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# Gene Expression Analysis of *Salmonella enterica* Serovar Typhimurium Following Gamma Radiation under Different Oxygen Levels

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**Abstract** Damage to biomolecules by low linear energy transfer (LET) radiation such as gamma (γ) rays is mostly mediated by the indirect action of reactive oxygen species (ROS), hence the biological effect of γ-radiation is enhanced in the presence of oxygen. To analyze changes in gene expression depending on oxygen levels, total RNAs from *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) irradiated with a sublethal dose (100 Gy) were subjected to oligonucleotide microarrays. Under low-oxygen conditions, 124 genes exhibited significant changes in expression, while the number of genes affected by γ-irradiation more than tripled to 378 under high-oxygen conditions, showing the oxygen effect in the cellular response to radiation. Genes belonging to the SOS regulon were up-regulated regardless of oxygen levels, but heat shock proteins were induced preferentially under high-oxygen conditions. This suggests that the extent of protein damage is greater in high-oxygen than in low-oxygen conditions. Of the four prophages, the whole Fels-1 genes were induced by γ-radiation under high-oxygen conditions, which might trigger prophage induction. Most of the down-regulated genes were associated with virulence determinants, such as *Salmonella* pathogenicity island 1 (SPI-1) and flagella, which are required for the invasion and locomotion of *S.* Typhimurium, respectively. Significant repression of the virulence genes was observed in high oxygen conditions. Taken together, these results imply that γ-radiation cannot increase the virulence of surviving *S.* Typhimurium after irradiation, especially when radiation treatment is given under high-oxygen conditions.

**Key words:** Gamma radiation, *Salmonella*, Microarray analysis, prophage, SOS response, Heat shock protein

## 1. INTRODUCTION

Ionizing radiation (IR) can be defined as a flow of subatomic particles (alpha, beta, and neutrons) or electromagnetic waves (X-rays and gamma rays), which are capable of freeing electrons from a substance, causing the substance to become ionized (or charged) [1,2]. When these IRs encounter living cells, cellular macromolecules, such as DNA, lipids, and proteins, are modified or damaged by direct and indirect ionization [1,2]. Direct ionization occurs when charged particles, e.g., electrons, interact with cellular components without an intermediary step, and indirect ionization occurs when non-charged particles, e.g., photons, interact with cellular water, i.e., via the reactivity of the high-energy species originating from water radiolysis [1,2]. Even photons (X-rays and gamma rays) can ionize atoms directly through the Photoelectric effect and the Compton effect, but indirect ionization is much more significant [3].

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The water radiolysis events can be divided into three consecutive stages depending on time scales. During the first "physical" stage ($\leq 10^{-15}$ s), the energy deposition caused by the incident radiation leads to the formation of ionized water molecules ($H_2O^+$), ejected electrons ($e^-$), and excited water molecules ($H_2O^*$) [4-6]. The resulting species are extremely unstable and undergo fast reorganization, such as proton transfer, dissociative electron attachment, electron hydration, etc., in the second "physicochemical" stage ($10^{-15}$~$10^{-12}$ s) [4-6]. These processes produce various chemically reactive species, including hydroxyl radicals (•OH), hydrogen radicals (H•), aqueous electrons ($e^-_{aq}$), hydronium ions ($H_3O^+$), and hydroxide ($OH^-$), which react with each other and also with surrounding molecules in the third "chemical" stage ($10^{-12}$~$10^{-6}$ s) [4-6]. On a quantitative basis, the primary products of water radiolysis in anoxic or hypoxic conditions are radicals ($e^-_{aq}$, •OH, and H•) and molecular products ($H_2$ and $H_2O_2$) [6]. In the presence of oxygen, the reducing equivalents $e^-_{aq}$ and H• are rapidly converted into superoxide and hydroperoxyl ($O_2^{\bullet-}$/$HO_2$•) radicals [5]. It has been suggested that $O_2^{\bullet-}$ might account for the increased sensitivity of cells to damage from electromagnetic radiation in the presence of oxygen [7].

*Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a gram-negative facultative bacterium that can cause food poisoning. Hence, this pathogen has been long subjected to food irradiation to ensure microbiological safety [8-10]. On the other hand, however, *S.* Typhimurium also has served as a model organism for radiobiological studies because this species is related phylogenetically to *Escherichia coli*, which is one of the best-studied prokaryotic model organisms, and they share a large amount of the similar genetic materials [11-15].

In our previous studies, we investigated changes in gene expression in *S.* Typhimurium exposed to high (1 kGy) and low doses (10 Gy) of gamma radiation (γ-adiation) using DNA microarray analysis and found a discrepancy in the gene expression patterns in response to the dose of γ-radiation [16,17]. In this study, we analyzed the gene expression profiles of *S.* Typhimurium exposed to a medium dose (100 Gy) of γ-radiation under high-oxygen (shaking condition) and low-oxygen (standing condition without shaking) conditions using DNA microarrays to identify the effect of oxygen on the γ-radiation response of *S.* Typhimurium.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strains and growth conditions

*Salmonella enterica* serovar Typhimurium LT2 was cultivated at 37°C in a Luria-Bertani medium containing 1% bacto-tryptone, 0.5% yeast extract, and 1% NaCl. A seed culture that had been grown overnight (~14 h) with shaking was inoculated into fresh LB broth at a 1 : 100 dilution.

### 2.2. Irradiation

For irradiation, *Salmonella* cultures were prepared as previously described [16,17]. Fifty mL of cultures grown to exponential phase for 4 h were divided into two 250-mL Erlenmeyer flasks (each flask contains 25 mL) and then irradiated at room temperature under either shaking or standing conditions. The cultures irradiated on a magnetic stirrer for shaking (shaking speed: 250 rpm) were referred to as high-oxygen conditions, and cultures irradiated without shaking were referred to as low-oxygen conditions. Non-irradiated control (0 kGy) samples were handled in the same way except for irradiation. After irradiation, the cells were immediately harvested by centrifugation for RNA isolation. γ-radiation was performed 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 applied dose of irradiation was 100 Gy, and the dose rate was 10 Gy $min^{-1}$.

### 2.3. Oligonucleotide microarray (DNA chip)-based hybridization analysis

The features of the oligonucleotide microarray used in this study were the same as those mentioned in a previous paper [17]. A total of 4,391 gene-specific probes were designed, which cover ~96.2% of 4,463 protein-coding genes on the LT2 chromosome. RNA extraction, RNA labeling, and hybridization onto the microarray were performed as described previously [17]. The total bacterial RNAs were isolated, and then the quality and integrity of the purified total RNAs were confirmed. The prepared RNAs were amplified to generate the labeled cRNA probes and hybridized into the arrays. The hybridizations were conducted in an Agilent Hybridization oven according to the manufacturer's protocol (Agilent Technologies, Inc.). Microarrays' data normalization and statistical analysis were also performed as described previously [17]. After quantification of the signal intensities of each spot in the arrays, the normalization of gene

expression by a LOWESS regression was applied for 3 data obtained from 3 biological replicates. The genes were considered to be differentially expressed when the logarithmic gene expression ratios had more than a 2-fold difference in the expression level. The statistical significance of the data was determined by Student's t-test. *P*-values of less than 0.01 were taken to be statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1. Transcriptomic profiles of irradiated *S.* Typhimurium

To determine the genes whose expression was altered by 100 Gy of γ-radiation, first, the *S.* Typhimurium cells exposed to γ-radiation under low-oxygen conditions were harvested for RNA purification, and then the purified RNAs were subjected to the oligonucleotide microarray. Of a total of 124 genes showing at least a 2-fold change in expression relative to a non-irradiated counterpart, 86 genes were up-regulated and 38 genes were down-regulated by γ-irradiation (Tables S1 and S2). Under high-oxygen conditions, the number of genes affected by γ-irradiation more than tripled to 378, in which 170 and 208 genes were up- and down-regulated, respectively (Tables S3 and S4). This is in accord with the oxygen effect in radiobiology, where oxygen enhances the biological effect of low linear energy transfer (LET) radiations such as γ-ray via interaction with the radiation-induced free radicals [18].

