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Transcriptional response of selected genes of *Salmonella enterica* serovar Typhimurium biofilm cells during inactivation by superheated steam

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ABSTRACT

Superheated steam (SHS), produced by the addition of heat to saturated steam (SS) at the same pressure, has great advantages over conventional heat sterilization due to its high temperature and accelerated drying rate. We previously demonstrated that treatment with SHS at 200°C for 10 sec inactivated *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilm cells on the surface of stainless steel to below the detection limit. However, bacteria withstanding heat stress become more resistant to other stress conditions, and may be more virulent when consumed by a host. Herein, we studied the transcriptional regulation of genes important for stress resistance and virulence in *Salmonella* biofilms after SHS treatments. Genes encoding heat shock proteins and general stress resistance proteins showed transcriptional surges after 1 sec of SHS treatment at 200°C, with parallel induction of stress-related regulator genes including *rpoE*, *rpoS*, and *rpoH*. Interestingly, *Salmonella* biofilm cells exposed to SHS showed decreased transcription of flagella and *Salmonella* pathogenicity island-1 (SPI-1) genes required for motility and invasion of host cells, respectively, whereas increased transcription of SPI-2 genes, important for bacterial survival and replication inside host cells, was detected. When the transcriptional response was compared between cells treated with SHS (200°C) and SS (100°C), SHS caused immediate changes in gene expression by shorter treatments. Understanding the status of *Salmonella* virulence and stress resistance induced by SHS treatments is important for wider application of SHS in controlling *Salmonella* biofilm formation during food production.

1. Introduction

Salmonella enterica serovar Typhimurium (hereafter referred to as *S.* Typhimurium) is a leading cause of foodborne illnesses. It provokes salmonellosis with symptoms of diarrhea, nausea, abdominal cramps, and fever, yet most infected persons recover without treatment within 7 days. However, non-typhoidal salmonellosis results in life-threatening diseases in immunocompromised patients, the elderly, and infants, causing 155,000 deaths globally each year (Majowicz et al., 2010). *S.* Typhimurium is a Gram-negative, rod-shaped, facultative anaerobic bacterium, and is able to produce biofilms on biotic or abiotic surfaces (Donlan and Costerton, 2002; Hall-Stoodley et al., 2006).

Biofilms are structured bacterial communities enclosed with polymeric matrices of DNA, protein, and polysaccharides (Stoodley et al., 2002; Sutherland, 2001; Whitchurch et al., 2002), and protect bacterial cells against environmental stresses, detergents, antibiotics, and the host immune system (Bower and Daeschel, 1999; Costerton et al., 1999; Mah and O'Toole, 2001; Yasuda et al., 1994). Accordingly, in terms of food hygiene, biofilms of foodborne pathogens are crucial problems in food processing environments. They may form on a wide variety of abiological surfaces, including stainless steel, polyvinyl chloride, glass, and rubber, which are common materials used in food processing machinery (Prouty and Gunn, 2003; Ronner and Wong, 1993; Ryu et al., 2004), and lead to potential hygiene problems by concomitant bacterial transmission to food products (Shi and Zhu, 2009).

A lot of approaches have been carried out to inactivate biofilms, since conventional methods of controlling planktonic bacteria, including chemical detergents and physical treatments, often prove ineffective. Current procedures to remove biofilms include combinations of mechanical action, such as high pressure, and concurrent application of biocides (detergents (Gibson et al., 1999), matrix-hydrolyzing enzymes (Johansen et al., 1997), and oxidizing substances (Norwood and Gilmour, 2000)). The efficacy of biocides may be enhanced by the use of electric fields (Blenkinsopp et al., 1992) and ultrasound (Mott et al., 1998). However, these methods all have restrictions. High pressure spraying may spread live bacteria over the environment due to

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aerosol generation, and the use of oxidizing substances like chlorine may cause environmental pollution and pose health risks to humans. Furthermore, they are not applicable to high-throughput processing on a large-scale for the food industry.

Recently, we demonstrated that superheated steam (SHS) could be utilized to inactivate biofilms of foodborne pathogens on stainless steel and polyvinyl chloride (PVC) surfaces (Ban et al., 2014). SHS is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure, and hence, transfers a larger amount of heat to the subject of treatment than saturated steam (James et al., 2000; Topin and Tadrist, 1997). Moreover, the SHS process is an energy saving and environmentally friendly technology (Tang and Cenkowski, 2000). With its multiple advantages, SHS has been exploited in the disinfection and sterilization processes for food production (Bari et al., 2010; Phungamngoen et al., 2011).

