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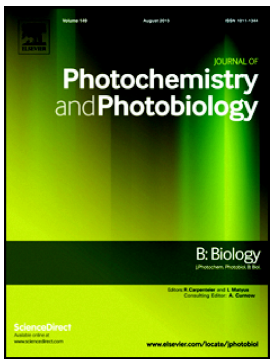

Bactericidal action of ferulic acid with ultraviolet-A light irradiation

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Title: Bactericidal action of ferulic acid with ultraviolet-A light irradiation

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Abstract

The bactericidal activity of ferulic acid (FA) against various microorganisms was remarkably enhanced by ultraviolet-A (UV-A) irradiation (wavelength, 365 nm). However, the bactericidal mechanism in the photo-combination system has not been evaluated. In the present study, this combined treatment was characterized by investigating associated changes in cellular functions of *Escherichia coli*, including assessments of respiratory activity, lipid peroxidation, membrane permeability, and damage to DNA and the cell surface. FA adsorbed onto and was incorporated into bacterial membranes, and the affinity resulted in decreased respiratory activity and enhanced lipid peroxidation in the cytoplasmic membrane with low-fluence (1.0 J/cm^2) UV-A irradiation. Flow cytometry analysis revealed that additional exposure (8 J/cm^2) combined with FA (1 mg/mL) induced increased cell permeability, yielding a 4.8-log decrease in the viable cell count. Morphologically, the treated cells exhibited a bacterial membrane dysfunction, producing many vesicles on the cell surface. However, despite this effect on the cell surface, plasmid DNA transformed into FA-treated *E. coli* maintained supercoiled integrity with negligible DNA oxidation. Our data strongly suggested that FA functions inside and outside the bacterial membrane; UV-A exposure in the presence of FA then causes increased oxidative modification and subsequent disruption of the bacterial membrane, without causing detectable genotoxicity.

Keywords: Bactericidal action; Ferulic acid; Ultraviolet-A; Oxidative stress; Membrane damage

1. Introduction

Ferulic acid (FA), one of the most abundant phenolic acids in plants such as rice, wheat, barley, citrus fruits, and tomatoes, occurs as esters conjugated covalently to mono-, di-, and polysaccharides and to cell wall lignin [1,2]. This molecule is a potential therapeutic agent with demonstrated antioxidant, hepatoprotective, and anti-inflammatory activities [2]. In Japan, FA is "generally regarded as safe" and has been approved for use as an antioxidant food additive and an UV protective agent in cosmetic products [3].

In a previous study, we demonstrated that FA enhances the photoantimicrobial activity of ultraviolet-A (UV-A) light (radiation with a wavelength of 350 to 385 nm) emitted by light-emitting diodes (LEDs), exhibiting activity against multiple food spoilers and pathogens (seven bacteria and four fungi species) [4]. It has been demonstrated that the bactericidal activity of the combination of FA and UV-A is related to the generation of reactive oxygen species (ROS). When polyphenols, including FA, are exposed to UV-A or violet light (wavelength range, 380-420 nm), photo-oxidation occurs at the hydroxyl groups on their aromatic rings, resulting in ROS generation through the reaction with environmental oxygen of electrons and protons derived from

the photo-oxidized polyphenols [5,6], as observed with autooxidation of phenolic acids [7]. In those papers, the generation of hydrogen peroxide and hydroxyl radicals was detected by electron spin resonance analysis. We also suggested that the strong synergy of UV-A light with FA reflects the production of hydrogen peroxide, as indicated by the fact that the observed photobactericidal activity was quenched by the addition of catalase [8]. An investigation of the photo-combination with gallic acid (GA) has elucidated excessive lipid peroxidation of the bacterial membrane in *Staphylococcus aureus*, yielding approximately 6-log decreases in cell viability [5]. In addition to the cytotoxic response, a genotoxic component was observed for the photobactericidal activation of caffeic acid (CA); this mechanism was demonstrated by an increase in 8-hydroxydeoxyguanosine (8-OHdG) formed by the oxidative reaction of DNA with ROS, yielding decreases in cell viability of greater than 5 logs [6]. For the photo-combination techniques using GA or CA, they were used with the concentrations of 0.68 or 1 mg/mL, respectively, at fluences of 72 J/cm^2 or above at a wavelength of 400 nm.

Notably, inactivation of bacteria by the combination of UV-A-irradiation with FA required much lower FA concentration and UV-A fluence; for some bacterial strains, irradiation with UV-A (1.0 J/cm^2) in the presence of 0.1 mg/mL FA resulted in a 6-log decrease in viable cell numbers [4]. A disinfection technology whereby FA and UV-A

are combined would be attractive for applications in the food industry, such as for food decontamination and postharvest disinfection: because FA is a safe natural compound and UV-A is a less-harmful type of light, compared with UV-B and -C. An UV-B at 312 nm can induce the formation of bipyrimidine photoproducts in the DNA of human skin at 0.2 J/cm^2; the yield of the formation per 10^6 normal bases per J/cm^2 within the whole skin is much higher than that of UV-A [9]. Cossu et al. found that no significant change in endogenous total phenolic content of spinach leaves was observed after UV-A light exposure [10], although UV-C induced decreases in the antioxidative activity of polyphenolic acids in fruit products [11]. As a novel approach for non-thermal decontamination of fresh produce, disinfection using a combination of a fog deposited FA and UV-A light eliminated microorganisms, *E .coli* O157:H7 and *Listeria innocua*, inoculated on spinach leaves [10]. However, the inactivation mechanism whereby UV-A-activation of FA kills bacteria remains unclear, even though a cytotoxic parameter affecting bacterial viability after UV-A exposure was demonstrated in those previous reports [4,10].

Therefore, in the work presented here, our aim is to assess the inactivation mechanism responsible for photobactericidal activity of the combination of FA and UV-A. A set of associated changes in cellular functions of *E. coli*, namely, respiratory activity, lipid peroxidation, membrane permeability, and damage to DNA and the cell

surface by UV-A disinfection combined with FA were measured. The results of this study can aid in developing an UV-A disinfection technology in combination with FA for applications in the food industry.

2. Materials and methods

2.1. Chemistry

FA was purchased from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). An FA stock solution was prepared at 200-fold of the assay concentration by dissolving the compound in 80% dimethylsulfoxide (DMSO). All other experimental materials were purchased from commercial sources.

2.2. Light source and irradiation

All photobactericidal experiments were performed using a device equipped with a UV-A LED (NCSU033B; Nichia Corp., Anan, Japan), as previously described [4]. The wavelength range was 350-385 nm and the peak was 365 nm. The irradiance was adjusted by changing the distance of the sample from the LED; irradiance was measured

using a laser power and energy meter (Nova II; Ophir Optronics Solutions, Ltd., Saitama, Japan) equipped with a photodiode sensor (PD-300-UV; Ophir Optronics Solutions, Ltd.). All bactericidal assays with or without UV-A exposure were performed at 30°C in an incubator box, using bacterial suspensions in plastic petri dishes (90 mm × 15 mm, Ecopetri ELS; Sansyo Co., Ltd., Tokyo, Japan) or in 48-well culture plates (AGC Tecno Glass Co., Ltd., Tokyo, Japan). For the petri-dish assay, the bacterial sample dish was placed on a magnetic stirrer (to provide continuous stirring) and 4 LEDs were positioned over the dish at a distance of 30 mm. The total irradiance was 18 mW/cm^2, based on an irradiance of 4.50 mW/cm^2 per LED. In the case of assays using 48-well culture plates, one LED was positioned below the plate at a distance of 23 mm, providing an irradiance of 8.58 mW/cm^2. The total fluence was calculated by multiplication of the irradiance by exposure time (sec) and expressed in units of J/cm^2.