### 3.2. Identification and classification of up-regulated genes

Bacteriophages, the viruses that infect bacteria, can be divided into lytic and temperate virus groups depending on their life cycle [19]. Temperate phages, also named prophages, are bacteriophages integrated into bacterial chromosomes and can propagate lytically under some circumstances [19]. The *S.* Typhimurium LT2 strain used in this

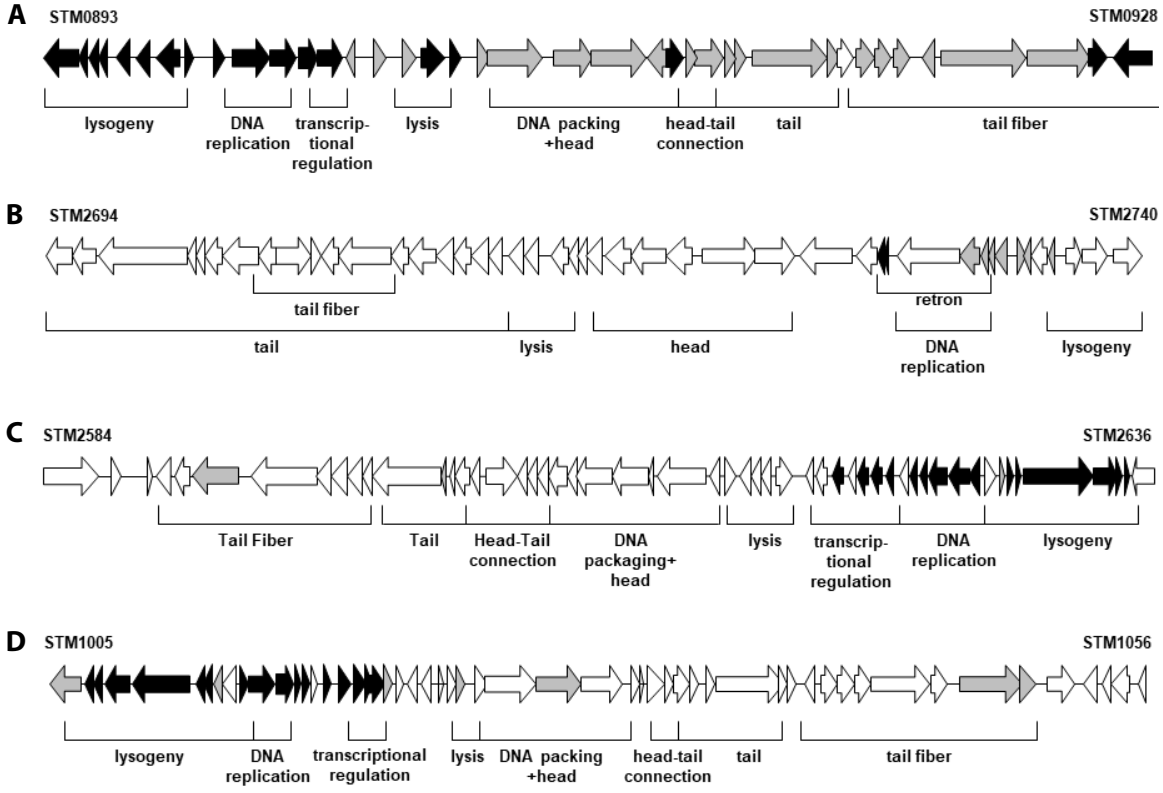


**Fig. 1.** Gene organization of *Salmonella* prophages. Arrow boxes denote the direction of transcription of the genes indicated. The modular structure of the prophages is indicated by the annotated brackets under the prophage genome maps; (A) Fels-1, (B) Fels-2, (C) Gifsy-1, and (D) Gifsy-2. Arrow boxes in black represent genes with high expression in both high- and low-oxygen conditions, and arrow boxes in grey represent genes with high expression in high-oxygen conditions. Locus tags of the first and the last gene of prophages are shown.

