Journal of Radiation Industry 15 (2) : 93 ~ 98 (2021) https://doi.org/10.23042/radin.2021.15.2.93

Lon Protease is Needed to Resume Growth of *Escherichia coli rpoH* Mutant Following Gamma Radiation

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Abstract - Heat shock proteins (HSPs) such as chaperones and proteases are upregulated to ensure correct protein folding and to prevent the formation of toxic protein aggregates in response to heat shock or other insults that increase cellular protein misfolding. In *Escherichia coli*, which has been used as a model organism in research in microbial genetics and physiology, HSPs are induced by temperature upshift from 30°C to 42°C, and their induction is regulated by the alternative sigma factor, σ^{32}, encoded by the *rpoH* gene. The observation that post-irradiation incubation of *E. coli* at temperatures higher than 37°C resulted in increased survival rates has led to the proposal that HSPs are required to survive stress from ionizing radiation. We have investigated this hypothesis by examining the effects of *rpoH* mutation on cell survival and post-irradiation recovery. The wild-type *E. coli* strain (WT) exposed to gamma radiation (γ-radiation) exhibited a clear decrease in survival relative to non-irradiated counterparts, but there was no significant difference in the survival outcomes between WT and an *rpoH* mutant strain (Δ*rpoH*). When cells irradiated with doses of 1, 2, and 3 kGy were re-inoculated into fresh media, the lag phase of Δ*rpoH* became longer than that of WT in a dose-dependent manner. Expression of the heat shock protease Lon could partially restore the growth defect observed in irradiated Δ*rpoH*. These findings suggest that Lon may play a role in the post-irradiation recovery period by degrading its primary substrate SulA, which inhibits cell division.

Key words : Gamma radiation, *Escherichia coli*, *rpoH*, Growth resumption, Lon protease

INTRODUCTION

Bacteria live in environments that undergo changes in temperature, pH, nutrients, and oxidation, and hence various global regulatory networks have evolved to cope with these environmental stresses. One of the most important and also first discovered regulatory systems is the heat-shock response, which is a fundamental response of living cells and is conserved practically amongst all living organisms (Craig 1985; Grover 2002). *Escherichia coli*, a rod-shaped Gram-negative bacterium, has been used extensively as a model organism in many areas of biological research. In *E. coli*, the heat shock response to temperature upshift from 30°C to 42°C consists of rapid induction of heat shock proteins (HSPs) (Arsène *et al*. 2000). Many of the HSPs are molecular chaperones including DnaK, DnaJ, GroEL, and GroES, and ATP-dependent proteases including ClpXP, DegP, FtsH, HslUV, and Lon (Arsène *et al*. 2000). These HSPs are important for *E. coli* survival in response to heat stress. DnaK and GroE are the two major chaperone systems in *E. coli* and are involved in protein folding and prevention of protein aggregation (Lund 2001). The heat-induced proteases such as ClpXP contribute to the *E. coli* heat shock response by eliminating damaged cellular proteins (Meyer and Baker 2011).

The synthesis of HSPs is controlled by the heat shock sig-

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ma factor, σ^{32}, encoded by the *rpoH* gene, which guides RNA polymerases to the promoters of heat shock genes (Yura *et al*. 2000). The σ^{32} levels are induced by heat shock and are regulated at the transcriptional, translational, and posttranslational levels (Arsène *et al*. 2000; Schumann 2016). At low temperature (30°C), the intracellular levels of σ^{32} are fewer than 10∼30 molecules per cell due to protein instability and proteolysis: most free σ^{32} molecules are degraded by the FtsH and ClpXP proteases. Upon a rapid shift to 42°C, significant stabilization occurs immediately, resulting in 15∼20-fold increased σ^{32} levels within 5 min (Straus *et al*. 1987; Yura *et al*. 2000; Schumann 2016).