2.3. Photobactericidal assay

All bactericidal assays were performed with *E. coli* NITE Biological Resource Center (NBRC) 12713; source and culture conditions were as described previously [4]. Bactericidal activity against NBRC 12713 was determined by counting of the number of colony-forming units (CFU) as reported previously [4]. The activity was expressed as

the log survival ratio, such that the log survival ratio = log (N_t / N_0), where N_0 is the initial colony count before bactericidal treatment and N_t is the colony count after the treatment for time t.

E. coli suspensions were prepared as described previously [4] in sterile ion-exchanged water or Dulbecco's phosphate-buffered saline (D-PBS(-), abbreviated hereafter as PBS; Nacalai Tesque, Inc., Kyoto, Japan) at the prescribed cell density, and then transferred to 48-well plates at 0.995 mL/well. A stock solution of FA was added to the bacterial suspension by 200-fold dilution to the prescribed concentration before initiating UV-A irradiation. To determine viable cell counts after treatments with or without UV-A irradiation, aliquots (0.15 mL) of each suspension were diluted 10-fold with SCDLP broth (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) followed by serial 10-fold dilutions in 0.8% (w/v) physiological saline containing 0.7% (w/w) Tween 80 (Kanto Chemical Co., Inc., Tokyo, Japan). Aliquots (0.1 mL) of each dilution were applied to SCDLP agar (Nihon Pharmaceutical Co., Ltd.) plates and incubated at 37°C for 48 h. For samples exposed to UV-A irradiation alone (without FA) and control samples (in the absence of both FA and irradiation), DMSO was added to a final concentration of 0.4%.

2.4. Photobactericidal assay against E. coli pretreated with FA

Photobactericidal activity following FA pretreatment (permitting adsorption and incorporation of FA into the bacterial membrane) was investigated by a modification of the previously published method [6]. An aliquot of an *E. coli* suspension (0.995 mL, approximately 2×10^8 CFU/mL) was transferred to a 1.5-mL microtube and FA was added to a final concentration of 1 mg/mL. The mixture was incubated for 10 min in the dark and then subjected to centrifugation (6500*g*, 3 min, 20°C). The resulting bacterial pellet was resuspended in 1 mL of sterile water and then diluted approximately 100-fold in a petri dish to yield a 10-mL suspension at $3\text{-}4\times10^6$ CFU/mL. The suspension was exposed to a total irradiance of 18 mW/cm^2 for 37 min (40 J/cm^2 as a total fluence). Simultaneously, samples for FA treatment alone (without UV-A), UV-A irradiance alone (without FA), and control (in the absence of both FA and irradiation) were prepared as described above. Bacterial viability then was measured as described above.

2.5. Staining assay for flow cytometry

The 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining assay (Bacstain-CTC Rapid Staining Kit; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was conducted to examine respiratory activity after treatment with FA. An *E. coli* suspension

(approximately 3×10^8 CFU/mL in PBS) was treated with 1 mg/mL FA for 1.9 min in the dark and then subjected to centrifugation (6500*g*, 3 min, 20°C). The resulting cell pellet was washed twice with PBS and then diluted 100-fold in PBS. After dual staining with CTC and DAPI (Bacstain DAPI solution at 1 mg/mL; Dojindo Molecular Technologies, Inc.), the stained cells were analyzed using a FACS Verse (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer.

The change in membrane permeability was assessed by measuring emission of red fluorescence by propidium iodide (PI). A suspension of *E. coli* (approximately 3×10^8 CFU/mL in PBS) was distributed to the individual wells of a 48-well culture plate and treated with a combination of FA (1 mg/mL) and UV-A (2 and 8 J/cm^2). Following treatment, the suspension was diluted 100-fold into 1 mL of PBS. The diluted suspension was stained with DAPI solution (1 μL) and PI solution (1 μL of Bacstain PI solution at 1 mg/mL; Dojindo Molecular Technologies, Inc.). The resulting mixture was subjected to FC analysis. Assay samples included cells treated with FA alone and cells treated with UV-A alone (8 J/cm^2). For the FA-alone sample, cells were subjected to a 15.5-min incubation in the dark, an interval corresponding to the exposure time of cells irradiated with UV-A at 8 J/cm^2. Both samples were analyzed by FC as above.

The fluorescence levels were expressed as arbitrary units (a.u.) normalized to the mean fluorescence values of control samples. For all staining assays, aliquots of the cell

suspension after each treatment were spread on SCDLP agar plates to determine viable cell counts.

2.6. Lipid peroxidation

Lipid peroxidation in *E. coli* cells was determined using the Fluorescence TBARS Microplate Assay Kit (Oxford Biomedical Research, Rochester Hills, MI, USA) as described previously [12]. Briefly, cell suspensions (2.0×10^9 CFU/mL in PBS) were distributed to a 48-well culture plate; FA was added to a final concentration of 0.1 mg/mL and the mixture was exposed to UV-A irradiation (1.0 J/cm^2) for 1.9 min. For comparison to the experimental (combined) treatment, cells treated with UV-A or FA alone were prepared in parallel. Control samples were generated by adding an equivalent volume of DMSO 0.4% in PBS and incubating them for 1.9 min in the dark. Cells were collected by centrifugation as above and lipid peroxidation of the cells was measured. Additionally, aliquots of the cell suspension after each treatment were spread on SCDLP agar plates to determine viable cell counts and survival ratios.

2.7. Scanning electron microscopy (SEM)

A suspension of *E. coli* (2×10^8 cells/mL) was treated with FA (1 mg/mL), UV-A (8 J/cm^2), or a combination of the two. Processing of the treated cells and SEM observation using a S-4700 (Hitachi, Japan) were performed as described previously [13].

2.8. Plasmid DNA cleavage in E. coli

DNA integrity was evaluated with the protocol of Liang et al. [14]. The assay was performed using *E. coli* XL1 Blue competent cells (Cosmo Bio Co., Ltd., Tokyo, Japan) transformed with pUC19 plasmid DNA (2,686 bp; Nippon Gene Co., Ltd., Tokyo, Japan). Transformed cells were grown in LB broth (supplemented with 50 μg/mL of ampicillin; to maintain the plasmid selection marker) at 37°C. Cells were collected by centrifugation as above and washed twice with PBS. Washed cells were adjusted to a density of approximately 2.0×10^8 cells/mL in PBS and distributed at 995 μL per well in a 48-well culture plate. FA solution was distributed at 5 μL per well to yield a final FA concentration of 0.5 mg/mL, and suspensions were exposed to UV-A at 2, 4, and 10 J/cm^2. Following irradiation, an aliquot (28 μL) of the contents of each well was combined with 3 μL of 10× Loading Buffer (Takara Bio Inc., Shiga, Japan) followed by

mixing with 30 μL of PCI reagent (Nacalai Tesque Inc.). After vortexing and centrifugation (15000*g*, 5 min, 20°C), 10 μL of the resulting blue aqueous phase was loaded into a well in a 1% agarose gel and subjected to electrophoresis and DNA staining with ethidium bromide. The intensities of the detected DNA bands were determined using a gel imager instrument (Gel Doc^{TM} EZ Imager; Bio-Rad Laboratories, Inc., CA, USA). DNA staining and analysis also were performed for intact plasmid (purified from the transformed cells) and linearized plasmid (digested with *Eco*RI (Takara Bio, Inc.))