study harbors four temperate prophages, Fels-1, Fels-2, Gifsy-1, and Gifsy-2 [20]. About 52% (88 out of 170) and 59% (51 out of 86) of the up-regulated genes observed in high- and low-oxygen conditions, respectively, belonged to the prophage genes (Tables S1 and S3). In addition, most of the highly induced genes with an expression fold-change greater than ten were located on the prophages (Tables S1 and S3). Since the lytic cycle of these phages was induced by DNA-damaging agents hydrogen peroxide and mitomycin C [21], it is reasonable to assume that the genotoxic effect of γ-radiation leads to the remarkable elevation of the prophage gene expression regardless of oxygen tension. The increased expression of these prophage genes was also observed in *S.* Typhimurium exposed to high (1 kGy) and low doses (10 Gy) of γ- radiation [16,17].

Under both high- and low-oxygen conditions, genes in the lysogeny module and the regulatory region controlling phage DNA replication and transcription were commonly up-regulated in Fels-1, Gifsy-1, and Gifsy-2 but not in Fels-2 (Fig. 1). Because Gifsy-1, Gifsy-2, and Fels-1 are lambdoid phages, and Fels-2 is a member of the P2 family of phages [21], the regulatory pathway of Fels-2 for the IR-dependent induction might be different from that of the lambdoid phages. Interestingly, all of the Fels-1 phage genes, including the tail, head, DNA packaging, and assembly genes, were induced by γ-radiation under high-oxygen conditions except for *stm0920* (Fig. 1), suggesting that *S.* Typhimurium can release a functional Fels-1 following γ-radiation treatment under high-oxygen conditions. Because the release of viral particles can kill the host, prophage induction might partly contribute to the increased lethality of *S.* Typhimurium irradiated in the air [22].

The SOS response refers to the production of a large number of enzymes following DNA damage, in which the RecA and LexA proteins play central roles [23]. When DNA damage produces single-stranded DNA (ssDNA), the RecA protein forms RecA-ssDNA filaments that trigger auto-cleavage of the LexA repressor protein [24]. The cleavage of LexA stimulates the expression of genes (called the SOS regulon) that encode enzymes, which are primarily involved in DNA repair [23]. Seventeen genes belonging to the SOS regulon including *recA* and *lexA* were up-regulated by γ-radiation under high-oxygen conditions, and 15 out of the 17 genes, except for *ruvB* and *uvrD*, were detected under low-oxygen conditions (Fig. 2A). Prophage induction is also stimulated by LexA inactivation [23]. Considering that the prophage genes were highly up-regulated by γ-radiation under both low- and high-oxygen conditions (Fig. 1), this result shows that the SOS response is activated by γ-radiation regardless of oxygen tension.

However, genes encoding heat shock proteins (HSPs) were induced preferentially under high-oxygen conditions (Fig. 2B). The HSPs can be grouped into molecular chaperones involved in protein folding and prevention of protein aggregation, and proteases involved in the elimination of damaged cellular proteins [25]. Under high-oxygen conditions, 10 of the 13 genes induced by γ-radiation were genes encoding chaperones, and the others (*hslU*, *hslV*, and *lon*) were genes encoding ATP-dependent proteases (Fig. 2B). This suggests that more protein damage occurs when cells are irradiated under high-oxygen conditions compared with low-oxygen conditions. During irradiation, molecular