Survival of microorganisms following treatment with ionizing radiation (IR) is dependent on growth conditions after IR treatment. Certain procedures applied after irradiation have been regarded as promoting cell restoration per se. In some investigations in which *E. coli* was used, post-irradiation incubation at temperatures higher than 37°C resulted in increased survival rates (Alper and Gillies 1958). This is in agreement with the observation by Alper (1978), who showed that an incubation temperature of 45°C allowed better recovery of *E. coli* than 37°C. Also, it was found that *E. coli* exposed to gamma radiation (γ-radiation) showed significant induction of the chaperones GroES, GroEL, and DnaK compared with non-irradiated controls (Caillet *et al*. 2008). These previous studies suggest that the *E. coli* heat-shock response, which is characterized by the synthesis of more than 20 HSPs under the control of σ^{32}, plays a role in the restoration of *E. coli* exposed to IR. In this study, we investigated the effect of *rpoH* mutation on the post-irradiation growth of *E. coli* and found that the Lon protease, one of the HSPs, was necessary for the recovery of *E. coli* after irradiation.

MATERIALS AND METHODS

1. Bacterial strains and growth conditions

A wild-type *Escherichia coli* strain MC4100 and its isogenic *rpoH* mutant strain SR701 (Shin *et al*. 2001) were used in this study. The *E. coli* cells were routinely cultivated at 30°C in Luria-Bertani (LB) broth containing 1% tryptone, 0.5% yeast extract, and 1% NaCl. A culture that had been grown overnight was used as the seed culture, and the seed culture was inoculated into fresh LB broth at a 1 : 100 dilution. Ampicillin (100 $\mu g \cdot mL^{-1}$) was used when necessary.

2. Cell growth and survival assays

E. coli cultures grown to the stationary phase with shaking were subjected to irradiation and then re-inoculated into fresh LB broth at a 1 : 50 dilution immediately. Cell growth was monitored over time by measuring the optical density at 600 nm (OD_{600}) of the broth culture with a spectrophotometer (GeneQuant *pro* UV/Vis spectrophotometer; Biochrom Ltd., Cambridge, UK). For a survival assay, irradiated cells were serially diluted tenfold in saline (0.85% NaCl) and then spotted on LB plates. The plates were incubated for 48 h prior to enumeration of colonies. The survival rates were determined by dividing the number of colonies of irradiated cells by the number of colonies of non-irradiated cells.

3. Irradiation

E. coli cultures were irradiated at room temperature using a cobalt-60 gamma irradiator (AECL, IR-79; MDS Nordion International Co. Ltd., Ottawa, Canada) at the Advanced Radiation Technology Institute in the Republic of Korea. The source strength was approximately 233 kCi at a dose rate of 50 $Gy \cdot min^{-1}$, and the actual doses were within ± 2% of the target dose. The applied doses of irradiation were 0.5, 1.0, 1.5, 2.0, and 3.0 kGy.

4. Plasmid construction

The *lon* gene was PCR amplified from *E. coli* MC4100 genomic DNA using the forward (5′-tgt cat ctg ggt acc tgg cgg aaa-3′) and reverse (5′-ggc aag ccc gaa ttc gcc tgc cag-3′) primers, which were designed to include BamHI and EcoRI sites, respectively. The amplified product was digested with the restriction enzymes and then ligated into a pEGFP plasmid (CLONTECH Lab., Palo Alto, CA, USA) carrying the *lac* promoter/operator for gene expression. The resulting plasmid, named pLon, was transformed into the wild-type and *rpoH* mutant strains.

RESULTS AND DISCUSSION

1. *rpoH* mutation delays growth resumption after irradiation

First, we examined γ-radiation resistances of the *E. coli* wild-type (WT) and *rpoH* mutant (Δ*rpoH*) strains. Follow-

ing irradiation, cells were diluted and spotted on plates to measure the survival fraction. WT showed a clear decrease in survival relative to non-irradiated cells in a dose-dependent manner: doses of 0.5, 1.0, 1.5, and 2.0 kGy resulted in approximately 0.6-log, 2.2-log, 3.1-log and 3.8-log unit reduction in survival, respectively (Fig. 1). No significant difference was observed in survival rates between WT and Δ*rpoH* under the experimental conditions tested here (Fig. 1). However, we observed that the growth of Δ*rpoH* was delayed compared with that of WT after γ-radiation treatment. WT colonies were noticeably formed after overnight incubation regardless of γ-radiation treatment, but visible colonies of irradiated Δ*rpoH* were observed later than their WT counterparts (data not shown).