Additionally, aliquots of the cell suspensions before and after UV-A exposure were spread on SCDLP agar plates to determine viable cell counts and survival ratios.

2.9. Measurement of DNA oxidation

Oxidation of DNA was quantified using a competitive enzyme-linked immunosorbent assay (ELISA) kit (Highly Sensitive 8-OHdG Check; Japan Institute for the Control of Aging, Nikken SEIL Corp., Fukuroi, Japan) as described previously [12]. This ELISA kit detects 8-OHdG formed by the oxidative reaction of DNA with ROS. Bacterial cells were treated under the same conditions as those used for the membrane permeability assay described above. Control samples were generated by adding an

equivalent volume of DMSO 0.4% in PBS and incubating them for 15.5 min in the dark.

2.10. Statistical analysis

All experiments were performed as three or four independent experiments, and the results are presented as the mean with error bars showing the standard deviation. Statistical analyses were performed using a two-tailed, unpaired Student's *t*-test (Microsoft Excel 2013; Microsoft Corporation, Redmond, WA, USA). *P* values <0.05 were considered significant.

3. Results

3.1. Photobactericidal effect on bacterial cells pretreated with FA

UV-A exposure at 40-J/cm^2 fluence of *E. coli* cells (initial density of 10^6 CFU/mL) pretreated with FA at a concentration of 1 mg/mL yielded a 2.5-log decrease in the viable cell count (Fig. 1). Bacterial cells subjected to the same treatment in the absence of FA exhibited a decrease in viability of only 0.9 logs. Therefore, pretreatment with FA

significantly potentiated the bactericidality of UV-A irradiation (P <0.01). No significant change in viability was seen for cells pretreated with FA but not irradiated, nor in control samples that were maintained for an equivalent interval (37 min) in the absence of both FA and UV-A.

3.2. Effect of FA treatment on respiratory activity in E. coli

Respiratory activity in *E. coli* was assessed using FC after dual staining with CTC and DAPI; tested samples included cells maintained in the dark in the presence or absence of FA. When *E. coli* was incubated with or without FA for 1.9 min, the decrease in viable cell count was negligible, as evidenced by a log survival ratio of close to zero (Fig. 2A). In contrast, when *E. coli* was exposed to FA for 1.9 min, a 0.84-fold decrease in fluorescent intensity was observed (Fig. 2B); this effect was significant compared to the control sample incubated in the absence of FA (P <0.01) (Fig. S1).

3.3. Phototreatment-induced lipid peroxidation

The TBARS assay was used to assess bacterial lipid membrane peroxidation. Figures 2C and 2D show bactericidal activity and malondialdehyde (MDA) equivalents,

respectively, for each treatment. None of the treatments, including incubation with FA at a 0.1 mg/mL for 1.9 min, UV-A exposure at 1.0 J/cm^2, and the combination of the two, exhibited bactericidal activity, as indicated by the negligible changes in viable cell counts compared to initial cell counts. The combination treatment, but not either individual treatment, yielded an increase in MDA equivalents. Specifically, MDA equivalents were 27 ± 4.8 nM with the combination treatment, a significant increase compared to the value of 17 ± 3.2 nM seen in the control sample (in the absence of both FA and irradiation) (P <0.01). On the other hand, MDA equivalents formed by treatment with FA or UV-A alone were 14 ± 2.9 or 11 ± 3.0 nM, respectively, values that were not statistically different from that of the control sample.

3.4. Effects of membrane integrity

The combination of 1 mg/mL of FA and 8 J/cm^2 UV-A exhibited bactericidality, yielding a 4.8-log decrease in the viable cell count (Fig. 2E). As shown in Fig. 2F, this combination yielded a significant 2.5-fold increase in PI fluorescence compared to the control sample (P <0.01), suggesting an increase in membrane permeability. Combination treatment at a lower UV fluence, using 1 mg/mL of FA and 2 J/cm^2 UV-A, provided a smaller decrease in cell viability (0.38-log, P <0.05), but did not yield a

significant change in PI fluorescence compared to the control sample. Similarly, cells exposed to FA alone (and incubated in the dark for an interval equivalent to that used for 8 J/cm^2 UV-A exposure) or treated with UV-A alone at 8 J/cm^2 also showed no significant change in PI fluorescence compared to the control. Neither of these procedures (FA alone or UV-A alone) caused significant changes in viable cells counts. Therefore, a bactericidal activity-dependent increase in fluorescence level was observed only in cells treated with the combination of FA and UV-A (Fig. S2).

3.5. SEM investigation of E. coli cells

SEM images confirmed the photobactericidal activity of the FA + UV-A combination treatment against *E. coli*. Comparatively smooth bacterial surfaces were seen for cells subjected to treatment by 15.5-min incubation with 0.4% DMSO (as a control), 1 mg/mL FA for 15.5 min, or 8 J/cm^2 UV-A light, respectively, as shown in Figures 3A-C. In contrast, combined treatment with FA and UV-A yielded damaged cells, with multiple distinct vesicles (Fig. 3D) and uneven surfaces (Fig. 3E) observed in the micrographs of the cells.

3.6. Cleavage assay of plasmid DNA transformed in E. coli

Photo-induced DNA breaks were investigated by testing the effect of treatment on plasmid DNA transformed into *E. coli*. Disinfection of the transformed cells was observed with UV-A exposure at 2 J/cm^2 in the presence of FA, resulting in a 1.4-log decrease in cell number compared with initial viability (P <0.01; Fig. 4A). When the fluence in the combination treatment was elevated above 2 J/cm^2, the bactericidal effect was significantly elevated (P <0.05). At the maximum tested UV-A fluence of 10 J/cm^2, the viability was decreased by 4.8 logs compared to the initial viability. While the viability decreased gradually as UV-A fluence increased from 2 to 10 J/cm^2, the supercoiled plasmid DNA bands from cells subjected to each of these fluences did not exhibit significant differences (corresponding to lanes 4-7 in Fig. 4C), as confirmed by band image analysis (Fig. 4B). Lane 4 in Fig. 4C shows the band of intact supercoiled plasmid DNA extracted from *E. coli* prior to UV-A irradiation. Lanes 5-7 show the DNA bands after the treatment with each of these fluences (2-10 J/cm^2).