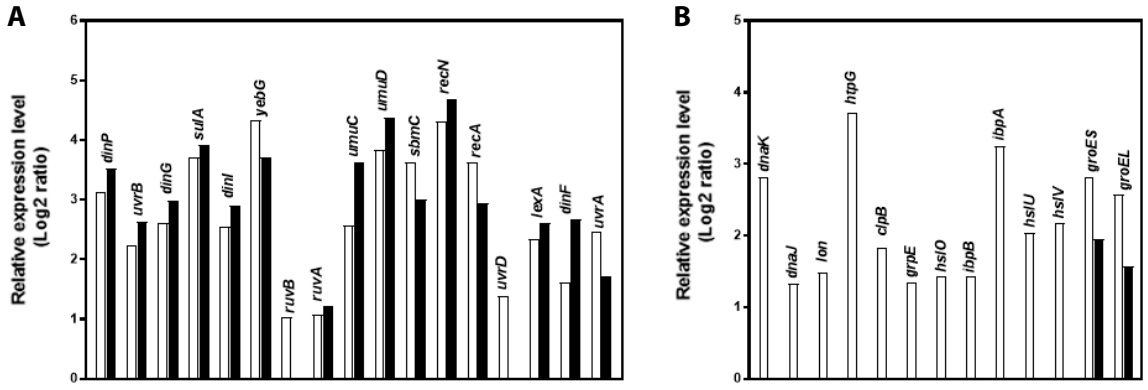


**Fig. 2.** Up-regulation of the SOS regulon (A) and heat shock proteins (B) following γ-radiation. Relative gene expression levels in the microarray analysis are expressed in the $\log_2$ scale. White and black bars represent expression fold changes in high-oxygen and low-oxygen conditions, respectively. Gene name is shown above bars.

oxygen efficiently scavenges $e^-_{aq}$ and forms $O_2^{\bullet -}$ [6], and its scavenging significantly increases $H_2O_2$ formation [26]. $O_2^{\bullet -}$ is relatively inert compared to •OH, but its reactivity is high for selected targets, i.e., small inorganic prosthetic iron-sulfur (Fe-S) groups of some proteins [27]. In the presence of the free iron released from the Fe-S clusters, $H_2O_2$ can additionally produce •OH radicals via Fenton chemistry, which can enhance damage [5]. Hence, the increased levels of $O_2^{\bullet -}$ and $H_2O_2$ might cause higher levels of protein damage, thereby leading to the increased expression of HSP genes under high-oxygen conditions.

### 3.3. Identification and classification of down-regulated genes

The oxygen effect on gene expression change was also observed in the down-regulated genes. It has been reported that the expression of genes within *Salmonella* pathogen islands (SPIs), which are large gene cassettes required for virulence, was reduced by γ-radiation [28]. Of the 36 genes within SPI-1, 34 genes were down-regulated under high-oxygen conditions, while only 3 genes were detected under low-oxygen conditions (Fig. 3A). Reduction of SPI-1 gene expression was observed in *S.* Typhimurium exposed to high levels (2 mM) of $H_2O_2$ [21]. The concentration of $H_2O_2$ increases with an increase in the dissolved oxygen concentration because the radicals $O_2^{\bullet -}$ and $HO_2$• generated in the presence of oxygen recombine to form $H_2O_2$ [29]. The down-regulation of all SPI-1 genes can be attributed to the additional formation of $H_2O_2$ under high-oxygen conditions. Flagella are cell surface appendages involved in a cell's locomotion and are a virulence factor of *S.* Typhimurium [30]. Of more than 50 genes required for the flagellar synthesis, down-regulation of 22 genes was observed only in high-oxygen conditions (Fig. 3B).

A list of the top 10 genes most significantly down-regulated in *S.* Typhimurium exposed to γ-radiation under high-oxygen conditions also included virulence determinants. Because maltose and maltodextrins are present in high concentrations in the intestinal tracts of animals, their transport and metabolism are important for intestinal colonization of *Salmonella* [31]. *lamB*, encoding the specific pore protein for maltodextrins, was the most repressed gene (~10-fold reduction compared with the level of non-irradiated cells). The expression of *malEFGKM* genes encoding the maltose/

