4 °C, the antibacterial effect reached 90% when the ε-PL concentration was 50 μg/mL. These results indicated that the antibacterial effect of ε-PL was dependent on temperature, where the median lethal dose of *E. coli* O157:H7 was 1 and 5 μg/mL at 37 and 4 °C, respectively. The difference between treatments at 37 and 20 °C was not significant ($p > 0.05$) when the concentration of ε-PL was increased to 5 μg/mL. Therefore, treatment at 37 °C and ε-PL concentration of 5 μg/mL was chosen in the succeeding experiments to study the effect of different pH levels on *E. coli* O157:H7. The highest inhibition effect of ε-PL was achieved when the culture was at pH 6 (Supplementary Fig. S1B). Inhibition effect of ε-PL decreased with increase in pH, indicating that pH plays a role on the inhibition effect of ε-PL as it facilitates the decrease in charge intensity of ε-PL in alkaline environments. Hiraki (2000) [19] also reported that under alkaline conditions, the antimicrobial activity of ε-PL was significantly lower. The influence of treatment duration on ε-PL antibacterial activities were also studied under optimal antibacterial conditions determined above. The apparent difference in mortality of *E. coli* O157:H7 between two different treatment durations (15 and 4 h) was observed at 1 μg/mL to 100 μg/mL of ε-PL (Supplementary Fig. S1C). At lower ε-PL concentration, longer treatment time was needed to generate high antibacterial effects.

3.2. Membrane lesions of E. coli O157:H7 caused by ε-PL

To confirm if cell membrane integrity and cell morphology of *E. coli* O157:H7 was affected by ε-PL, PMA-PCR, AFM, and TEM were performed. PMA can enter membrane-compromised cells and subsequently bind to cellular DNA, yielding no PCR products when DNA from dead cells is the only available template. In this study, PMA was added after ε-PL treatment and prior to DNA extraction in order to bind DNA in cells that died from ε-PL treatments. The higher the ratio of membrane disruption of cells, the more the PMA binds to DNA. When the concentration of ε-PL was above 10 μg/mL, PCR products were barely seen (Fig. 1A), suggesting that the membrane of *E. coli* O157:H7 was damaged completely after treatment with ε-PL. However, when the concentration of ε-PL was at 5 μg/mL and mortality was 90%, PCR products were obtained, indicating that some cells had integral cell membranes.

TEM confirmed that the outer membrane of *E. coli* was stripped from the cytoplasm by ε-PL treatment [11]. However, details on changes in the shape of *E. coli* O157:H7 cells have not been reported. In this study, AFM was combined with TEM to conduct a

