Contribution of SOS Genes to H_2O_2-induced Apoptosis-like Death in Escherichia Coli

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Research Article

Keywords: Hydrogen peroxide, Escherichia coli, SOS response, Apoptosis-like death, Reactive oxygen species

Posted Date: May 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-520509/v1

Version of Record: A version of this preprint was published at Current Genetics on August 25th, 2021. See the published version at https://doi.org/10.1007/s00294-021-01204-0.

Abstract

Hydrogen peroxide (H_2O_2) is a debriding agent that damages the microbial structure and function by generating various reactive oxygen species (ROS). H_2O_2-produced hydroxyl radical (OH•) also exert oxidative stress on microorganisms. The spread of antibiotic resistance in bacteria is a serious issue worldwide, and greater efforts are needed to identify and characterize novel antibacterial mechanisms to develop new treatment strategies. Therefore, this study aimed to clarify the relationship between H_2O_2 and *Escherichia coli* and to elucidate a novel antibacterial mechanism(s) of H_2O_2. Following H_2O_2 exposure, increased levels of 8-hydroxyldeoxyguanosine and malondialdehyde indicated that H_2O_2 accelerates oxidation of bacterial DNA and lipids in *E. coli*. As oxidative damage worsened, the SOS response was triggered. Cell division arrest and resulting filamentation were identified in cells, indicating that *LexA* was involved in DNA replication. It was also verified that *RecA*, a representative SOS gene, helps self-cleavage of *LexA* and acts as a bacterial caspase-like protein. Our findings also showed that *dinF* is essential to preserve *E. coli* from H_2O_2-induced ROS, and furthermore, demonstrated that H_2O_2-induced SOS response and SOS genes participate differently in guarding *E. coli* from oxidative stress. As an extreme SOS response is considered apoptosis-like death (ALD) in bacteria, additional experiments were performed to examine the characteristics of ALD. DNA fragmentation and membrane depolarization appeared in H_2O_2-treated cells, suggesting that H_2O_2 causes ALD in *E. coli*. In conclusion, our investigations revealed that ALD is a novel antibacterial mode of action(s) of H_2O_2 with important contributions from SOS genes.

Introduction

Hydrogen peroxide (H₂O₂) is a colorless, toxic liquid that causes oxidative stress in many organisms. This reactive biochemical molecule readily passes through the plasma membrane of cells via aquaporin, and due to its high oxidative property, it alters the membrane potential, generates new molecules, and changes the intracellular redox balance of cells, as well as triggers tissue impairment in multicellular organisms (Bienert, et al. 2007, Lennicke, et al. 2015, Zhu, et al. 2005). Moreover, H₂O₂ is an apoptosis inducer because it exerts a broad cytotoxic efficacy on most types of cells (Xiang, et al. 2016). It induces apoptosis by acting as an apoptotic signaling molecule, exerting its effect through changes in pro- and anti-apoptotic protein expression, DNA damage, mitochondrial cytochrome *c* release, and caspase-9/caspase-3 activation (Saito, et al. 2006, Singh, et al. 2007). In particular, H₂O₂ is widely used as an antimicrobial chemical in that it is effective in removing pathogenic microorganisms (Cooke, et al. 2015, Garcez, et al. 2011). A high concentration of H₂O₂ is involved in the stress response of fungi, which causes cell death, and thereby serves as a fungicide (Glass and Dementhon 2006). It also exhibits bactericidal activity by either inhibiting cell proliferation or oxidizing bacterial cell walls (Linley, et al. 2012). Therefore, proper concentration of H₂O₂ is known to cure chronic skin wounds, and the application of an appropriate H₂O₂ concentration helps to treat bacterial infections (Mueller, et al. 2012, Zhu, et al. 2017).

When DNA is damaged by reactive oxygen species (ROS) or antibiotics in *Escherichia coli*, the DNA damage repair network is activated so the cell can survive the resulting genotoxic damage (Memar, et al. 2020). In *E. coli*, the DNA repair pathway, known as the SOS response, has been studied for decades, and it was recently discovered that the system also exists in other types of bacteria (Baharoglu and Mazel 2014). DNA damage is a critical issue in cells that can lead to cell death. The SOS response has >40 SOS genes that participate in the DNA repair process. In addition to SOS genes, they are accompanied by a number of proteins and molecules that help maintain DNA integrity. The key regulating protein of this coordinated cellular response are typically *LexA* and *RecA*. *LexA* is a dual-function repressor protease that suppresses the transcription of SOS gene under no-stress conditions (Simmons, et al. 2008). However, when bacteria are stressed, *RecA* combines with single-stranded DNA (ssDNA) and forms an activated nucleoprotein filament (*RecA**), which in turn stimulates *LexA* to autoproteolyze (self-cleavage), thereby resulting in the downstream activation of SOS effector genes. After stress disappear, the level of *RecA** decreases, whereas that of the *LexA* repressor increases, resulting in repression of the SOS response. In addition to *LexA* and *RecA*, it was reported that the damage inducible (*din*) gene is expressed in response to DNA damage-inducible antibiotics, although the function of *dinF* remains unknown (Rodríguez-Beltrán, et al. 2012).

Apoptosis is the programmed cell death (PCD) for eliminating unnecessary cells in multicellular organisms to maintain tissue function and homeostasis. In general, these apoptotic pathways appear in eukaryotes and are known to exist in yeast, which is a unicellular organism (Carmona-Gutierrez, et al. 2010, Madeo, et al. 2004). However, there is considerable evidence that apoptosis-like PCD is also present in prokaryotes, and this process is defined as apoptosis-like death (ALD) (Engelberg-Kulka, et al. 2006, Lee and Lee 2019a, Ramsdale 2008). ALD has phenotypic hallmarks similar to those of eukaryotic mitochondrial apoptosis (Dwyer, et al. 2012). When the cell death occurs in *Xanthomonas campestris*, both DNA degradation and production of a caspase-like protein occurs (Bayles 2014). In *Streptococcus pneumonia*, cell shrinkage, DNA condensation, DNA fragmentation, and membrane depolarization occur. Moreover, several studies have reported that DNA-damaging agents and antibiotics induce ALD in *E. coli* and there is evidence that *RecA* acts as a bacterial caspase-like protein involved in processes such as chromosomal condensation and extracellular exposure of phosphatidylserine (PS) (Hakansson, et al. 2011, Lee and Lee 2014, Yun, et al. 2018). However, because of a poor understanding of the signals, homologous proteins, and underlying mechanisms of bacterial ALD, continued research is needed.

The previously described SOS response has recently been deemed as an ALD in *E. coli*, and efforts have begun to target the bacterial SOS response as a new therapeutic strategy for the development of antibiotics (Erental, et al. 2014, Mo, et al. 2016). Therefore, in the current study, we determined whether H_2O_2 stimulates SOS genes in *E. coli* for DNA repair and whether a H_2O_2-induced SOS response prompts ALD in *E. coli* similar to apoptosis in eukaryotes. Our investigation also explored the effects and underlying mechanism of H_2O_2 in ALD.

Materials And Methods

Bacterial strains, compound preparation, and cell culture conditions

Escherichia coli (ATCC 25922) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and *ΔLexA*, *ΔRecA*, and *ΔdinF* mutants were obtained from the *E. coli* ASKA collection (library of *E. coli* ORF clones). H_2O_2 and norfloxacin was purchased from Sigma (St. Louis, MO, USA) and dissolved in H_2O and acetic acid, respectively. Sodium pyruvate (10 mM, Sigma), a scavenger of H_2O_2, was dissolved in H_2O and thiourea (150 mM, Sigma), a scavenger of OH•, was also dissolved in H_2O according to previous studies (Choi and Lee 2012, Franco, et al. 2007). For all experiments, bacterial cells were grown in Luria-Bertani (LB, BD Pharmingen, San Diego, CA, USA) agar plates at 37 ℃ and inoculated in LB broth under aerobic conditions at 37 ℃ and 120 rpm. Bacterial strains in the exponential phase were harvested and then resuspended in phosphate-buffered saline (PBS, pH 7.4).