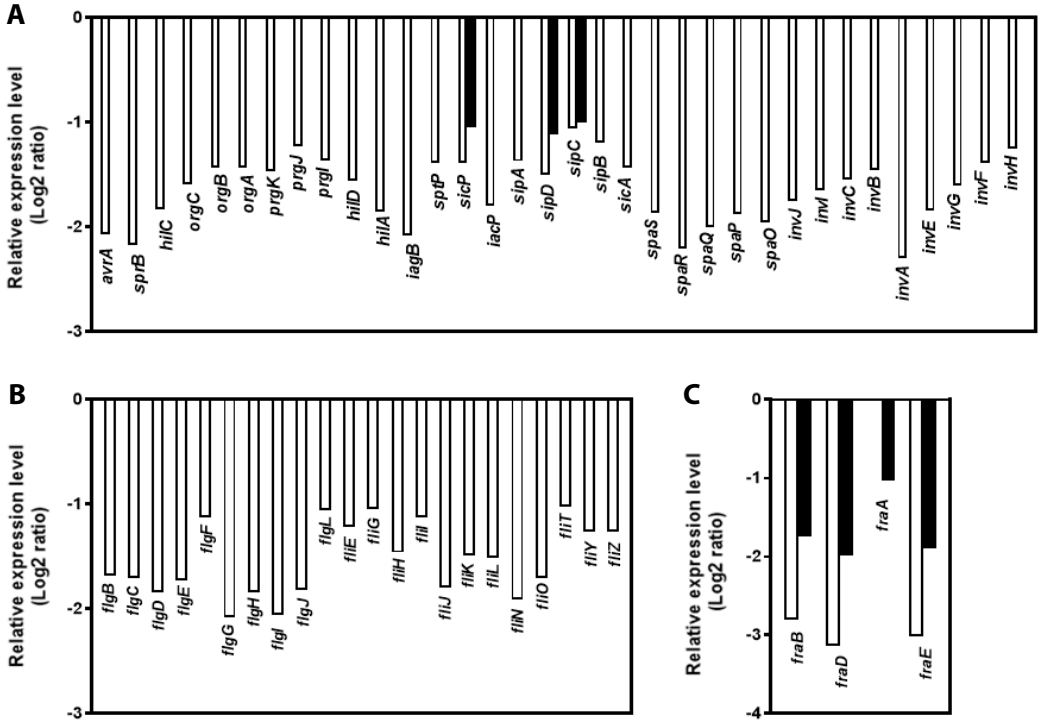


**Fig. 3.** Down-regulation of *Salmonella* pathogenicity island 1 (A), flagella (B), and *fra* genes (C) following γ-radiation. Relative gene expression levels in the microarray analysis are expressed in the $\log_2$ scale. White and black bars represent expression fold changes in high-oxygen and low-oxygen conditions, respectively. Gene name is shown below bars.

maltodextrin translocation complex was also reduced 2- to 5-fold (Table S4). The *fraBDAE* operon encodes proteins required for the utilization of fructose-asparagine, which is the primary nutrient used by *Salmonella* during growth in the inflamed intestine [32]. The *fraBDAE* genes were listed in the top 10 genes exhibiting decreased expression under both low- and high-oxygen conditions (Tables S2 and S4), but they were more greatly reduced under high-oxygen conditions than low-oxygen conditions (Fig. 3C). The *lsr ACDBFGE* operon encodes proteins associated with transport and degradation of small cell-to-cell signaling molecule autoinducer-2 (AI-2). AI-2 is phosphorylated by LsrK and then inactivates the transcriptional repressor LsrR [33]. We observed that all of the *lsr* operon genes were down-regulated under high-oxygen conditions (Table S4), suggesting a low level of internal AI-2 following γ-radiation. Since LsrR relieved from AI-2 inactivation represses not only the *lsr ACDBFGE* operon but also SPI-1 and flagella expression [33], the decreased expression of SPI-1 and flagellar genes observed in high-oxygen conditions may be dependent on LsrR. Taken together, the down-regulation of virulence genes in *S.* Typhimurium irradiated with a non-sterilizing dose (100 Gy) dispels the concern about surviving pathogens with increased virulence after irradiation.

## SUPPORTING INFORMATION

The supplementary tables generated during the current study are available from the corresponding author on reasonable request.

## ACKNOWLEDGMENTS

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