In this study, the effects of SHS treatment on the virulence and resistance of *Salmonella* Typhimurium were investigated. *Salmonella* cells surviving sublethal injury have been shown to become more resistant to subsequent stress (Pin et al., 2012), increasing their virulence (Sirsat et al., 2011). We aimed to understand the transcriptional responses of *Salmonella* to SHS treatment, to support the use of SHS for biofilm inactivation in food processing facilities.

2. Materials and methods

2.1. Bacterial strains and culture preparation for biofilm formation

The *Salmonella enterica* serovar Typhimurium LT2 (ATCC 19585) strain used in this study was obtained from the bacterial culture collection at Seoul National University (Seoul, Korea). Stock cultures were stored at −80°C in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 15% glycerol. For working cultures, the stock cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37°C for 24 h, and stored at 4°C. For the preparation of *Salmonella* cultures for biofilm formation, bacteria were grown in 10 ml of tryptic soy broth at 37°C for 24 h, collected by centrifugation at 5,000 g at 4°C for 15 min, and washed three times with phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The final bacterial pellets were resuspended in sterile PBS to make approximately 10^7–10^8 colony-forming units (CFU)/ml.

2.2. Biofilm formation

Type 316 stainless steel coupons with No. 4 finish (5 cm × 2 cm) were immersed in 70% ethanol for 60 min to disinfect the surface, and rinsed with sterile distilled water. The washed stainless steel coupons were dried in a laminar flow biosafety hood (22 ± 2°C) for 3 h and sterilized by autoclaving before use. Each prepared stainless steel coupon was submerged in the prepared 30 ml bacterial suspensions (10^7–10^8 CFU/ml) in a sterile 50 ml conical centrifuge tube (SPL Lifesciences, Pocheon, Korea) and incubated at 4°C for 24 h to facilitate bacterial attachment to the surfaces. The coupons were then removed with sterile forceps and rinsed in 300 ml of sterile distilled water (22 ± 2°C) by stirring gently for 5 sec. Rinsed coupons were deposited in 50 ml conical centrifuge tubes containing 30 ml of fresh TSB, then incubated at 25°C for 6 days. This method was adapted from that used by Kim et al. (Kim et al., 2006).

2.3. Saturated steam (SS) and superheated steam (SHS) treatment

Once *Salmonella* biofilms were formed on the stainless steel coupons after incubation for 6 days at 25°C, the coupons were removed and rinsed briefly in 300 ml of sterile distilled water (22 ± 2°C), as described previously, and then exposed to SS or SHS on both sides for 1, 3, 5, 10, and 20 sec, respectively. During the SS or SHS treatments, the temperature was controlled automatically by a temperature sensor and an intelligent power module in the steam generator (Ban et al., 2012, 2014). SS treatments were conducted at 100°C, and SHS treatments were performed at 200°C.

2.4. Enumeration of live bacteria

After SS and SHS treatments, the stainless steel coupons were placed in sterile 50 ml conical centrifuge tubes containing 30 ml of PBS at 25°C and 3 g of sterile glass beads (425–600 μm; Sigma-Aldrich, St. Louis, MO, USA), and then agitated for 1 min with a bench-top vortex mixer set at maximum speed in order to detach cells of the bacterial biofilm from the coupons (Kim et al., 2006). Cell suspensions in the tubes were serially diluted tenfold in buffered peptone water (BPW; Difco), and 0.1 ml of the undiluted cell suspension and diluents was spread-plated onto Xylose Lysine Desoxycholate Agar (XLD; Difco) plates. When low bacterial numbers were anticipated, 250 μl of the respective undiluted cell suspensions was plated onto four agar plates. The plates were incubated at 37°C for 24–48 h, after which the colonies were counted.

2.5. RNA preparation and quantitative real-time PCR (qRT-PCR)

Stainless steel coupons covered with *Salmonella* biofilm cells were submerged in a solution containing PBS and RNAprotect Bacteria Reagent (Qiagen) at a ratio of 1:2 immediately after heat treatments. The bacterial cells were scraped off the stainless steel coupons and collected by centrifugation at 10,000 g for 10 min. Bacterial cell pellets were lysed and processed with an RNeasy mini kit (Qiagen), as per the manufacturer's instructions. Residual chromosomal DNA was then removed with a TURBO DNA-free kit (Ambion), according to the manufacturer's recommendations. RNA was converted into cDNA using RNA to cDNA EcoDry Premix (Clontech), and cDNA corresponding to 10 ng of input RNA was used as a template in each quantitative real-time PCR (qRT-PCR), with SYBR green reagent to detect duplex DNA product (Power SYBR Green PCR Master Mix, Applied Biosystems). Primers specific to target genes were designed using Primer Express Software ver. 3.0 (Applied Biosystems), and their specificity of amplification was verified based on melt curve analysis. Primers used in qRT-PCR are listed in Table 1. qRT-PCR was carried out in 40 cycles of 95°C for 15 sec and 60°C for 1 min, following initial denaturation at 95°C for 10 min using a StepOnePlus real-time PCR instrument (Applied Biosysetms). RNA samples not subjected to cDNA synthesis did not produce significant amplification products in qRT-PCR during the 40 cycles. The expression ratio of each gene was presented as the average from at least three independent RNA samples, and was normalized to the level of *gyrB* (Parsons and Heffron, 2005; Yoon et al., 2009).