Cell viability measurement

E. coli cells (1×10^6 cells/mL) were incubated with H□O□ at four concentrations or with 1.25 μg/mL norfloxacin, including the minimum inhibitory concentration (MIC). H□O□ and norfloxacin concentrations were selected based on previously published reports (Kim and Lee 2020, Orrù, et al. 2010). The treatment concentrations of H□O□ applied were 25 μg/mL (1/4 MIC), 50 μg/mL (1/2 MIC), 100 μg/mL (MIC), and 200 μg/mL (2 MIC). After 2 h incubation, cultures were spread onto LB agar plates. For colony forming unit (CFU)/mL calculations, 100 μL of culture was centrifuged at 12,000 rpm for 5 min, washed twice with PBS, and serially diluted in PBS. Each dilution was plated onto LB agar and the plates were incubated at 37 ℃ for 24 h. Only those dilutions that yielded between 20 - 200 colonies were counted. CFU/mL is expressed as a percentage of survival using the following formula: [(CFU of sample treated with test compound)/(CFU of untreated control) × 100].

Detection of intracellular hydroxyl radical

3'-(p-hydroxyphenyl) fluorescein (HPF, Molecular Probes, Invitrogen, Carlsbad, CA, USA) was applied to detect hydroxyl radicals (OH•), which are decomposition product of H_2O_2. HPF was dissolved in N,N-dimethylformamide (DMF, JUNSEI Chemical Co., Tokyo, Japan). It is nonfluorescent until it reacts with OH•, becoming fluorescent upon exposure to OH•. Bacterial cells (1×10^6 cells/mL) were treated with H_2O_2, H_2O_2 pretreated with thiourea, or norfloxacin, and incubated for 2 h at 37 °C. Following incubation, cells were centrifuged at 12,000 rpm for 5 min and the supernatant was removed. Cell pallets were then suspended with 1 mL PBS and dyed with 5 μM HPF. After HPF staining, the fluorescence intensity of each sample was assessed by utilizing a FACSVerse flow cytometer (Becton Dickinson, NJ, USA).

Assessment of oxidative DNA damage

Under oxidative stress, the guanine base of DNA is readily damaged, forming 8-hydroxydeoxyguanosine (8-OHdG). Therefore, the level of 8-OHdG is used as a biomarker to determine the degree of DNA oxidative damage. Bacterial cells were incubated for 2 h at 37 ℃ with H_2O_2, H_2O_2 pretreated with 10 mM sodium

pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin. Following incubation, DNA was extracted in an extraction solution (containing 100 mM NaCl, 10 mM Tris-Cl , 1 mM EDTA, 1 % SDS, 2 % Triton X-100, and 10 mg/mL proteinase K) as described in our previous study (Lee and Lee 2017). The 8-OHdG levels were estimated by competitive enzyme immunoassay method, using an Oxiselect Oxidative DNA Damage ELISA Kit (Cell Biolabs Inc., San Diego, CA, USA), according to the manufacturer's instructions. The absorbance of 8-OHdG was measured using an ELISA microplate reader (BioTek Instruments, Winooski, VT, USA) at 450 nm.

Evaluation of lipid peroxidation

The thiobarbituric acid-reactive substances (TBARS) assay was employed to detect malondialdehyde (MDA), which is the final product of lipid peroxidation. MDA levels were evaluated from standard curves (created based on the suggestions of the manufacturer, Sigma). Bacterial cells were treated with H_2O_2, H_2O_2 pretreated with sodium pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin. Following 2 h incubation at 37 °C, cells were centrifuged at 12,000 rpm for 5 min, and cell pellets were then mixed with lysis buffer (pH 8.0, 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 2 % Triton-X 100, and 1 % SDS). Next, cells were sonicated on ice using an ultrasonic sonicator (10 pulses of 1 min each at an amplitude of 38) (Sonics, Newtown, CT, USA) followed by centrifugation. The supernatant was mixed with thiobarbituric acid in 5 % trichloroacetic acid. The mixture was heated at 95 ℃ for 30 min, and then cooled on ice. Absorbance of the reaction mixture was measured at 532 nm.

Determination of cell division arrest and cell filamentation

4',6-diamidino-2-phenylindole (DAPI, Sigma) was used to ascertain bacterial cell division arrest. *E. coli* wild-type and *ΔLexA* cells were first treated with H_2O_2, H_2O_2 pretreated with sodium pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin and incubated for 2 h at 37 °C. For chromosomal staining, cells were washed with PBS and then incubated with 1 μg/mL DAPI in a darkroom for 20 min. The intensity of DAPI fluorescence was analyzed with a spectrofluorophotometer (Shimadzu RF-5301PC; Shimadzu, Japan), using excitation and emission wavelengths of 340 nm and 488 nm. To identify whether cell filamentation occurred, bacterial cells (wild-type and *ΔLexA*) were incubated for 2 h at 37 °C with H_2O_2, H_2O_2 pretreated with sodium pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin. Next, cells were harvested by centrifugation and suspended in PBS. Cell filamentation was observed using an Eclipse Ti-s microscope (Nikon, Tokyo, Japan).

Estimation of caspase-like protein activation

To investigate prokaryotic caspase homologs, the CaspACE^{TM} FITC-VAD-FMK In Situ Marker (Promega, Fitchburg, WI, USA) was applied. The marker is a fluorescent analog of the pan-caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) and intends for monitoring caspase activation. *E. coli* wild-type and *ΔRecA* cells were treated for 2 h at 37 °C with H_2O_2, H_2O_2 pretreated with sodium pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin. Following incubation, cells

were washed twice with PBS and stained with 5 μM FITC-VAD-FMK for 30 min. The total volume was adjusted to 1 mL with PBS, and fluorescence intensity of caspase-like protein activity was analyzed utilizing a FACSVerse flow cytometer.

Measurement of intracellular reactive oxygen species

Intracellular ROS levels were evaluated using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes). This probe passively diffuses through membranes, remaining in the cell after cleavage by esterases. When oxidation occurs by ROS, nonfluorescent H_2DCFDA changes to fluorescent 2',7'-dichlorofluorescein (DCF). *E. coli* wild-type and *ΔdinF* cells were incubated for 2 h at 37 °C with H_2O_2, H_2O_2 pretreated with sodium pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin. After incubation, the cells were centrifuged at 12,000 rpm for 5 min and the supernatant was eliminated. Then, cells were resuspended in PBS and 10 μM H_2DCFDA (dissolved in DMSO) was added to the suspension. Following incubation for 1 h at 37 ℃, cells were washed with PBS, and fluorescence intensity was assessed by utilizing a FACSVerse flow cytometer.

Analysis of DNA fragmentation

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was employed to assess DNA cleavage. This assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Basel, Switzerland). To identify fragmented DNA, the 3'-OH termini of nucleotides were enzymatically labeled and attached to DNA strand breaksby terminal deoxynucleotidyl transferase. *E. coli* cells were incubated for 2 h at 37 °C with H_2O_2, H_2O_2 pretreated with sodium pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin. Following incubation, cells were washed with PBS and then fixed with 2 % paraformaldehyde for 1 h on ice. Thereafter, the fixed cells were incubated with permeabilization solution (0.1 % Triton X-100 and 0.1 % sodium citrate) on ice for 2 min followed by incubation with TUNEL reaction mixture for 1 h at 37 °C. Fluorescence was assessed by utilizing a FACSVerse flow cytometer.

Detection of membrane depolarization and PS exposure

To assess membrane depolarization, bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC(3), Molecular Probes) was employed. *E. coli* cells were treated for 2 h at 37 °C with H_2O_2, H_2O_2 pretreated with sodium pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin. Following incubation, cells were suspended in PBS and stained with 5 μg/mL DiBAC(3). The fluorescence intensity was analyzed using a FACSVerse flow cytometer. To detect PS exposure, an Annexin V–FITC Apoptosis Detection kit (BD Pharmingen) was used. Cells were incubated for 2 h at 37 °C with H_2O_2, H_2O_2 pretreated with sodium pyruvate, H_2O_2 pretreated with thiourea, or norfloxacin. After incubation, cells were harvested and resuspended in 100 μL of 1× Annexin V binding buffer, followed by the addition of 50 μL/ml Annexin V–FITC. The mixtures were incubated for 15 min in the dark and fluorescence was measured by utilizing a FACSVerse flow cytometer.

Statistical analysis

All experiments were performed in triplicate and values were expressed as mean ± standard deviation. After confirming normality of distributions using the Shapiro-Wilk test, statistical comparisons between various groups were carried out by analysis of variance followed by Tukey's post-hoc test for three-group comparisons using SPSS software (version 25, SPSS/IBM. Chicago, IL, USA). Intergroup differences were considered statistically significant at $p < 0.05$.