3.7. DNA oxidation

Formation of 8-OHdG, a marker of DNA oxidation, was assessed as a marker of DNA damage using cells subjected to each treatment (Fig. 5). As shown in Fig. 5, the

8-OHdG oxidation product was detected at 3.38 ng/mL in the control sample (15.5-min incubation in the dark), and at 3.24 ng/mL in cells treated with UV-A irradiation alone (15.5 min at 8 J/cm^2); this difference was not significant (P >0.05). FA treatment alone for a 15.5-min incubation yielded a significant decrease in the 8-OHdG level (1.87 ng/mL) compared to the control sample (P <0.05). When cells were subjected to combined disinfection (UV-A at 8 J/cm^2 in the presence of FA at 1 mg/mL), 8-OHdG was detected at 2.55 ng/mL. The 8-OHdG concentration was a significant elevation compared to the concentrations detected for cells treated with FA alone (P <0.05). However, the concentration with the combination treatment was lower than those of the control and UV-A treatment-alone samples, despite the 4.8-log decrease in cell viability seen with the combination treatment (Fig. 2E). These results suggested that the degree of DNA oxidative damage did not correlate with the bactericidal effect of the combination procedure.

4. Discussion

This study tested the inactivation mechanism of the combination of FA with UV-A irradiation by investigating various cellular parameters, including effects on cell viability, respiratory activity, lipid peroxidation, membrane permeability, cell surface

damage, and DNA damage.

The bactericidality based on photo-irradiation has been suggested to result from the generation of ROS by photo-oxidation of polyphenolic compounds; phenolic acids, including FA, and polyphenols [5,6,8,15]. Hydroxyl radical is one of the primary agents causing oxidative damage to bacterial cells, given that the hydroxyl radical is a more-highly reactive oxidative agent than is hydrogen peroxide. The highly reactive nature of the hydroxyl radical is derived from its very short half-life (estimated to be around 10^{-9} sec) and the very close diffusion distance (estimated to be around 6 nm) [16,17]. The highly oxidative reaction, which is believed to be responsible for the photobactericidality of FA, is presumed to occur at the surface or the inside of bacterial cells.

In the present study, to evaluate the photoinactivation mechanism, *E. coli* cells were treated with FA at a concentration of 1 mg/mL (except for the measurement of lipid peroxidation), because the minimum inhibitory concentration of FA was more than 1 mg/mL against *E. coli* [4] and the cell viability decreased significantly as UV-A fluence increased from 2 to 8 J/cm^2 in the combination with 1 mg/mL FA, as shown in Fig. 2E.

To confirm the mechanistic importance of the adsorption or incorporation of FA into bacterial cells, *E. coli* cells were pretreated with FA at a concentration of 1 mg/mL for 10 min in the dark. The cells then were harvested and resuspended with sterile water at

a 100-fold lower density, such that FA would be retained only if adsorbed onto the bacterial cells or incorporated into the bacterial membrane. Results of this experiment showed that the cells pretreated with FA were eliminated after UV-A exposure. This observation suggested that the bactericidal action of the combined treatment requires that FA is adsorbed onto or incorporated into the bacterial membrane, facilitating effective oxidative damage to cells upon UV-A irradiation.

FC analysis was further applied to evaluate FA's action on bacterial membranes. Bacterial cells with respiratory activity reduce the CTC substrate to yield the red-fluorescing lipophilic compound CTC-formazan, which accumulates within bacterial membranes [18]. A 2-hour incubation with resveratrol, an analogue of FA, at the MIC has been shown to cause a significant decrease in *Arcobacter* respiratory activity, detected as a decrease in the count of CTC-stained cells by FC analysis [19]. This decrease in respiratory activity further suggests a function of resveratrol in the bacterial membrane, given that the CTC reduction reaction occurs in the electron transport system *via* oxidative phosphorylation carriers localized to the cytoplasmic membrane. The application of 1 mg/mL FA against *E. coli* resulted in a reduced growth rate compared to a control grown in the absence of FA (Fig. S3). Thus, treatment with the concentration of FA appears to affect some cell functions. Indeed, exposure to 1 mg/mL FA for only 1.9 min caused a significant decrease in the CTC fluorescence level

(0.84-fold). The results of our CTC analysis implied that this lowering of respiratory activity is caused by adsorption and incorporation of FA on/into the bacterial membrane, suggesting that the photobactericidal activity of FA functions initially at the bacterial membrane. The fluorescence decrease observed during the short incubation period presumably reflects the high affinity of FA for the bacterial membrane. This affinity also may facilitate more efficient reaction of ROS with membrane components (e.g., lipid peroxidation), leading to the first oxidative damage to bacteria under UV-A irradiation.

Previous work has used the TBARS assay to demonstrate that the uptake of ZnO and TiO_2 nanoparticles into bacterial cells induces an increase in the formation of MDA, a marker of lipid peroxidation. The increase in MDA was consistent with the generation of intracellular ROS [20]. If ROS are generated within the membrane where both the incorporation of FA and the absorption of UV-A energy by FA molecules occur, lipid peroxidation is expected to initiate at the location at which unsaturated lipid molecules are available. Treatment with the combination of FA and UV-A yielded extensive peroxidation, even with a short incubation time with the low-fluence UV-A (1 J/cm^2), yet this combination showed little bactericidal activity. These results indicated that lipid peroxidation is the first photoreaction mediated by FA, suggesting that the propagation triggers disturbances of membrane and cellular functions, eventually leading to cell death.

While the combination of FA and UV-A showed a high photobactericidal activity, yielding a 4.8-log reduction in viable cell count, a significant increase also was seen in PI fluorescence. The corresponding red dye is known to enter cells with permeabilized cytoplasmic membranes and then to intercalate with dsDNA [21]. This observation is consistent with an increase in membrane permeability that would be lethal to cells. In *E. coli*, UV-A light has been reported to cause permeabilization of the cytoplasmic membrane, and finally cell death [22]. The effect has been shown to yield an approximately 2-log reduction in viable cell number following illumination with 130 J/cm^2 UV-A, correlating with a 20% elevation in the percentage of PI-stained cells [22]. In the present study, the final stage of inactivation of cellular functions during combined disinfection (FA + UV-A) induced increased membrane permeability, similar to the inactivation mechanism demonstrated with a higher fluence of UV-A light alone. More detailed analyses of the cellular functions that are compromised before the lethal permeabilization event are expected to elucidate the inactivation mechanism responsible for photobactericidal activity of the FA + UV-A combination. Notably, this combination system permits loss of membrane integrity by irradiation at the relatively low fluence of 8 J/cm^2; the level of membrane damage observed here was comparable to that seen by Berney et al. with high-fluence UV-A [22].

To determine whether the photoactivation of FA resulted in a collapse of bacterial

structure, we used SEM to observe cell structure in bacteria subjected to combination treatment with FA + UV-A. In SEM micrographs, the combination of FA and UV-A produced uneven cell surfaces on which multiple vesicles had formed. This phenotype was similar to that observed following exposure of bacterial cells to a quaternary ammonium salt [23]. The vesicle formation and the destruction of the cell surface after treatment with the combination of FA and UV-A in the present work was inferred to correspond to the increase in membrane permeability detected by FC analysis of PI-stained cells. Direct damage to the cell membrane could result not only from oxidative stress but also from photoisomerization of FA to the *cis*-isomer. Notably, *cis*-cinnamic acid, an analogue of FA, is isomerized by UV exposure, and this isomer has been shown to cause a disruption in the bacterial cell wall [24].