Thus, we monitored cell growth of WT and Δ*rpoH* by measuring the OD_{600} values over time after irradiation. The timing of growth resumption was delayed in irradiated WT relative to non-irradiated cells (Fig. 2). For instance, the growth of WT exposed to doses of 2 and 3 kGy resumed at approximately 8 h and 13 h post-inoculation, respectively (Fig. 2), which is likely attributable to the decrease in inoculum size by irradiation (Fig. 1). When irradiated, Δ*rpoH* showed a longer lag phase after inoculation into a fresh medium and reached the stationary phase at a lower optical density compared with WT (Fig. 2). The duration of the lag period was markedly longer in Δ*rpoH* than in WT at 2 and 3 kGy doses of γ-radiation (Fig. 2). This longer lag phase is not associated with the inoculum size because parallel determination of viable counts showed that the cell viability of Δ*rpoH* was uncompromised as compared with WT (Fig. 1).

Exposure of living systems to IR induces the immediate formation of reactive oxygen species (ROS), including superoxide ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH \cdot$), etc., which result in oxidative damage in various cellular compartments such as DNA, proteins, and lipids (Riley 1994). To prevent the harmful effects of such ROS, the expression levels of antioxidant proteins are upregulated in response to the oxidative threat. These include not only ROS scavenging enzymes, such as catalase, superoxide dismutase (SOD), and thioredoxin-associated proteins but also heat shock proteins such as DnaJ, DnaK and GroES (Greenberg and Demple 1989; Delaney 1990; Han and Lee 2006; Yang *et al*. 2014). Therefore, it is reasonable to assume that IR can induce the *E. coli* chaperone proteins via ROS generation, which is supported by the IR-dependent induction of GroES, GroEL, and DnaK in *E. coli* (Caillet *et al*. 2008). UV radiation is also capable of inducing GroEL and DnaK in a σ^{32}-dependent manner (Krueger and Walker 1984). Although it has not been known that the IR-dependent chaperone induction requires σ^{32}, the chaperones likely help protect cells against protein aggregation during irradiation and/or the post-irradiation recovery period. A mutation in *rpoH* caused a prolonged lag phase after irradiation without compromising survival outcomes under the experimental conditions tested (Figs. 1 and 2).

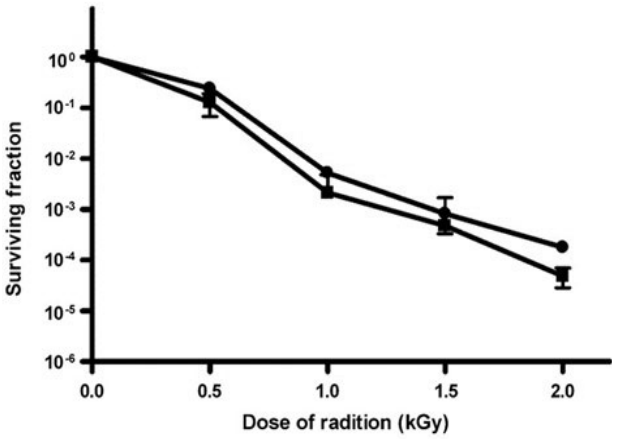

Fig. 1. Survival curves for *E. coli* after irradiation. The wild-type (●) and *rpoH* mutant (■) strains grown to the stationary phase in the LB medium were exposed to doses of 0.5, 1.0, 1.5, and 2 kGy of γ-radiation. Samples were removed from each culture, and serial dilutions were plated to assess survival. Values are means of two independent experiments. Standard deviation bars of the mean are given except where they are smaller than the symbol size.