Results

Cell growth and hydroxyl radical generation

It is established that the MIC of H_2O_2 and norfloxacin in *E. coli* is 100 µg/mL and 1.25 µg/mL, respectively. Based on these values, cell viability was measured at 1/4, 1/2, 1, and 2 MIC of H_2O_2. It was found that cell viability was noticeably reduced at 1/2 MIC of H_2O_2, and the extent of reduction was similar to that found with 1.25 µg/mL norfloxacin (Fig. 1A). Both H_2O_2 and norfloxacin are known to produce intracellular ROS such as OH•, therefore, experiment was conducted using HPF to figure out whether H_2O_2 forms OH• in *E. coli*, using norfloxacin for comparison as a known OH• generator. As shown in Fig. 1B, the fluorescence intensity of cells treated with H_2O_2 or norfloxacin was 61.49 % and 84.77 %, respectively, compared with 12.45 % in untreated cells. However, H_2O_2-treated cells pretreated with thiourea have a fluorescence intensity similar to untreated cells. Thus, these findings suggest that H_2O_2 forms OH• within *E. coli*, and that OH• created by H_2O_2 decomposition may trigger oxidative damage to bacteria.

Oxidative DNA damage has accelerated

Exposure to H_2O_2 and OH• activates mutagenesis and results in damage to DNA bases in *E. coli* (Krohn, et al. 2007). 8-OHdG is one of the representative DNA adducts generated by DNA oxidation and is considered as an oxidative stress indicator (Valavanidis, et al. 2009). For this reason, to confirm whether H_2O_2 and H_2O_2-induced OH• oxidize the *E. coli* DNA, the concentration of intracellular 8-OHdG was measured. As a result, the intracellular 8-OHdG concentration of H_2O_2- or norfloxacin-treated cells was higher than in untreated cells (Fig. 2). In contrast, H_2O_2-treated cells, pretreated with either sodium pyruvate (a H_2O_2 scavenger) or thiourea, had a lower concentration than only H_2O_2-treated cells. In our investigation, this result assumes that both H_2O_2 and H_2O_2-induced OH• promote oxidation in DNA bases of *E. coli*, acting as oxidative stress factors.

Membrane lipid peroxidation has expedited

It was previously reported that excess production of H_2O_2 and OH• negatively impacts biomembranes, resulting in lipid peroxidation of membrane polyunsaturated fatty acids (Su, et al. 2019). In general, estimating MDA production is the method to detect lipid peroxidation. MDA is the end product of

biological membrane peroxidation, so it is useful to measure damages by ROS (Siddique, et al. 2012). MDA levels in *E. coli* were measured to determine whether H_2O_2 and H_2O_2-induced OH• elicit oxidative damage to bacterial cell membranes. As shown in Fig. 3, MDA levels have increased in H_2O_2- or norfloxacin-treated cells, but pretreatment of sodium pyruvate or thiourea in H_2O_2-treated cells reduced MDA levels and was similar to that found in untreated cells. Therefore, this data indicates that H_2O_2 and OH• function as an oxidative stress factors in *E. coli*, causing lipid peroxidation in bacterial cell membranes.

LexA is associated with filamentation due to cell division arrest

DAPI was used to confirm whether cell division was arrested in *E. coli* wild-type and *ΔLexA* cells following exposure to H_2O_2. In Fig. 4, the fluorescence intensity of DAPI in wild-type cells treated with H_2O_2 or norfloxacin was more than double compared with untreated cells. Moreover, pretreatment of sodium pyruvate or thiourea reduced fluorescence intensity in H_2O_2-treated cells similar to that found in untreated cells. On the other hand, in *ΔLexA* cells, the pretreatment of sodium pyruvate or thiourea in H_2O_2-treated cells as well as those treated with H_2O_2 or norfloxacin was no different in untreated cells. Furthermore, as shown in Fig. 5, filamentation caused by cell division arrest also exhibited similar pattern as in DAPI assay. Thus, these results imply that H_2O_2 suppresses the *E. coli* division and characterizes SOS response such as filamentation. Furthermore, the absence of *LexA* indicates that *LexA* is a SOS gene involved in DNA replication, given that no cell division arrest and filamentation is observed.

Caspase-like protein, *RecA*, is expressed and activated

Caspases are proteases that mediated PCD, acting in the initiation and execution of apoptosis. It is known that caspases exists in some bacteria as well, and it has recently been revealed that *RecA* is a caspase-like protein that functions as a potential coprotease in *E. coli* (Lee and Lee 2019a). FITC-VAD-FMK was employed to figure out whether H_2O_2 activates caspase-like protein and whether *RecA* acts as a caspase-like protein in *E. coli*. In wild-type *E. coli*, cells treated with H_2O_2 or norfloxacin accounted for 76.49 and 90.32 % of caspase-like protein activation, respectively, compared with 10.33 % in untreated cells. Whereas the pretreatment of sodium pyruvate and thiourea in H_2O_2-treated cells showed levels similar to untreated cells (Fig. 6A). In *ΔRecA* cells, each sample did not show caspase-like protein activation with a clear difference from the untreated cells (Fig. 6B). Thus, these data demonstrate that H_2O_2 exposure leads to caspase-like protein activation in *E. coli*, and that *RecA* functions as a caspase-like protein.

dinF is conducive to protecting cells from oxidative stress

H_2O_2 can result in ROS overproduction under the presence of iron ions, eliciting an oxidative stress environment (Collin 2019). Further, the SOS gene, *dinF* is a known oxidative stress resistance protein (Rodríguez-Beltrán, et al. 2012). Hence, our study also focused on investigating how *dinF* is related to H_2O_2-induced intracellular ROS in *E. coli*. To examine whether H_2O_2 generates intracellular ROS, H□DCFDA

was applied in *E. coli* wild-type and *ΔdinF* cells. As shown in Fig. 7, the treatment of H_2O_2 and norfloxacin in both wild-types and *ΔdinF* cells generated ROS, and the pretreatment of sodium pyruvate or thiourea in H_2O_2-treated cells resulted in a rapid decrease in ROS production. Moreover, compared to each wild-type cells, our finding represented that the ROS generation has increased further in *ΔdinF* cells. Thus, given that the overproduction of ROS is more likely to occur in the absence of *dinF*, our findings suggest that *dinF* plays a role in protecting *E. coli* from intracellular ROS.

ALD is confirmed via PS exposure

Generally, exposure of PS on the outer plasma membrane is considered a distinct marker of apoptotic cells (Mariño and Kroemer 2013). Therefore, to assess whether H□O□ treatment results in ALD, we examined PS exposure in *E. coli* using Annexin V/PI double staining, in which Annexin V^+/PI^- indicates early apoptosis and Annexin V^+/PI^+ indicates necrosis. As shown in Fig. 8, while the PS exposure rate of untreated cells was 0.99 %, H□O□- or norfloxacin-treated cells exhibited 45.76 and 68.27 %, respectively. However, pretreatment of H□O□ or OH• scavenger in H□O□-treated cells decreased the fluorescence intensity of PS exposure similar to that found in untreated cells. Our observation demonstrates that early apoptosis progresses as H□O□ promotes PS exposure in *E. coli* and also suggests that H_2O_2 induces ALD in *E. coli*.

The hallmarks of ALD are occurred

Typical characteristics or indicator of ALD are DNA fragmentation and membrane depolarization (Erental, et al. 2014). First, the experiment was conducted through TUNEL assay to identify whether DNA fragmentation occurs as a result of oxidative DNA damage caused by H□O□. In contrast to untreated cells (10.17 %), 74.71 % and 91.06 % of fluorescence intensity were observed in cells treated with H□O□ or norfloxacin, respectively (Fig. 9). But in H□O□-treated cells pretreated with sodium pyruvate or thiourea, the fluorescence intensity decreased by a level similar to that of untreated cells. AS shown in Fig. 10, similar results were also found for membrane depolarization using DiBAC□(3). These examinations suggest that H□O□ induces DNA fragmentation and membrane depolarization, and that blocking the H□O□ and OH• produced by H□O□ are causative factors of ALD in *E. coli*.

Discussion

H_2O_2 is an oxygenating compound that is toxic to various types of cells. However, in that its pharmacological activities are effective in treating microbial infection, it is necessary to elucidate the therapeutic effects of H_2O_2 in greater detail (Amanna, et al. 2012, McDonnell 2009). Moreover, with the advent of antibiotic resistant bacteria, it has become particularly important to discover novel antibacterial mode of action to overcome previously known mechanisms of conventional antibiotics (Frieri, et al. 2017, Pourmand, et al. 2017). Although it was recently discovered that PCD-like response occurs in bacteria similar to eukaryotic cells, there remains a lack of research toward a better understanding of the

relationship between H_2O_2 and bacterial-PCD as well as the underlying mechanisms. Therefore, the aim of our study was to manifest the novel antibacterial mode of action of H_2O_2 in *E. coli*.