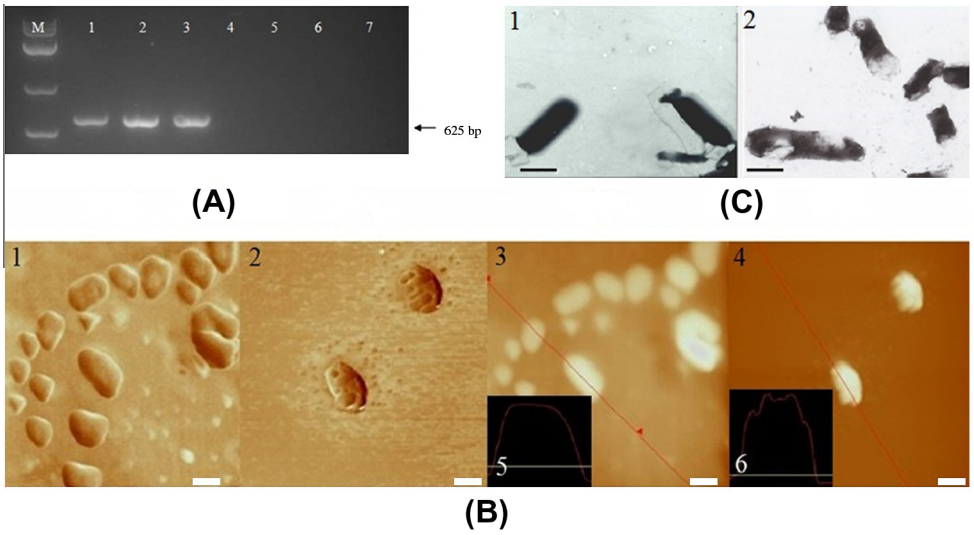


Fig. 1. The effect of ε-poly-L-lysine (ε-PL) on the cell surface of *E. coli* O157:H7. (A) Electrophoresis pattern of propidium monoazide (PMA)-PCR products of *E. coli* O157:H7 treated by ε-PL. Concentrations of ε-PL for lanes 1–7 are 0, 1, 5, 10, 50, 100, and 500 μg/mL, respectively. M represents Marker (500–1,500 bp; Takara, Kyoto, Japan). (B) AFM image of cells treated with (B_2, B_4, B_6)/without (B_1, B_3, B_5) 50 μg/mL ε-PL. (B_1, B_2) Phase image, (B_3, B_4) height image, (B_5, B_6) section analysis. (C) TEM image of cells treated with(C_2)/without(C_1) 50 μg/mL ε-PL for 4 h. Lateral scale bar = 1 μm.

detailed analysis of the effect of ε-PL on the cell surface. As showed in Fig. 1B, the untreated surface of the control*E. coli* O157:H7 in the phase image was bright and smooth (Fig. 1B_1 and B_3), whereas the membrane of treated *E. coli* O157:H7 was rough, with wrinkles and cracks, and surrounded by cellular debris (Fig. 1B_2 and B_4). One cell of untreated *E. coli* O157:H7 (Fig. 1B_3) or treated *E. coli* O157:H7 (Fig. 1B_4) was used to prepare a height image and section analysis in order to glean information on cell morphology. The surface of untreated bacteria appeared very smooth (Fig. 1B_5), whereas that of treated one was rough and had an uneven shape (Fig. 1B_6). Arithmetic average roughness increased from 11.40235 nm to 30.10581 nm after ε-PL treatment, as did the root mean square roughness, from 15.33474 nm to 46.00695 nm. This anomaly in the observed shape of ε-PL-treated *E. coli* O157:H7 by AFM has not been reported. To observe the integrity of the cell membrane, ε-PL-treated *E. coli* O157:H7 was examined under TEM (Fig. 1C). Untreated *E. coli* O157:H7 cells presented smooth borders, integral outer membranes, and a homogeneous cytoplasmic structure (Fig. 1C_1). Meanwhile, cells exposed to ε-PL showed collapsed, lysis and rough membranes, a cavitied cell shape, and non-integral membranes (Fig. 1C_2).

3.3. Oxidative stress induced by ε-PL

Oxidation-sensitive fluorescent probe DCFH-DA was used to detect the level of ROS generated in ε-PL-treated*E. coli* O157:H7. This non-fluorescent probe is hydrolyzed by esterase to form non-fluorescent DCFH in the cytoplasm, which is immediately transformed into the fluorescent product DC only in the presence of ROS. As showed in Fig. 2A, when the concentrations of ε-PL increased from 0 μg/mL to 50 μg/mL, the fluorescence intensity gradually increased, indicating a high ROS level. At these conditions, bacteria mortality was as high as 90%. Thus, it appears that the bactericidal effect of ε-PL may be a result of the production of high ROS concentration.

To confirm the relationship between ROS generation and the antibacterial function of ε-PL further, antioxidant N-acetylcysteine (NAC) was used to inhibit the formation of ROS during ε-PL treatment. As shown in Fig. 2B, in the presence of NAC, the mortality of cells decreased to only 3%, 12%, and 35%, respectively, after 15 h incubation; whereas in the presence of ε-PL at a concentration range of 5 μg/mL to 50 μg/mL, cell mortality was as high as 95% when NAC was not present.

3.4. Gene expression profile of *E. coli* O157:H7 in response to ε-PL treatment

In order to validate the action of ε-PL through ROS and DNA damage-related mechanisms, and thereby ascertain the antibacterial basis of ε-PL at the molecular level, RT-qPCR was used to detect the expression of relevant genes in *E. coli* O157:H7 (Fig. 3). Transcription levels of two oxidative stress-related genes, namely, *sodA* and *oxyR* [16] were up-regulated by 4- and 16-fold, respectively. *SodA* is a key structural gene in *E. coli* that expresses superoxide dismutase under superoxide stress to protect cells [20]. *OxyR* regulon is induced by hydrogen peroxide stress in *E. coli* to enhance antioxidative defenses, which involve activities of catalases and peroxidases [21]. The up-regulation of these two genes indicated that*E. coli* O157:H7 initiated adaptive responses to oxidative stress in order to drive down ROS levels. The DNA damage response (SOS response) regulon genes *recA* and *lexA* [22] were also affected by ε-