In *E. coli*, de novo protein folding is mainly performed by three highly conserved chaperone systems: trigger factor (TF), which is the only ribosome-associated bacterial chaperone, DnaK/DnaJ/GrpE system, and GroEL/GroES system (Bhandari and Houry 2015). The GroEL/ES system is essential at all temperatures, but DnaK is required at growth temperatures above 37°C and below 15°C (Bhandari and Houry 2015). The *rpoH* mutant strain SR701 used in this study expresses a high level of GroEL due to a DNA insertion in the *groE* upstream region (Kusukawa and Yura 1988; Kim *et al*. 1999). Although the chaperone systems are not perfectly complementary to each other, the GroEL/GroES system is known to assume a broader role in protein folding in the absence of TF and DnaK (Kerner *et al*. 2005). This suggests that the noticeable growth delay observed in irradiated Δ*rpoH* may not be entirely attributable to dysregulation of chaperone synthesis.

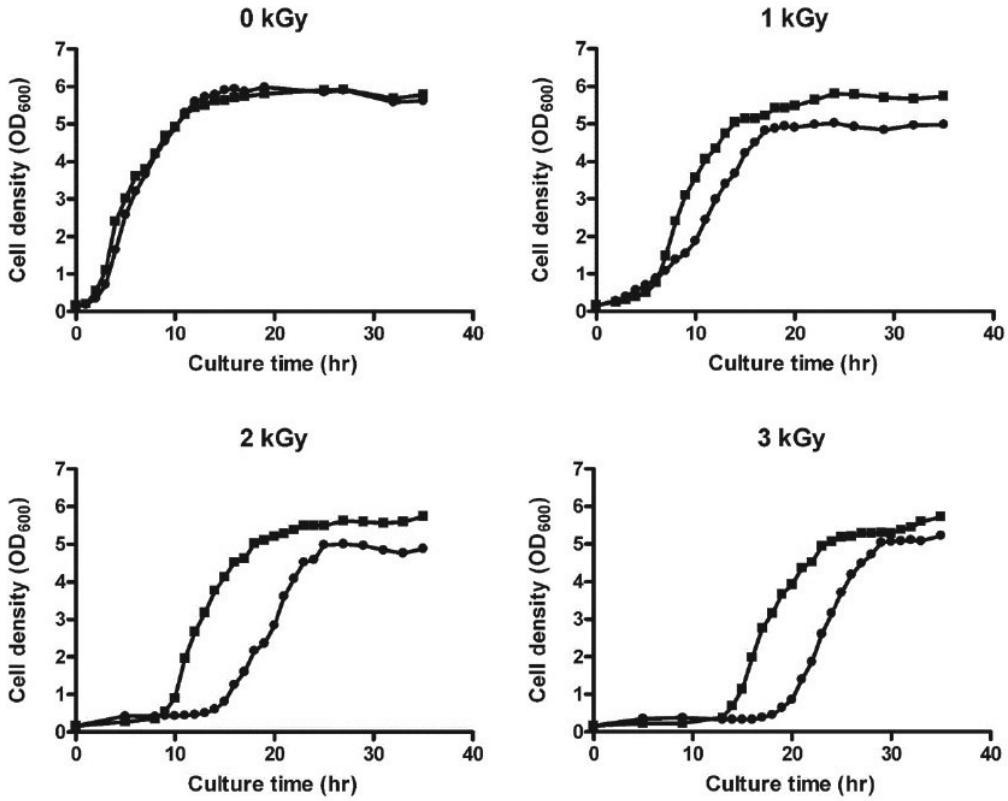

Fig. 2. Growth curves for *E. coli* after irradiation. The wild-type (■) and *rpoH* mutant (●) strains grown to the stationary phase in the LB medium were exposed to doses of 1, 2 and 3 kGy of γ-radiation. After re-inoculation of irradiated or non-irradiated cells at 1 : 50 into a fresh LB medium, samples were harvested at the indicated times from cultures of cells. The results are representative of two independent experiments.