Our experiments revealed that the survival rate of *E. coli* at 1/2 MIC H_2O_2 was drastically reduced, and we then investigated further to identify factors other than H_2O_2 that affect survival rate. Most notably OH• is a decomposition product of H_2O_2, a process which is catalyzed in cells by intracellular enzymes of a metal ions such as Fe^{2+} or Cu^{2+} (Lee, et al. 2013). In general, OH• is produced through the Fenton-reaction and it is known to act as a more powerful oxidative stress factor than H_2O_2 (Watts, et al. 2003). It is also widely known that the Fenton-reaction occurs in *E. coli* using iron ions and that norfloxacin is useful as a positive control as it is known generator of OH• in several types of cells (Bisaccia, et al. 2019, Takeda, et al. 2010). Our findings confirm that intracellular OH• levels in *E. coli* increase following H□O□ treatment, suggesting that both H□O□ and OH• act as oxidative stress factors in *E. coli*.

H□O□ and OH• cause oxidative damage to nucleic acids, lipids, and proteins of microorganisms, making bacteria unable to perform structural or physiological functions. The ROS accumulation frequently leads to modification of nucleotide bases and DNA strand breaks (Srinivas, et al. 2019). Especially in nucleotide bases, guanine is reported to be vulnerable to oxidative stress and easily form an 8-OHdG, which disturbs bacterial genome integrity (Brierley and Martin 2013, Delaney, et al. 2012). Additionally, lipid peroxidation alters membrane permeability, adversely affecting membrane fluidity, which in turn suppresses the solute particle transport system (Smith, et al. 2013). Therefore, collapse of the phospholipid bilayer destroys the cell itself, but also affects the function of membrane-attached organelles. As a result, it becomes difficult to maintain cellular homeostasis, which leads to plasma membrane instability. In this study, H□O□ and H□O□-induced OH• increase the intracellular 8-OHdG and MDA levels in *E. coli*, thereby illustrating that H□O□ and OH• accelerate oxidative damage to bacterial DNA and lipid.

Prokaryotes have several strategies to adapt to environmental changes. They synthesize DNA repair enzymes to counter extreme genetic damage caused by DNA-damaging molecules such as ROS and antibiotics (Memar, et al. 2020). In *E. coli*, the SOS response is a well-known instance of DNA repair system. The exposure of cells to ~ 50 μM H_2O_2 induces expression of DNA repair genes as well as cell division arrest (Santa-Gonzalez, et al. 2016). These genes block DNA replication, resulting in the SOS response and cell filamentation (Cox, et al. 2000, Justice, et al. 2006). Our findings revealed that H_2O_2 inhibits the cell division in *E. coli*, and in addition, leads to filamentation as a result of cell division arrest. Our data intimates that the SOS response is activated in response to oxidative DNA damage in H_2O_2-treated cells.

LexA is a transcriptional repressor that contains DNA-binding domain and binds to SOS box. In the absence of DNA damage, *LexA* downregulates the SOS response genes while sustaining an appropriate basal level of *LexA* expression. However, when *E. coli* is exposed to endogenous oxidative compound, self-cleavage of *LexA* is stimulated by *RecA**, initiating derepression of SOS genes (Žgur-Bertok 2013). In the DNA repair process, *LexA* also acts as a cell division inhibitor, helping the *RecA*-ssDNA complex pause

the replication fork (Erill, et al. 2007). Our paper observed cell division arrest and filamentation in *E. coli* wild-type and *ΔLexA* cells. As a result, cell division arrest due to H_2O_2 and H_2O_2-induced OH• and the resulting filamentation were detected in wild-type cells. However, such changes were not detected in *ΔLexA* cells, suggesting that *LexA* is involved in cell division inhibition and filamentation in the SOS response of *E. coli*.

RecA is 38 kilodalton protein that participates in several reactions related to DNA repair. In *E. coli*, it is an essential for genetic recombination as much as *LexA* and regulates SOS response. Except for that *RecA* has a variety of activities and it has been recognized in some recent papers that *RecA* is considered as a caspase-like protein in *E. coli* (Asplund-Samuelsson 2015, Lee and Lee 2017, Yun and Lee 2016). Some bacteria have identified the presence of caspase, but not in *E. coli*, and it was only reported that *RecA* functions similar to eukaryotic caspases (Asplund-Samuelsson, et al. 2012). In addition, as eukaryotic caspases mediate PCD such as apoptosis, it was determined that *RecA* causes ALD in *E. coli* (Lee and Lee 2019b). In our experiments, there was caspase-like protein activation in H_2O_2-treated wild-type cells, but not in *ΔRecA* cells. Therefore, this data demonstrates that *RecA* serves as bacterial caspase-like protein and that H_2O_2 promotes caspase-like protein activation in *E. coli*.

The *E. colidinF* gene is part of the *LexA* transcription operon and located downstream of the *LexA* gene (Fernández de Henestrosa, et al. 2000). This gene regards as DNA damage inducible protein. Although the role of *dinF* in the SOS response is poorly understood, it has been reported to protect *E. coli* from oxidative stress (Rodríguez-Beltrán, et al. 2012). H_2O_2 itself is a ROS, but it can also generate other more powerful ROS within cells. In present study, ROS production was measured using H_2DCFDA, and levels were compared in wild-type and *ΔdinF* cells to determine how much cells are protected from oxidative stress. In consequence, the amount of ROS produced in *ΔdinF* cells was greater than wild-type cells, and it was shown that *dinF* is gene related to the production of ROS in *E. coli*. In addition, this result indicated that H_2O_2 facilitates ROS production in *E. coli*, and our finding illustrates that, under condition of ROS overproduction, *dinF* protects *E. coli* from oxidative stress.

The SOS response is the most studied DNA repair system used by bacteria in response to DNA damage. The *LexA-RecA* genes involved in this reaction also participate in the DNA repair pathway, but it has recently been known that these genes response to extreme DNA damage and result in PCD in *E. coli* (Erental, et al. 2014). The novel PCD created by an intense SOS response in *E. coli* is termed ALD, which is similar to the eukaryotic mitochondrial apoptosis hallmarks. The representative ALD characteristics include DNA fragmentation and membrane depolarization, as well as high-level OH• formation through the Fenton-reaction. In addition, it was previously reported that the SOS-related protein, *LexA* and *RecA*, are involved in *E. coli* ALD (Lee and Lee 2019b). As a result, we conducted additional experiments to reveal evidence to support the occurrence of ALD in *E. coli*.

It is well known that H_2O_2 causes cell death in mammalian cells, and it induces apoptosis in different types of cancer cells depending on the concentration. PS is an abundant phospholipid in the plasma membrane. Under normal conditions, PS is kept inside the cell by the flippase, but during apoptosis, it is

exposed to the cell surface by a calcium-dependent scramblase (Nagata, et al. 2016). This PS translocation is considered an important biochemical and physiological characteristic of apoptosis. Prior to investigating the characteristics of ALD, we monitored whether H_2O_2 treatment leads to PS exposure in *E. coli*. In our investigation, H_2O_2 triggered the PS exposure in *E. coli*, and the scavenger of H_2O_2 or OH• decreased this effect, indicating that H_2O_2 and H_2O_2-induced OH• can prompt ALD in *E. coli*. Furthermore, these results also demonstrate that ALD in *E. coli* occurs after the initial SOS response induced by H_2O_2 becomes extreme.

There is a close relationship between typical apoptosis features and ROS (Simon, et al. 2000). Commonly known responses to oxidative damage in cells is DNA degradation and lipid peroxidation. In terms of DNA, apoptosis is accompanied by chromosomal DNA degradation and the formation of oligonucleosomal fragments by endogenous endonuclease (Zhang and Ming 2000). Regarding lipid peroxidation, it inherently alters membrane potential, and the resulting plasma membrane depolarization is considered to be an early event of apoptosis (Sen, et al. 2006). We investigated these distinct ALD hallmarks in *E. coli* and found that H_2O_2-treated cells exhibited both DNA fragmentation and membrane depolarization. As a result, these examinations show that indicative markers of ALD were caused by H_2O_2, providing greater mechanistic insight of ALD in *E. coli*.