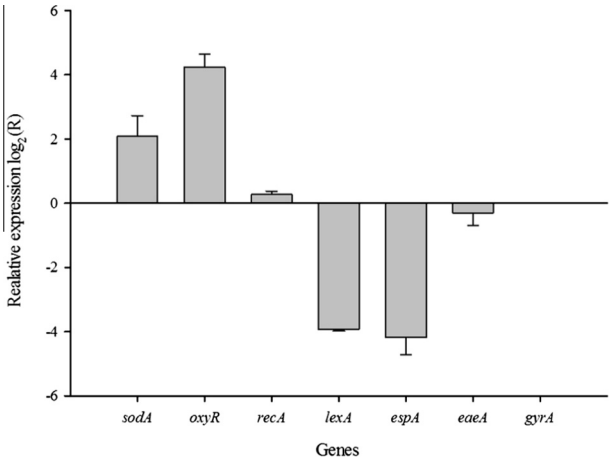
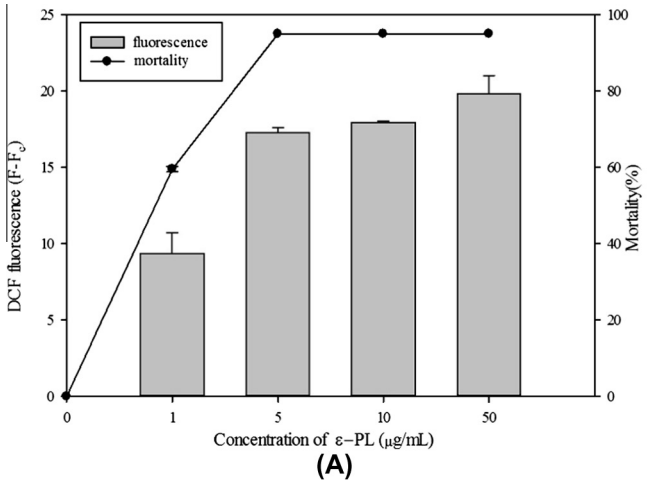


Fig. 3. Relative gene expression levels of *E. coli* O157:H7 treated with/without ε-PL. Transcripts of the relative genes were quantified by real-time quantitative PCR (RT-qPCR), and data were analyzed using the comparative critical threshold $2^{-\Delta\Delta CT}$ method. The relative expression ratio was presented as a log2 value in the histogram. A ratio greater than zero indicated up-regulation of gene expression, whereas a ratio below zero indicated down-regulation. Error bars indicated standard deviations for three replicates.



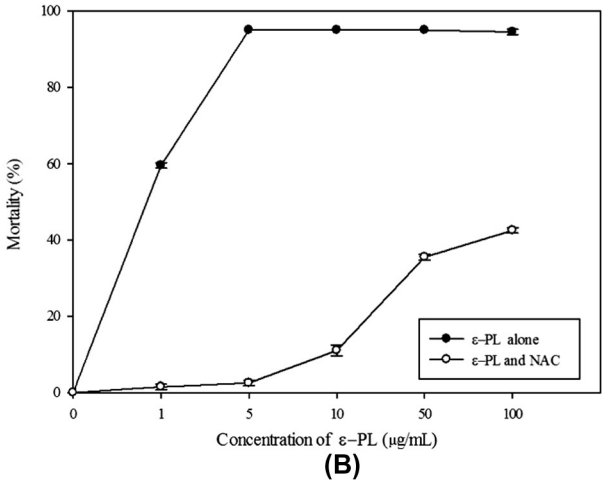


Fig. 2. Effect of reactive oxygen species (ROS) and antioxidant *N*-acetylcysteine (NAC) on the oxidative stress of ε-PL against *E. coli* O157:H7. (A) *E. coli* O157:H7 be treated with ε-PL at final concentrations of 0, 1, 5, 10, and 50 μg/mL. (B). *E. coli* O157:H7 was treated with ε-PL at final concentrations at 0, 1, 5, 10, 50, and 100 μg/mL, with or without 10 mM NAC, and incubated at 37 °C for 15 h. Percentage of cell mortality was calculated as the ratio of cell counts of *E. coli* O157:H7 in the treated group with ε-PL to those in the control group. Parallel test was performed in triplicate, and the average value and error bars were analyzed using the statistical program SigmaPlot 11.0.