To further characterize the mechanism of photoactivation of FA, we next investigated other bactericidal roles of ROS in photobactericidal systems. Specifically, the photoactivation of riboflavin by blue light has been shown to destroy intracellular supercoiled plasmid DNA in a manner that was dependent on irradiation time [14]; the authors of that study proposed that the riboflavin intercalated into DNA, such that photoactivation yielded ROS generation in proximity to the DNA. In the present work, we investigated whether the structure of intracellular plasmid DNA was destroyed after treatment with the combination of FA and UV-A. Notably, plasmid DNA recovered after

UV-A irradiation of FA-exposed cells still exhibited a supercoiled structure, although transformed cells exposed to UV-A showed an irradiation time-dependent decrease in viability. Results similar to those described here were previously reported for ZnO and Ti_2O nanoparticles. Specifically, the oxidative activity of ZnO and Ti_2O nanoparticles did not cause DNA fragmentation (as assessed by agarose gel electrophoresis) in *E. coli* even at nanoparticle concentrations sufficient to generate ROS in the cells [20]. In the present study, FC analyses using CTC and PI stains and SEM imaging suggested that photoactivated FA functions at the cytoplasmic membrane. Therefore, the integrity of plasmid DNA observed in cells subjected to the combined treatment, under conditions that nonetheless yielded high photobactericidality, indicated that lethality does not result from DNA damage. Indeed, even if the intact plasmid DNA, purified from transformed *E. coli*, was dissolved in 0.5 mg/mL FA and exposed to UV-A at 10 J/cm^2, no damage to the DNA structure was observed in comparison to a pre-irradiation sample (Fig. S4). Instead, we infer that photoactivation of FA causes fatal oxidative damage to the bacterial membrane, for which FA possesses high affinity; damage to the bacterial membrane clearly precedes any potential destructive activity by FA against DNA. The photobactericidal system employing FA was expected to exhibit low genotoxicity, given the low affinity of FA for nucleic acids. The low genotoxicity predicted for FA was consistent with the minimal oxidative DNA damage observed in the present work,

where the level of 8-OHdG seen with the combination of FA and UV-A was lower than that seen in control cells, even under conditions that yielded a 4.8-log decrease in viability. Thus, this mechanism differs from the photobactericidal mechanism of riboflavin and polyphenols such as CA [6,14].

Treatment with polyphenolic compounds combined with UV-A or violet light may be useful for the improvement of photo-disinfection technology. In the present study, it was suggested that FA is adsorbed onto or incorporated into bacterial membrane. Thus, the high affinity of FA for bacterial membrane can facilitate the accumulation of FA in microorganisms. The organic composites, microorganisms and polyphenols photo-irradiated artificially, may turn into pollutants which cause the death of organisms and the changes in soil environmental, which even cause great threats to human health through the food chain. Compound contamination in soil is a serious problem which attracts increasing attention on a global scale [25]. In future, it is important to assess the biological safety of the organic composites after disinfection by photo-combination with polyphenols.

In conclusion, our findings confirmed that FA adsorbed onto and was incorporated into bacterial membranes, and that this affinity plays an important role in the photobactericidal activation of FA upon UV-A exposure. The rapid interaction, related to FA's affinity for the bacterial membrane, resulted in decreased respiratory activity and

enhanced lipid peroxidation in the cytoplasmic membrane, even under conditions that did not significantly impair viability. Subsequent amplified generation of ROS by continued UV-A photoactivation of FA resulted in acute damage to the bacterial membrane, as evidenced by increased permeability and the formation of an uneven cell surface, subsequently leading to cell death. The oxidative activity of photoactivated FA may be exerted completely at the cytoplasmic membrane, before the DNA suffers significant oxidative damage. These results suggest that the disinfection activity of the combination of FA and UV-A derives from high cytotoxicity rather than from genotoxicity.

Conflict of Interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Author Contributions

A. Shirai designed this work and the two authors including Y. Yasutomo collected the data; A. Shirai wrote the manuscript. All authors have approved the manuscript.

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Fig. S1. Dual-parameter dot plots of CTC-stained cells evaluated by flow cytometry after incubation with 0.4% DMSO (control, A) or 1 mg/mL FA (B) for 1.9 min. The lower panel is a histogram showing the distribution of CTC fluorescence intensity in treated cells (C): solid line, control; dotted line, FA treatment.

Fig. S2. Dual-parameter dot plots of PI-stained cells evaluated by flow cytometry after incubation with 0.4% DMSO (control; A), 1 mg/mL FA (B), 8 J/cm^2 UV-A (C), or the combination of FA and UV-A (D). The lower panel is a histogram showing the distribution of PI fluorescence intensity for cells subjected to each of the treatments (E): solid line, control samples; dotted line, FA treatment; long-dash dotted line, UV-A; long-dash line, combination of FA and UV-A.

Fig. S3. Effect of FA on the growth curve of *E. coli* at 37°C; in the presence of FA at a concentration of 1 mg/mL (dashed line) or DMSO 0.4% (without FA; control; solid line). Growth was monitored by measuring optical density at 650 nm (OD_{650}) every 5 min in a 96-well culture plate using a plate reader (Multiskan FC, Thermo Fisher Scientific, Inc.). The bacteria were cultivated in the plates in LB broth (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Shaking of the 96-well plate was paused every 10 sec during OD_{650} measurements. The

data are presented as the means of values obtained in two independent experiments (n = 2).

Fig. S4. Agarose gel electrophoresis of the pUC19 plasmid DNA: Lane 1, 20 kb DNA marker; lane 2, supercoiled plasmid DNA (intact); lane 3, linearized plasmid DNA (following digestion with restriction enzyme); lanes 4-7, plasmid DNA exposed to FA and irradiated with UV-A at 0, 2, 4, and 10 J/cm^2, respectively. The effect of the combination of FA with UV-A on intact plasmid DNA, purified from transformed *E. coli*, was assessed according to the previously described method [14].

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Fig. 1. Bactericidal activity of a 10-min pretreatment with FA against *E. coli*. Each cell suspension (initial density, 3-4×10^6 CFU/mL) was pretreated with FA or 0.4% DMSO without FA and then exposed at 40 J/cm^2 fluence UV-A or stored in the dark for an equivalent interval (37 min). Data are presented as mean ± SD (n = 3). Significant differences (**P <0.01; two-tailed, unpaired t test) are indicated relative to viability after incubation in the dark without FA (control sample).

Fig. 2. Analysis of cellular functions of *E. coli* after each treatment with three indicators. Figures A, C and E present the inactivation levels of *E. coli* after each treatment. (B) Intracellular CTC fluorescence level. (D) Lipid peroxidation; lipid peroxidation was determined by the TBARS assay and is presented as MDA equivalents per 10^9 cells. (F) Intracellular PI fluorescence level. Fluorescence levels for each treatment were expressed as a.u. of mean fluorescence, evaluated from flow cytometry analysis, compared to the value obtained from each control sample. All experiments were done at least three times. *P <0.05 and **P <0.01 (by two-tailed, unpaired t test) compared to each control sample. Where mean and error bars are not apparent, values are smaller than the graph scale.