To summarize, we discovered that a novel antibacterial mechanism of H_2O_2 in *E. coli* is ALD, which is mediated by H_2O_2-induced oxidative stress. Further, genes involved in the SOS response induced by H_2O_2 not only participate in the DNA repair system but also play important roles in ALD. *LexA* acts as a cell division inhibitor, inducing cell filamentation, and hence causes morphological changes (filamentation) that are characteristic of ALD. *RecA* acts as a caspase-like protein and, similar to eukaryotic caspases, provides cell death signaling to initiate ALD. *dinF* protects cells from H_2O_2-induced ROS overproduction, which is a characteristics of ALD. Furthermore, an extreme SOS response by H_2O_2 triggers ALD, and that some feature of ALD, such as caspase-like protein activation, PS exposure, DNA fragmentation, and membrane depolarization, express in *E. coli* cells exposed to H_2O_2. Also, the effects of H_2O_2 on these hallmark characteristics of ALD hallmarks were reduced when cells were pretreated with thiourea rather than sodium pyruvate, indicating that OH• produced by H_2O_2 acts as a stronger inducer of ALD than H_2O_2 itself.

In conclusion, oxidative stress induction has been of considerable interest in the search for new antimicrobial strategies because it is extremely effective in killing pathogens, and oxidative stress-induced antibiotics are now widely used. In this study, we demonstrate that H_2O_2-induced ALD may be a new approach in the treatment of bacterial infection, and that targeting the regulation of SOS genes may improve the effectiveness of antibiotics.

Declarations

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2020R1A2B5B01001905)

Availability of data and material

No mandated datasets were generated or analyzed during the current study.

Author contribution

H. Kim and D.G. Lee conceived the study and designed the experiment. H. Kim performed the experiments and collected the data. H. Kim and D.G. Lee analyzed the data. H. Kim wrote the manuscript.

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Figures

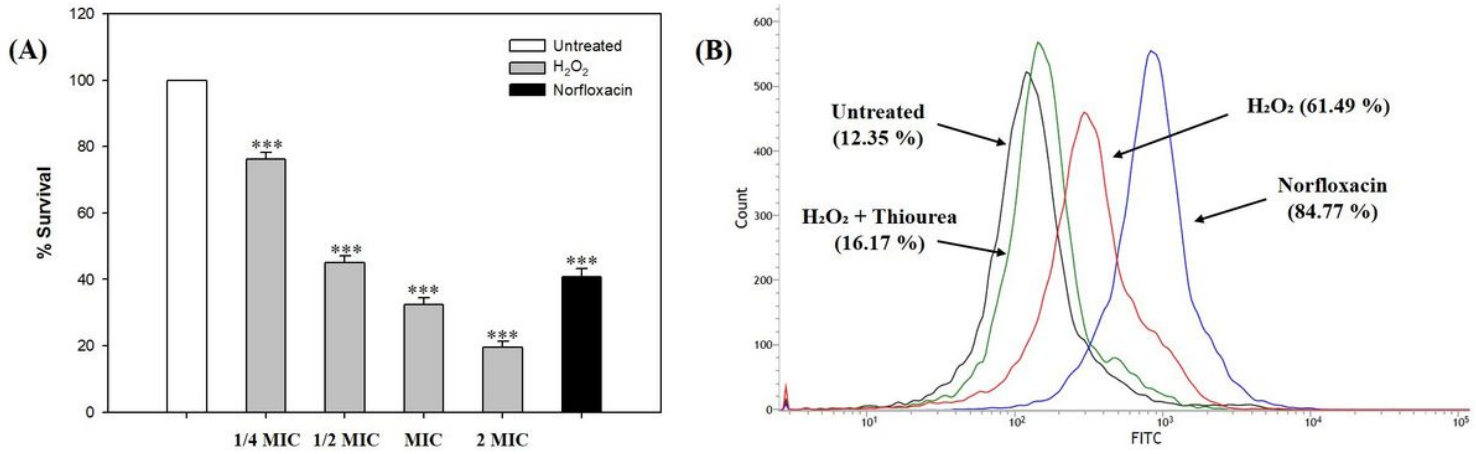

Figure 1

(A) Percentage of survival was evaluated by counting CFU after treatment with the compounds. The data represents the average, standard deviation, and p values from three independent experiments (***p< 0.01). (B) Hydroxyl radical formation was measured using HPF in E. coli. H□O□ was treated with 50

μg/mL, H□O□ and Thiourea was treated with 50 μg/mL and 150 mM Thiourea, and norfloxacin was treated with 1.25 μg/mL.

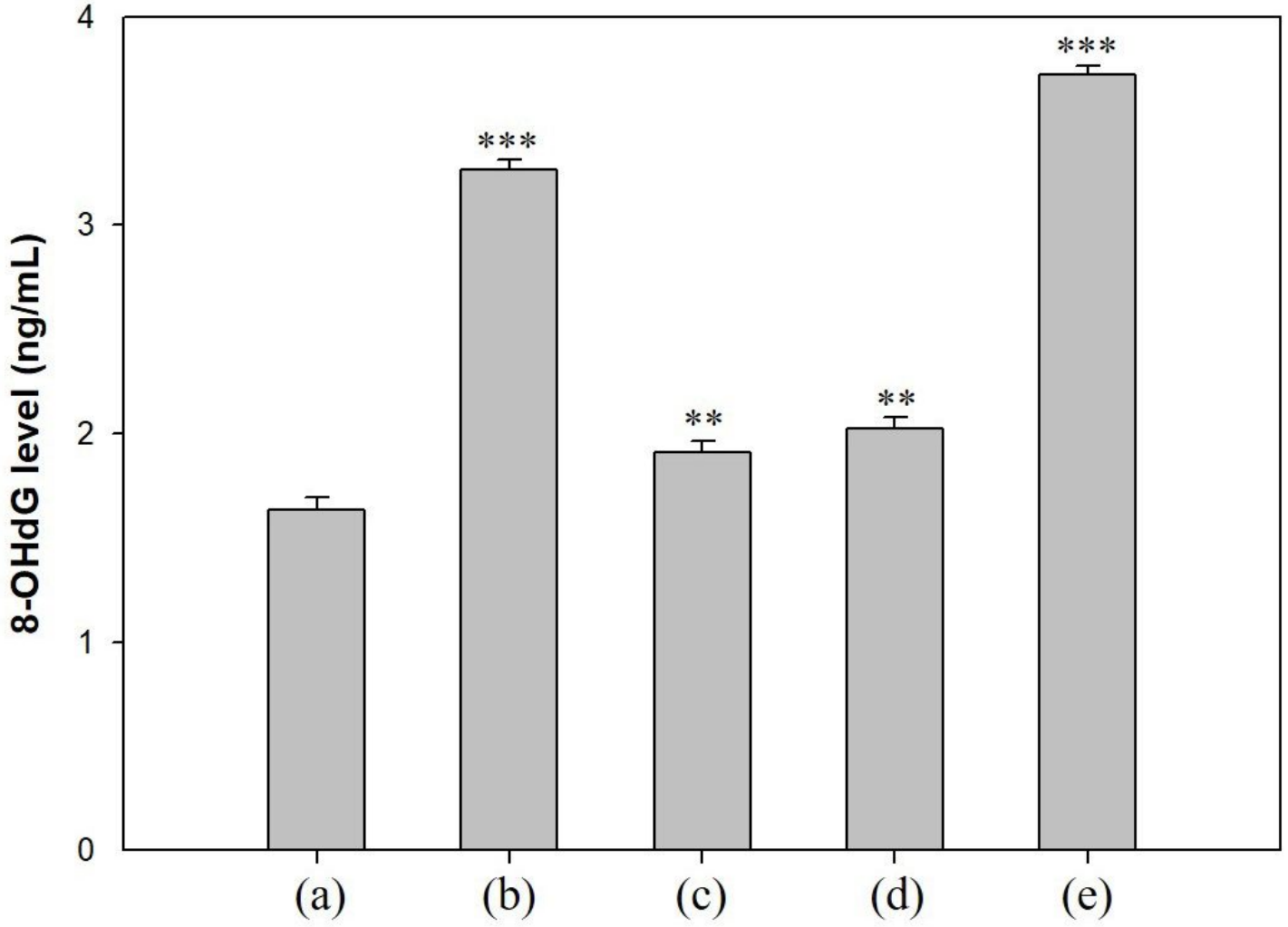

Figure 2

Spectrophotometric analysis of oxidative DNA damage was conducted by 8-OHdG quantitation in E. coli. (a) Untreated cells, (b) cells were treated with 50 μg/mL H□O□, (c) cells were pretreated with 150 mM Thiourea and treated with 50 μg/mL H□O□, (d) cells were pretreated with 10 mM Sodium pyruvate and treated with 50 μg/mL H□O□, and (e) cells were treated with 1.25 μg/mL norfloxacin. The data represents the average, standard deviation, and p values from three independent experiments (*p< 0.1; **p< 0.05; ***p< 0.01).