PL. *recA* expression was found to be slightly up-regulated, and*lexA* expression was evidently down-regulated. The bacterial recombinase *recA* is critical in SOS response to regulate DNA repair, which promotes cleavage of the transcriptional repressor *lexA* to promote DNA repair [23]. Differential expressions of *recA* and *lexA* after ε-PL treatment suggested that the SOS response genes of *E. coli* O157:H7 for encoding DNA-repair systems may stimulate the repair and synthesis of DNA after it has been damaged in the presence of ε-PL. The virulence genes, *eaeA* and *espA* [24], were down-regulated, caused by the loss of cell viability.

4. Discussion

PMA-PCR result showed that the concentration of ε-PL at 10 μg/mL could not obtain PCR product, suggesting that the cell membrane was compromised. However, In viable count result appears that 90% mortality is reached with a concentration of ε-PL at 5 μg/mL (Supplementary Fig. S1A), but at this concentration a PCR product was obtained suggesting a part of cells have integral cell membrane which might kill by other antibacterial action.

Thus, the antibacterial activity of ε-PL may involve other actions in addition to membrane disruption. ROS generation and oxygen stress have been reported as common anti-bactericidal episodes caused by many cationic antibacterial compounds. In this study, oxidation-sensitive fluorescent probe DCFH-DA was used to detect the level of ROS generated in ε-PL-treated*E. coli* O157:H7. Del Carlo and Loeser (2006) [25] reported that ROS produced by poly-L-lysine could induce significant cell death in chondrocytes and could be inhibited by antioxidants. In the present study, the presence of antioxidant could reduce the concentration of ε-PL-induced ROS and further inhibit antibacterial action, which indicates that ROS produced in *E. coli* O157:H7 in response to ε-PL is associated with the antibacterial activity of ε-PL.

Antibacterial compounds are reported to induce the expression of different genes related to oxidative/redox stress, SOS response, virulence, adenosine triphosphate biosynthetic process, and chemotaxis [26,27]. RT-qPCR results in this work showed that ε-PL induced the up-regulation of bacterial oxidative stress response, as indicated by ROS-related genes *oxyR* and *sodA*, elicited the SOS response by regulon genes *recA* and *lexA*, and reduced the transcription of virulence genes *eaeA* and *espA*.

Based on the results of AFM, TEM, and other molecular methods, the potential antimicrobial mechanism of ε-PL is depicted in Fig. 4. When a positive charge from ε-PL came in contact with the bacteria, it became bound to the membrane surface by electrostatic attraction. The accumulation of such interaction resulted in the disturbance of the cell membrane, and led to changes that resulted in fractures of the membrane structure. Thus, ε-PL entered the cytoplasm through the membrane fractures. Additionally, ε-PL induced the generation of ROS and SOS response, which also affected cell death and regulated the expression level of related genes. The synergy or buildup of these factors affected the cells and finally resulted in the death of the bacteria.

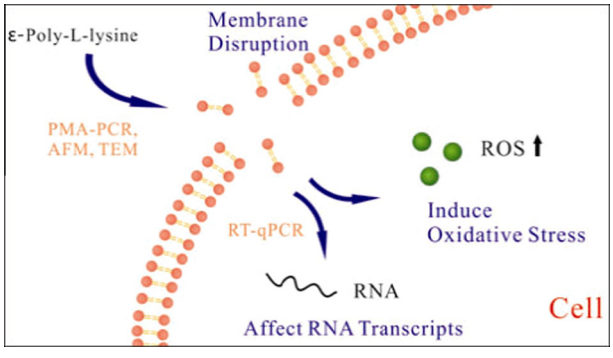


Fig. 4. Potential antibacterial mechanism of ε-PL against *E. coli* O157:H7. ε-PL attached to the membrane surface from electrostatic attraction, and resulted in the disturbance of the target membrane, as well as structural change and fracture of the membrane. ε-PL entered the cytoplasm through membrane pores, and subsequently, two parallel reactions took place, namely, increase of the level of ROS and interaction with genome DNA, which caused cell death.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.001.

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