Fig. 3. SEM of cells after each treatment: A, control (0.4% DMSO) for 15.5 min; B, 1 mg/mL FA for 15.5 min in the dark; C, 8 J/cm^2 UV-A; and D and E, the

combination of FA and UV-A. The multiple lumps on the cell surfaces (panel D) indicate membrane vesicles. All panels are micrographs at a magnification of × 30k. White scale bars represent 1 μm.

Fig. 4. Effects of FA treatment on *E. coli* transformed with the pUC19 plasmid: A, inactivation level; B, the intensity of bands of plasmid DNA extracted from the cells, evaluated from DNA-staining assay, compared to values obtained from samples before UV-A exposure; C, agarose gel electrophoresis of the plasmid DNA extracted from the cells treated under each condition. Lane 1, 20 kb DNA marker; lane 2, supercoiled DNA (intact); lane 3, linearized DNA (following digestion with restriction enzyme); lanes 4-7, plasmid DNA recovered from cells that had been exposed to FA and irradiated with UV-A at 0, 2, 4, and 10 J/cm^2, respectively. *P <0.05 and **P <0.01 (by two-tailed, unpaired t test) by comparison as indicated. The data are presented as mean ± SD (n = 3). Where error bars are not apparent, values are smaller than the graph scale.

Fig. 5. Changes in DNA oxidation of *E. coli* after each treatment. Different letters (a, b, and c) above the columns refer to significant differences (P <0.05 by two-tailed, unpaired t test) between different groups. The data are presented as mean ± SD (n = 4).

Highlights

- Bactericidal action of combined ferulic acid and ultraviolet-A was investigated.
- Respiratory activity was decreased by uptake of ferulic acid into bacterial membrane.
- Short UV-A exposure for the combination facilitated lipid peroxidation.
- The combination enhanced permeability and cell damage during UV-A irradiation.
- No genotoxicity, DNA oxidation, or DNA strand breaks were seen for the combination.

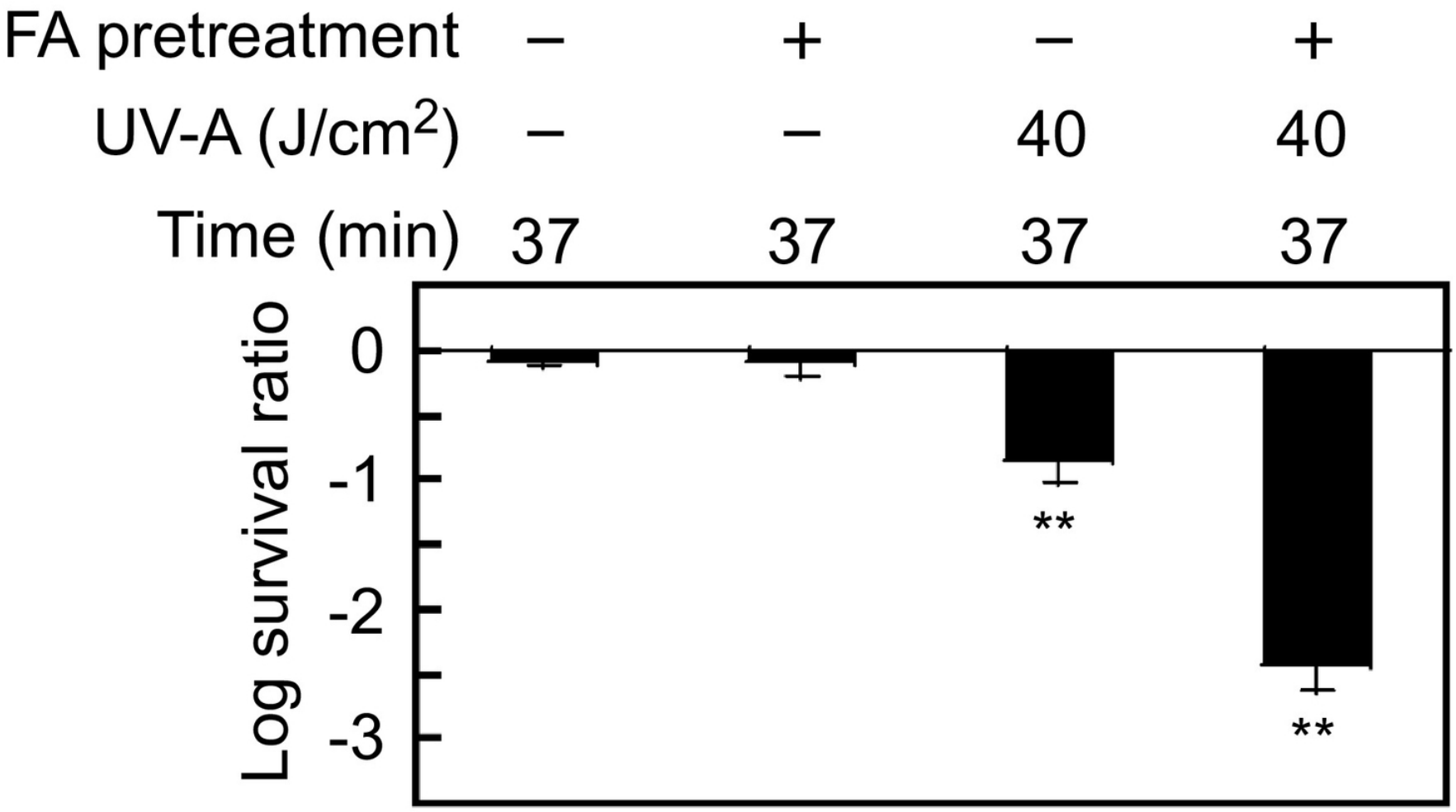
FA pretreatment – + – +
UV-A (J/cm2) – – 40 40
Time (min) 37 37 37 37
Log survival ratio
0
-1
-2
-3
**
**