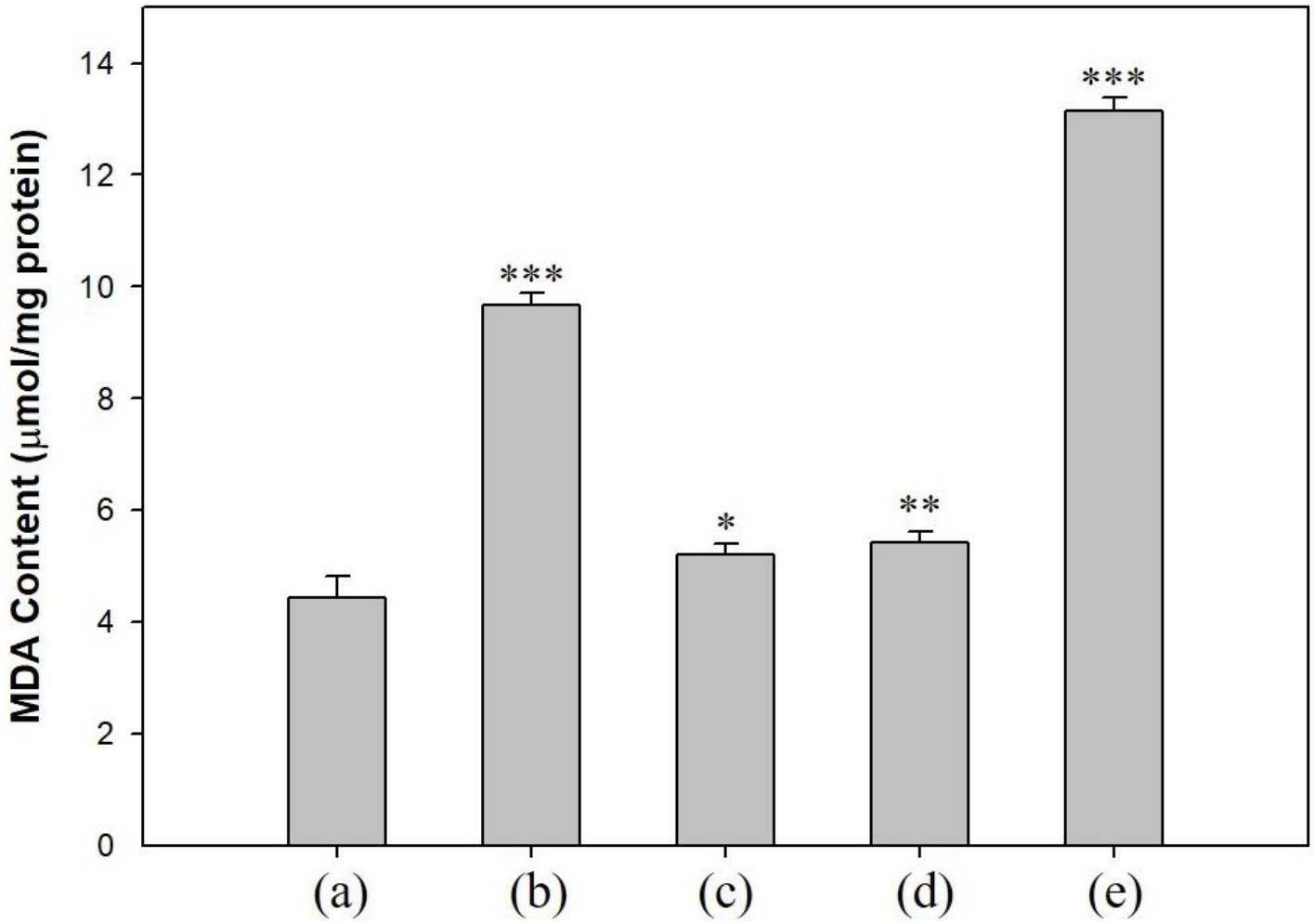

Figure 3

Lipid peroxidation was measured by the TBARS assay in E. coli and increase in MDA levels indicates peroxidation of lipids. (a) Untreated cells, (b) cells were treated with 50 µg/mL H₂O₂, (c) cells were pretreated with 150 mM Thiourea and treated with 50 µg/mL H₂O₂, (d) cells were pretreated with 10 mM Sodium pyruvate and treated with 50 µg/mL H₂O₂, and (e) cells were treated with 1.25 µg/mL norfloxacin. The data represents the average, standard deviation, and p values from three independent experiments (*p< 0.1; **p< 0.05; ***p< 0.01).

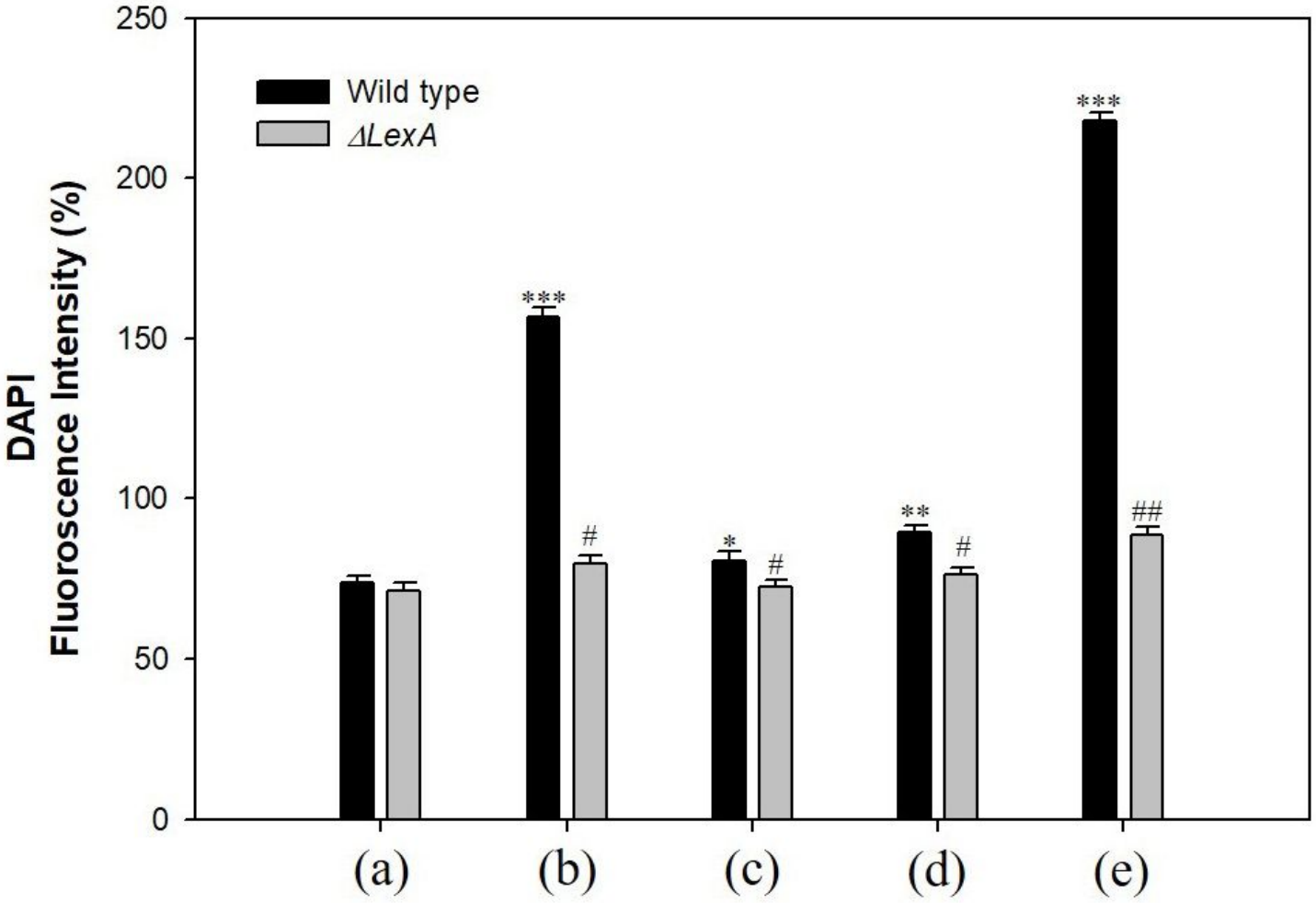

Figure 4

Spectrofluorophotometric analysis of cell division arrest was measured using DAPI in E. coli wild-type and ΔLexA cells. (a) Untreated cells, (b) cells were treated with 50 µg/mL H□O□, (c) cells were pretreated with 150 mM Thiourea and treated with 50 µg/mL H□O□, (d) cells were pretreated with 10 mM Sodium pyruvate and treated with 50 µg/mL H□O□, and (e) cells were treated with 1.25 µg/mL norfloxacin. The data represents the average, standard deviation, and p values from three independent experiments (*p< 0.1; **p< 0.05; ***p< 0.01 vs. untreated sample in wild-type; ##p< 0.05, #p< 0.1 vs. untreated sample in ΔLexA).