2. Expression of Lon protease partially rescues the impaired growth of Δ*rpoH*

DNA represents the most critical target of IR (Diehl 1995). IR produces a wide spectrum of DNA lesions, such as base and sugar damage and single and double strand breaks (Symons 1994). Since 2- or 3-kGy radiation certainly caused massive DNA damage in the *E. coli* genome, the surviving fractions might need to extend the lag phase, in which DNA replication ceases, to repair DNA damage (Fig. 2). *E. coli* has evolved an inducible system, termed the SOS response, which entails increased expression of over 50 proteins that carry out diverse functions to survive sudden increases in DNA damage (Maslowska *et al*. 2019). Many of the proteins induced as part of the SOS response are unusual substrates for the heat-shock proteases such as ClpXP, HslUV, Lon, etc. (Neher *et al*. 2006). One of those proteins is the division inhibitor SulA, which is synthesized in large amounts during the SOS response and inhibits cell division to give the bacterium time to complete the repairs (Butala *et al*. 2008). After the SOS response is turned off, SulA is degraded by Lon protease, allowing for cell division to resume (Maslowska *et al*. 2019; Burby and Simmons 2020).

The observation that strains with mutations in the *rpoH* gene are defective in proteolysis and cell division (Tsuchido *et al*. 1986; Straus *et al*. 1988) has led us to investigate whether the Lon protease is involved in the growth delay observed in the irradiated Δ*rpoH*. We constructed a plasmid expressing the Lon protein (pLon) and then transformed it into Δ*rpoH* to test whether Δ*rpoH* could be complemented by the plasmid-borne Lon. Providing Lon *in trans* did not fully restore the impaired growth of Δ*rpoH* irradiated at 2 kGy compared with WT but allowed cells to enter logarithmic growth in a shorter amount of time (Fig. 3). While Δ*rpoH* displayed a long lag phase until 13 h of post-irradiation incubation, the Δ*rpoH* harboring pLon restarted cell division within 10 h of post-irradiation recovery (Fig. 3). The *lon* expression had no effect on growth characteristics of WT (data not shown). These findings suggest that the long lag phase of Δ*rpoH* may be due to increased levels of SulA resulting from the low

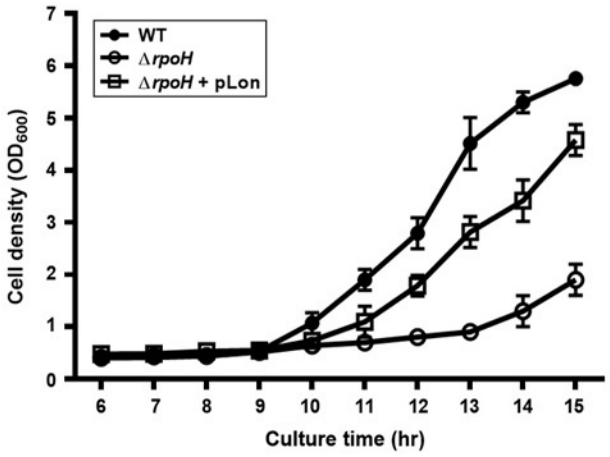

Fig. 3. Growth curves for *E. coli* after irradiation. The indicated strains grown to the stationary phase in the LB medium were exposed to a dose of 2 kGy. After re-inoculation of irradiated or non-irradiated cells at 1 : 50 into a fresh LB medium, samples were harvested at the indicated times from cultures of cells. Values are means of two independent experiments. Standard deviation bars of the mean are given except where they are smaller than the symbol size.

expression of *lon* after irradiation. However, although the evidence that Lon is responsible for the degradation of SulA *in vivo* is convincing, it has been reported that another heat-shock protease, HslUV, is capable of recognizing and degrading SulA and appears to act as a backup for Lon under certain conditions (Wu *et al*. 1999; Burby and Simmons 2020) Further research is needed to estimate the role of each heat-shock protease in post-irradiation recovery of *E. coli*.