Figure 1

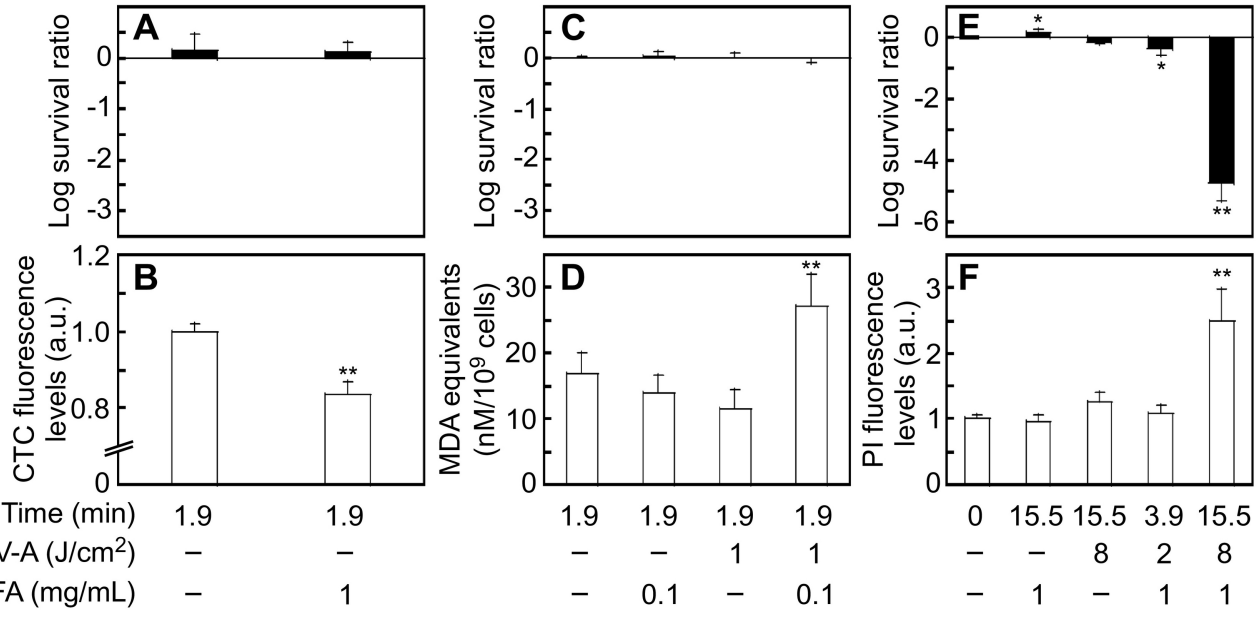
A
Log survival ratio
0
-1
-2
-3
B
CTC fluorescence levels (a.u.)
1.2
1.0
0.8
0
**
C
Log survival ratio
0
-1
-2
-3
D
MDA equivalents (nM/10^9 cells)
30
20
10
0
**
E
Log survival ratio
0
-2
-4
-6
*
*
**
F
PI fluorescence levels (a.u.)
3
2
1
0
**
Time (min) 1.9 1.9 1.9 1.9 1.9 0 15.5 15.5 3.9 15.5
UV-A (J/cm2) – – – – 1 1 – – 8 2 8
FA (mg/mL) – 1 – 0.1 – 0.1 – 1 – 1 1