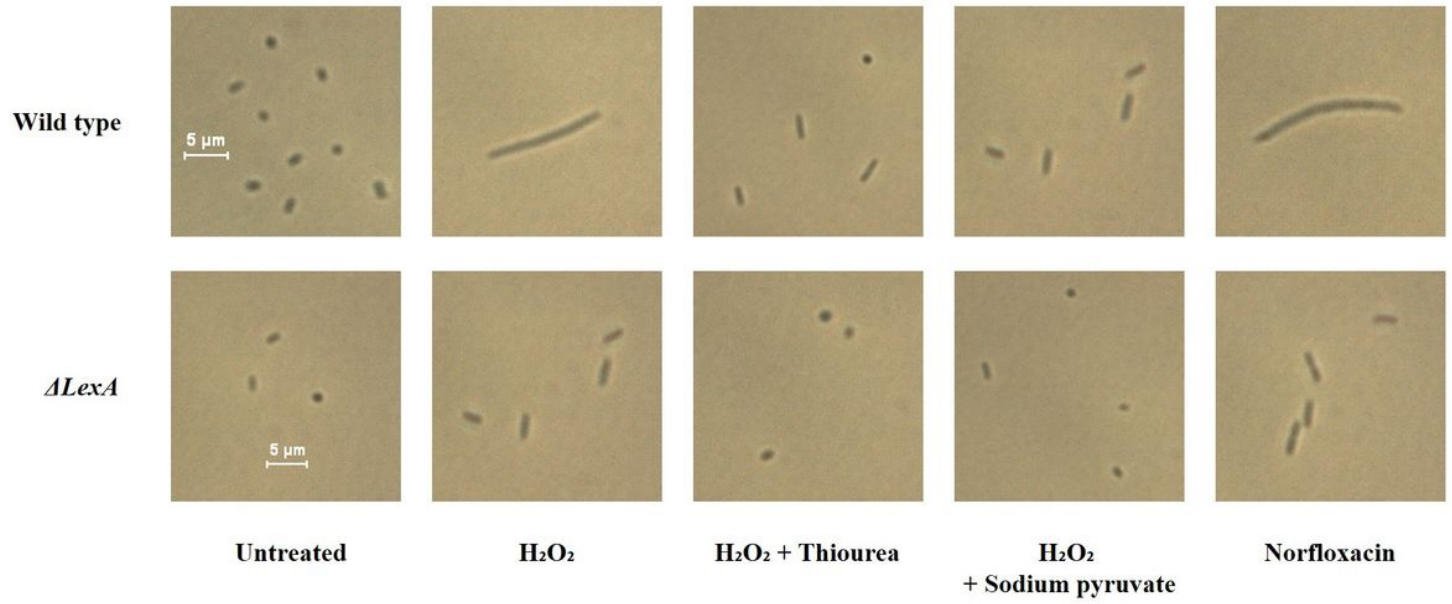

Figure 5

Cell filamentation in E. coli wild-type cells and ΔLexA cells.

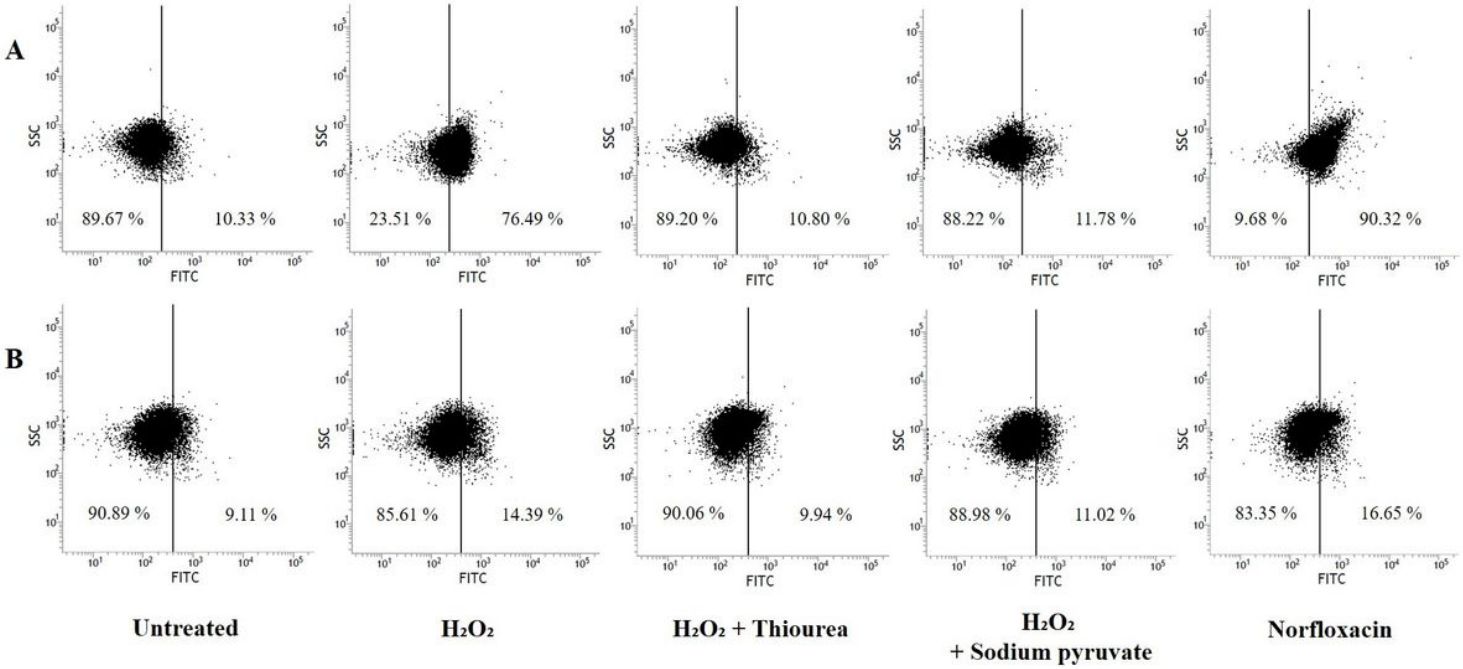

Figure 6

Caspase-like protein activation was measured by caspACE FITC-VAD-FMK. (A) E. coli wild-type cells and (B) E. coli ΔRecA cells.