CONCLUSION

IR exposure can modify (or damage) cellular macromolecules via both direct ionization and the reactivity of the high-energy ROS originating from water radiolysis (indirect effects of ionization), affecting an estimated 2000 primary ionization events (Reisz *et al*. 2014). Considering this global impact of IR on cells, IR likely triggers multiple signals in the affected cell to adapt to cellular changes, to repair lesions of biomolecules, and ultimately to survive IR. A series of studies examining bacterial responses to IR using high-throughput omics technologies showed that a low dose of γ-radiation (10 Gy) induces typical SOS genes such as *recA*, *sulA*, and *umuD*, but a relatively high dose of γ-radiation (1∼3 kGy) does not. Instead, the high doses appear to activate the oxidative stress responses preferentially (Lim *et al*. 2008; Lim *et al*. 2011; Lim *et al*. 2013). In this study, we showed that the heat shock response also plays a role in *E. coli* in response to IR. The partial overlap between these stress responses has been observed in *E. coli* exposed to $CdCl_2$ (VanBogelen *et al*. 1987). Based on previous and our findings, irradiated cells may prioritize one of the three responses, oxidative stress, SOS, and heat shock responses, and then activate them in a hierarchical way to cope with different damage levels, which are basically proportional to the γ-radiation dose.

ACKNOWLEDGEMENTS

This research was supported by the Nuclear R&D program of the Ministry of Science and ICT (MSIT), Republic of Korea.

REFERENCES

Alper T and Gillies NE. 1958. 'Restoration' of *Escherichia coli* strain B after irradiation: its dependence on suboptimal growth conditions. *J. Gen. Microbiol*. **18**:461-472.

Alper T. 1978. Mechanisms of cell death due to ionizing radiation. pp. 9-29 (Vol 2). *In*: Sterilization of Medical Products by Ionizing Radiation (Gaughran ERL and Goudie AJ eds.), Muitiscience, Montreal.

Arsène F, Tomoyasu T and Bukau B. 2000. The heat shock response of *Escherichia coli*. *Int. J. Food. Microbiol*. **55**:3-9.

Bhandari V and Houry WA. 2015. Substrate interaction networks of the *Escherichia coli* chaperones: trigger factor, DnaK and GroEL. *Adv. Exp. Med. Biol*. **883**:271-294.

Burby PE and Simmons LA. 2020. Regulation of cell division in bacteria by monitoring genome integrity and DNA replication status. *J. Bacteriol*. **202**:e00408-00419.

Butala M, Podlesek Z and Žgur-Bertok D. 2008. The SOS response affects thermoregulation of colicin K synthesis. *FEMS Microbiol. Lett*. **283**:104-111.

Caillet S, Millette M, Dussault D, Shareck F and Lacroix M. 2008. Effect of gamma radiation on heat shock protein expression of four foodborne pathogens. *J. Appl. Microbiol*. **105**:1384-1391.

Craig EA. 1985. The heat-shock response. *CRC Crit. Rev. Biochem*. **18**:239-280.

Delaney JM. 1990. Requirement of the *Escherichia coli dnaK* gene for thermotolerance and protection against H_2O_2. *J. Gen. Microbiol*. **136**:2113-2118.

Diehl JF. 1995. Biological effects of ionizing radiation. pp. 89-

132. *In*: Safety of Irradiated Foods. Marcel Dekker Inc., New York, NY.

Greenberg JT and Demple B. 1989. A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. *J. Bacteriol.* **171**:3933-3939.

Grover A. 2002. Molecular biology of stress responses. *Cell Stress Chaperones* **7**:1-5.

Han M-J and Lee SY. 2006. The *Escherichia coli* proteome: past, present, and future prospects. *Microbiol. Mol. Biol. Rev.* **70**:362-439.

Kerner MJ, Naylor DJ, Ishihama Y, Maier T, Chang HC, Stines AP, Georgopoulos C, Frishman D, Hayer-Hartl M, Mann M and Hartl FU. 2005. Proteome-wide analysis of chaperonin-dependent protein folding in *Escherichia coli*. *Cell* **122**:209-220.

Kim SY, Nam TW, Shin D, Koo BM, Seok YJ and Ryu S. 1999. Purification of Mlc and analysis of its effects on the pts expression in *Escherichia coli*. *J. Biol. Chem.* **274**:25398-25402.

Krueger JH and Walker GC. 1984. *groEL* and *dnaK* genes of *Escherichia coli* are induced by UV irradiation and nalidixic acid in an *htpR*$^{+}$-dependent fashion. *Proc. Natl. Acad. Sci. USA.* **81**:1499-1503.