Figure 2

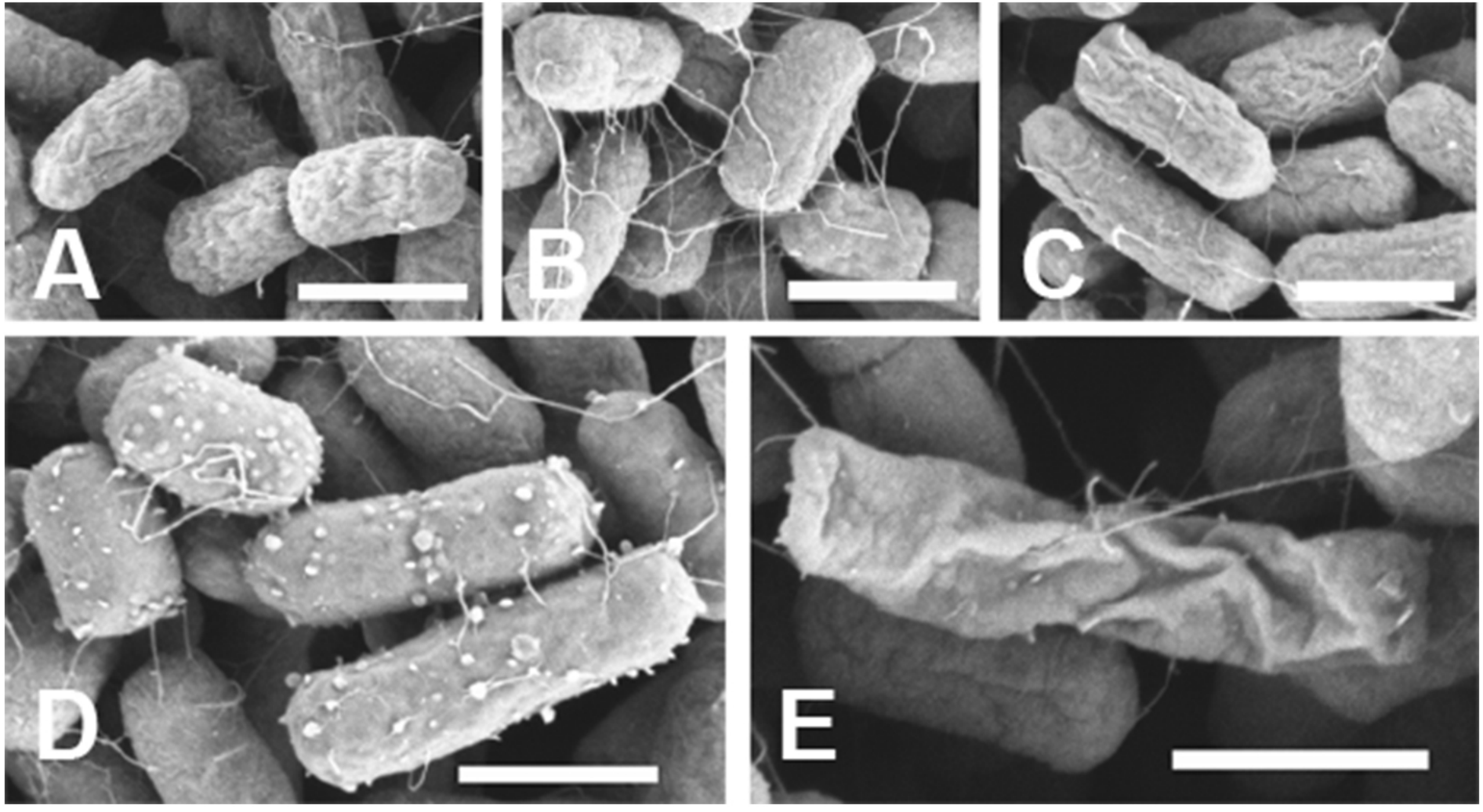

Figure 3

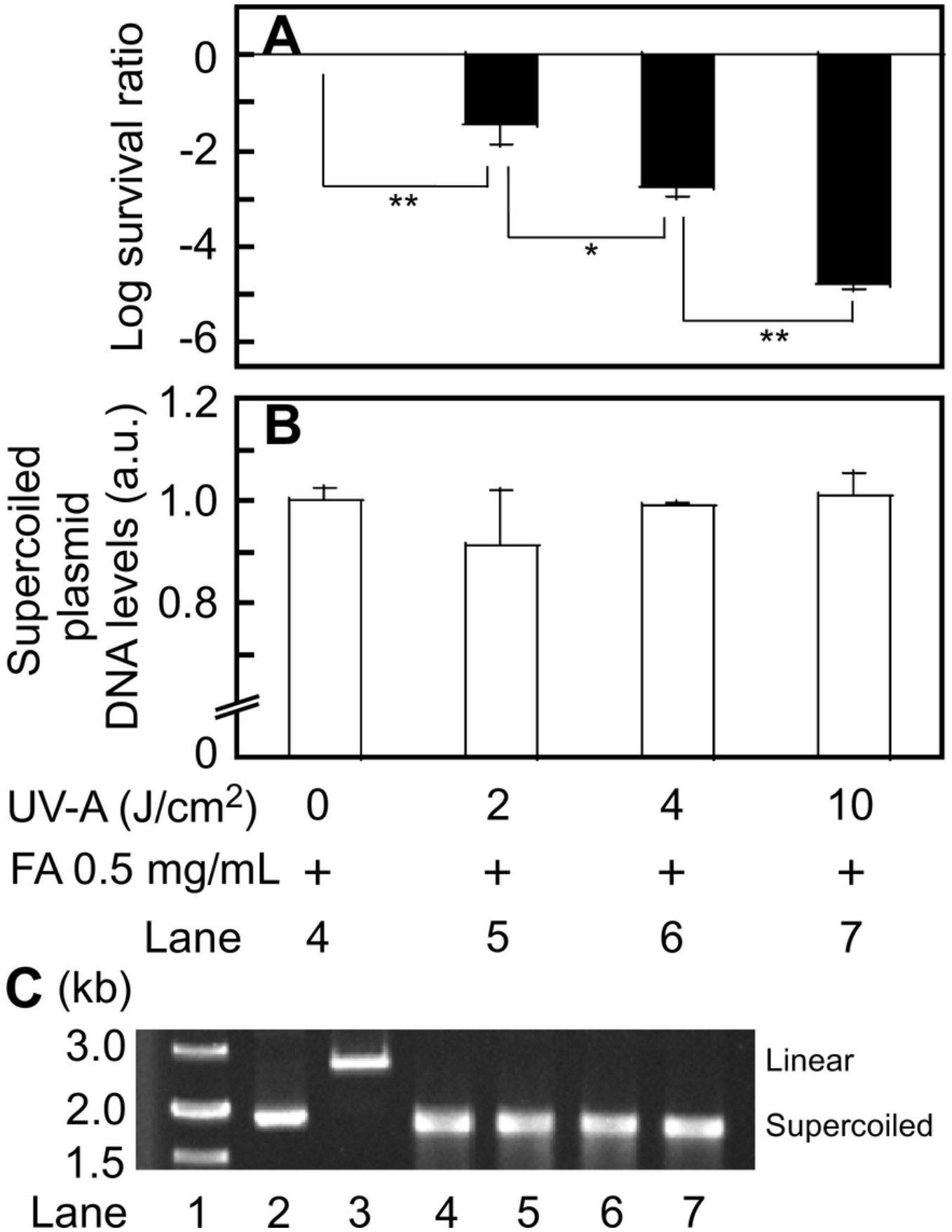
A
Log survival ratio
0
-2
-4
-6
**
*
**
B
Supercoiled plasmid DNA levels (a.u.)
1.2
1.0
0.8
0
UV-A (J/cm2) 0 2 4 10
FA 0.5 mg/mL + + + +
Lane 4 5 6 7
C (kb)
3.0
2.0
1.5
Linear
Supercoiled
Lane 1 2 3 4 5 6 7

Figure 4

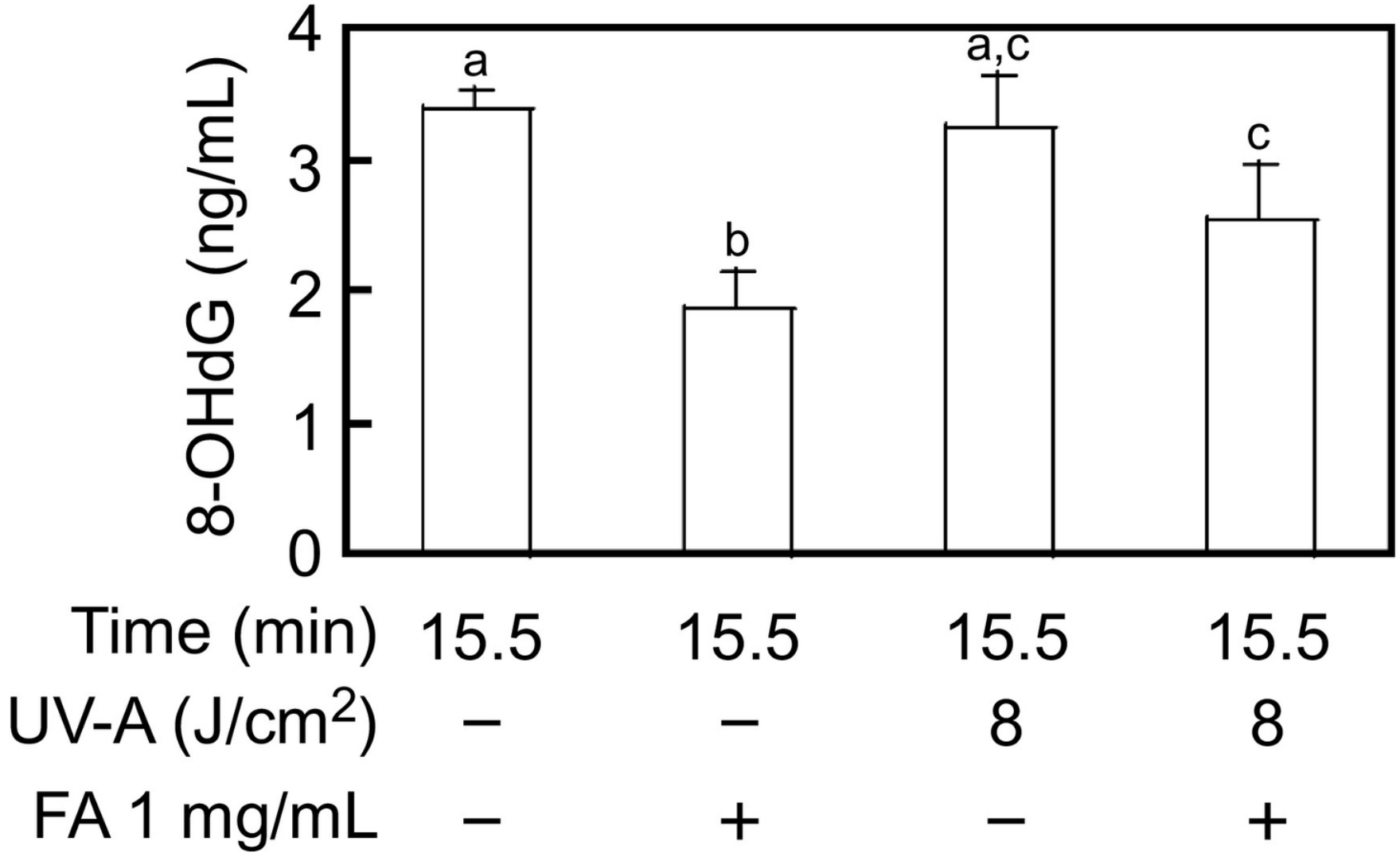
8-OHdG (ng/mL)
4
3
2
1
0
a
b
a,c
c
Time (min) 15.5 15.5 15.5 15.5
UV-A (J/cm2) – – 8 8
FA 1 mg/mL – + – +

Figure 5