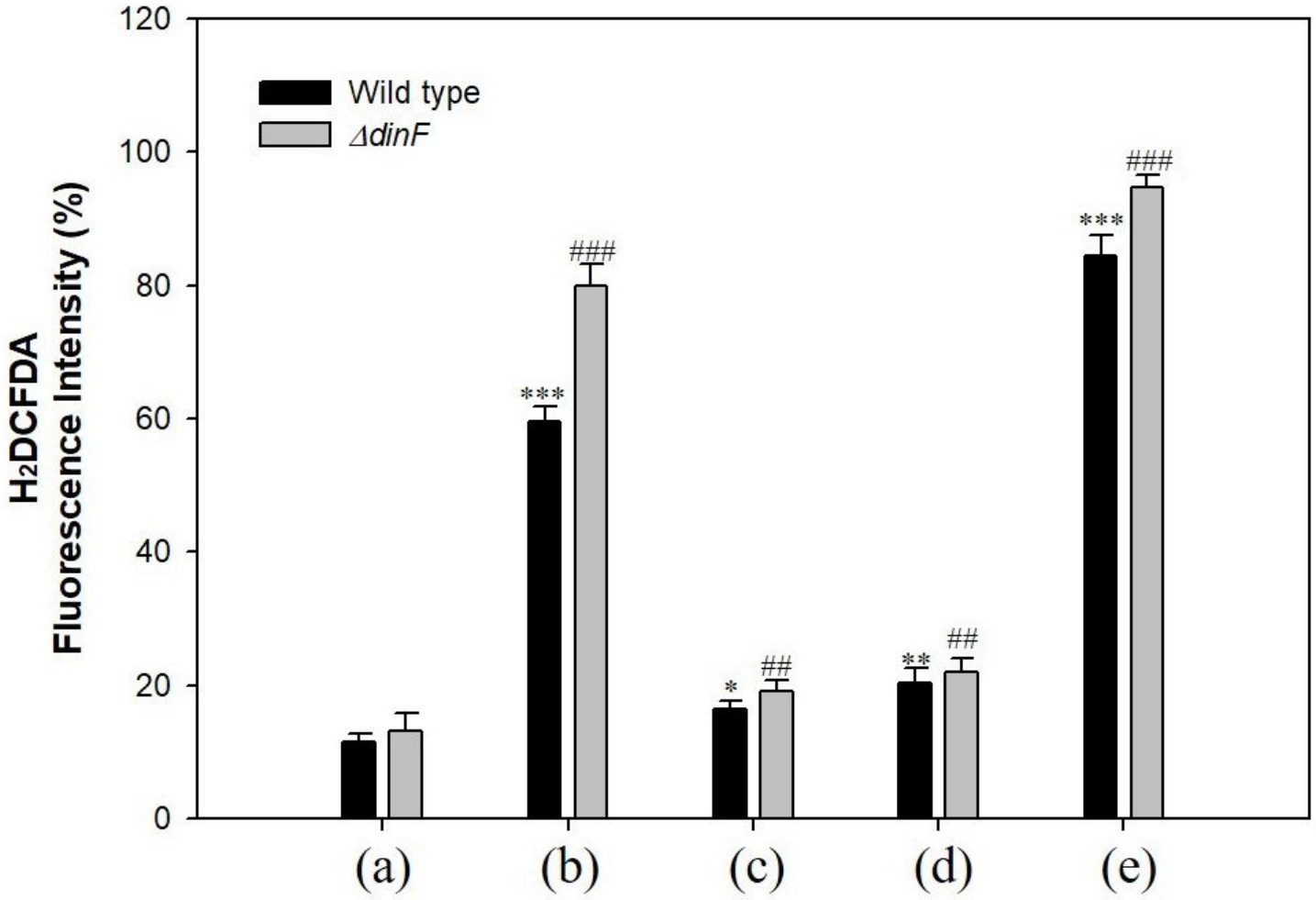

Figure 7

Flow cytometry analysis of ROS generation was measured using H₂DCFDA in E. coli wild-type and ΔdinF cells. (a) Untreated cells, (b) cells were treated with 50 µg/mL H₂O₂, (c) cells were pretreated with 150 mM Thiourea and treated with 50 µg/mL H₂O₂, (d) cells were pretreated with 10 mM Sodium pyruvate and treated with 50 µg/mL H₂O₂, and (e) cells were treated with 1.25 µg/mL norfloxacin. The data represents the average, standard deviation, and p values from three independent experiments (*p< 0.1; **p< 0.05; ***p< 0.01 vs. untreated sample in wild-type; ##p< 0.05, ###p< 0.01 vs. untreated sample in ΔdinF).

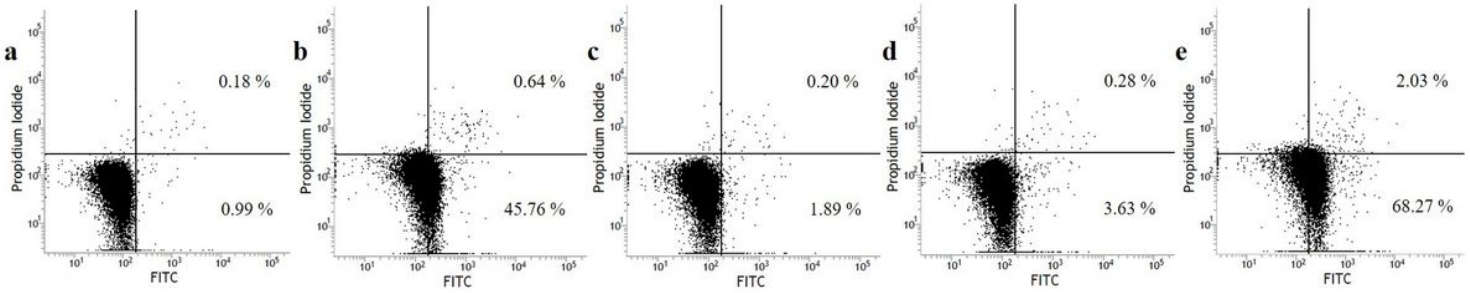

Figure 8

Flow cytometric analysis of phosphatidylserine exposure was measured using Annexin V/propidium iodide double staining in E. coli. (a) Untreated cells, (b) cells were treated with 50 μg/mL H□O□, (c) cells were pretreated with 150 mM Thiourea and treated with 50 μg/mL H□O□, (d) cells were pretreated with 10 mM Sodium pyruvate and treated with 50 μg/mL H□O□, and (e) cells were treated with 1.25 μg/mL norfloxacin.

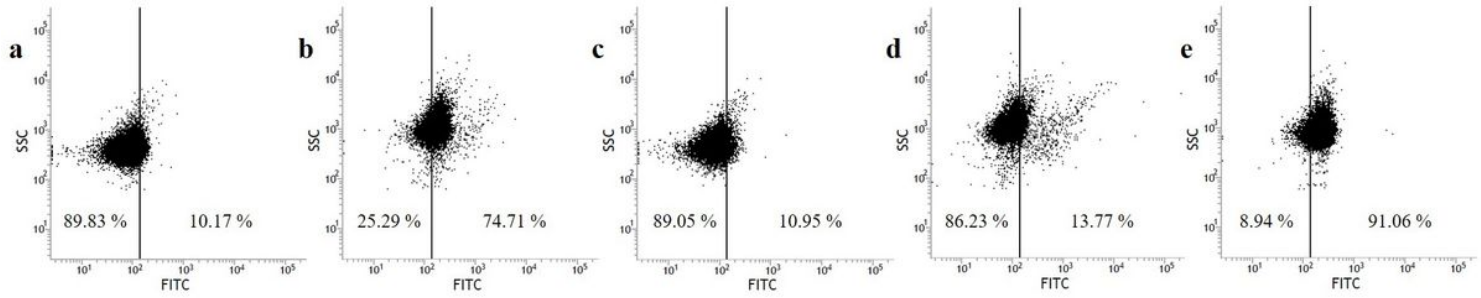

Figure 9

Flow cytometry analysis of DNA fragmentation was measured by TUNEL assay in E. coli. (a) Untreated cells, (b) cells were treated with 50 μg/mL H□O□, (c) cells were pretreated with 150 mM Thiourea and treated with 50 μg/mL H□O□, (d) cells were pretreated with 10 mM Sodium pyruvate and treated with 50 μg/mL H□O□, and (e) cells were treated with 1.25 μg/mL norfloxacin.

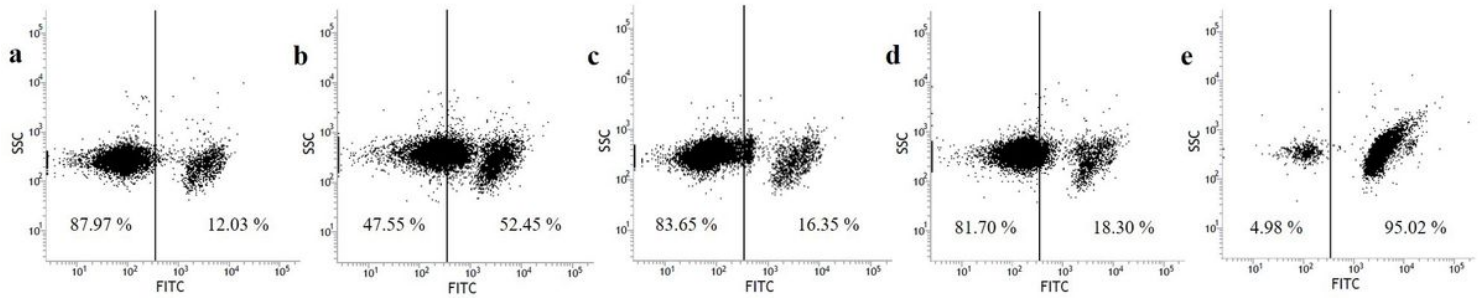

Figure 10

Membrane depolarization was measured using DiBAC□(3) in E. coli. (a) Untreated cells, (b) cells were treated with 50 μg/mL H□O□, (c) cells were pretreated with 150 mM Thiourea and treated with 50 μg/mL H□O□, (d) cells were pretreated with 10 mM Sodium pyruvate and treated with 50 μg/mL H□O□, and (e) cells were treated with 1.25 μg/mL norfloxacin.