Kusukawa N and Yura T. 1988. Heat shock protein GroE of *Escherichia coli*: key protective roles against thermal stress. *Genes. Dev.* **2**:874-882.

Lim S, Joe M, Seo H and Kim D. 2013. Induction of SOS genes by a low dose of gamma radiation, 10 Gy, in *Salmonella enterica* serovar Typhimurium. *J. Radiat. Ind.* **7**:109-113.

Lim S, Jung S, Joe M and Kim D. 2008. Gene expression profiles following high-dose exposure to gamma radiation in *Salmonella enterica* serovar Typhimurium. *J. Radiat. Ind.* **2**:111-119.

Lim S, Lee M, Joe M, Song H and Kim D. 2011. Proteome analysis of *Escherichia coli* after high-dose radiation. *J. Radiat. Ind.* **5**:1-5.

Lund PA. 2001. Microbial molecular chaperones. *Adv. Microb. Physiol.* **44**:93-140.

Maslowska KH, Makiela-Dzbenska K and Fijalkowska IJ. 2019. The SOS system: A complex and tightly regulated response to DNA damage. *Environ. Mol. Mutagen.* **60**:368-384.

Meyer AS and Baker TA. 2011. Proteolysis in the *Escherichia coli* heat shock response: a player at many levels. *Curr. Opin. Microbiol.* **14**:194-199.

Neher SB, Villén J, Oakes EC, Bakalarski CE, Sauer RT, Gygi SP and Baker TA. 2006. Proteomic profiling of ClpXP substrates after DNA damage reveals extensive instability within SOS regulon. *Mol. Cell.* **22**:193-204.

Reisz JA, Bansal N, Qian J, Zhao W and Furdui CM. 2014. Effects of ionizing radiation on biological molecules-mechanisms of damage and emerging methods of detection. *Antioxid. Redox Signal.* **21**:260-292.

Riley PA. 1994. Free radicals in biology: oxidative stress and the effects of ionizing radiation. *Int. J. Radiat. Biol.* **65**:27-33.

Schumann W. 2016. Regulation of bacterial heat shock stimulons. *Cell Stress Chaperones* **21**:959-968.

Shin D, Lim S, Seok YJ and Ryu S. 2001. Heat shock RNA polymerase ($E\sigma^{32}$) is involved in the transcription of *mlc* and crucial for induction of the Mlc regulon by glucose in *Escherichia coli*. *J. Biol. Chem.* **276**:25871-25875.

Straus DB, Walter WA and Gross CA. 1987. The heat-shock response of *E. coli* is regulated by changes in the concentration of sigma 32. *Nature* **329**:348-351.

Straus DB, Walter WA and Gross CA. 1988. *Escherichia coli* heat shock gene mutants are defective in proteolysis. *Genes Dev.* **2**:1851-1858.

Symons MCR. 1994. Direct and indirect damage to DNA by ionizing radiation. *Radiat. Phys. Chem.* **43**:403-405.

Tsuchido T, VanBogelen RA and Neidhardt FC. 1986. Heat shock response in *Escherichia coli* influences cell division. *Proc. Natl. Acad. Sci. USA.* **83**:6959-6963.

VanBogelen RA, Kelley PM and Neidhardt FC. 1987. Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*. *J. Bacteriol.* **169**:26-32.

Wu W-F, Zhou Y and Gottesman S. 1999. Redundant in vivo proteolytic activities of *Escherichia coli* Lon and the ClpYQ (HslUV) protease. *J. Bacteriol.* **181**:3681-3687.

Yang K, Yang L and Liu J. 2014. The molecular chaperone DnaJ is involved in the peroxide- induced oxidative stress response in *Escherichia coli*. *J. Exp. Micrbiol. Immunol.* **18**:41-47.

Yura T, Kanemor M and Morita MT. 2000. The heat-shock response: Regulation and function. pp. 3-18. *In*: Bacterial stress responses (Storz G and Hengge-Aronis R eds.), ASM press, Washington.

Received: 1 April 2021
Revised: 28 April 2021
Revision accepted: 13 May 2021