2.6. Subsequent heat treatment of SHS- or SS-injured cells

Cells of the biofilms treated with SHS or SS were subsequently subjected to thermal inactivation to measure bacterial resistance to additional heat treatment. Upon SHS or SS treatments, the stainless steel coupons with attached *Salmonella* biofilms were submerged in PBS at 25°C for 5 min, transferred to 50 ml conical centrifuge tubes containing PBS at 45°C, and then incubated at 45°C for 5 min. After additional heat treatments, the stainless steel coupons were immediately shifted to PBS at 25°C, containing sterile glass beads, and were processed for bacterial enumeration as described above.

2.7. Statistical analysis

All experiments were repeated at least three times, with independently prepared samples. Data were subjected to one-way ANOVA and Tukey's post-hoc analysis for statistical analysis. $P < 0.05$ was used to determine significant differences in the analysis.

Table 1
Forward and reverse primers used in qRT-PCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')
groES	CGAAATCATCGCTGTCGGTAA	TTAAAAATAACGATGTCGCCAACTT
groEL	GTTGCAAAAGCAGGCAAACC	GCCACGCATGGTGTTAACAA
clpB	CGGCGGCAACATTATCTCA	CGAATGCTGGATGCAGCTT
yrfH	ATGACGAACGGACGGTGATC	TTTCCGCCGTCTCTTCATACA
ibpA	TATCCCCGCTGTACCGTTCT	GAGGGTAGCCGCCATTACTTT
ibpB	AAACGAGCCCAAATGGTTACA	GCGCCGGAAACTTCCATATT
agsA	GATCCCTGAAAGCGAGAAACC	CGCTCCCATTAACGCGATTA
hslO	TGCCGCAACATGACCAATTA	TCTGTTGCAAGGTTTCCGAAA
danJ	ACGAGATTTTAGGCGTTTCCAA	TGCGGTCCGGATGATATTTC
dnaK	TTGAAGATCTGGTGAACCGTTCT	GGATCACGTCGTTGATATCAGACA
htpG	TCCGGCACCAAATCTTTCC	GGAGTAGAAGCCTACGCCAAAC
rpoE	ATTGAGTCCCTCCCGGAAGA	CACCGGACAATCCATGATAGC
rpoH	GATTTGATCCAGGAAGGCAACA	AATCCAGTGTACGGCGAAAGAA
rpoS	CAGCCGTATGCTTCGTCTCA	TTTTCATCGGCCAGGATGTC
ssaE	CCGCAGCAATATCAGCAAAA	AAGTGCGCTGTTATGGTAACGA
sseA	AAAGGCTGCGTTTAGTGAATATCG	TGACTCACCTTAGCCCGGATT
sscA	GGCTCGCTGCGTATGTTGTT	GCCGGCGAATTCTTTTACCT
ssaG	ATGATTTGCTCAACCCAGAA	TTTAGCAATGATTCCACTAAGCA
ssaH	TTCCCAGGTACATGCGATGTTA	TCATTTAAACCCGCCAACAATA
ssaN	GATGCAACGTCTGAGGCTGAA	GGCAACCACGCATTTAACAA
ssrB	CCGCAGGTGCTAATGGCTAT	TTGGGTCAATGTAACGCTTGTT
invF	ATCGATGGCGCAGGATTAGT	AGCGTTTACGATCTTGCCAAA
hilA	TTACTGTGCGCTGGCAGAAT	CGCCTTAATCGCATGTTCTTT
prgH	CCATGGCGGAGTAAATTTTGA	TTGCACCGAACGAGATTCAG
hilC	AATATTTCACAAGTCGCGACGAT	CGCATAAAGCTAAGCGGTGTAA
hilD	TGAAGAGGTCAATGGCCACAT	TTTGGTTTGCTGCTCGTTTG
spaO	TGCCCGTCAAACTGGAATTT	GCATTGGTCGGCAGTGATAA
sipB	TTGCCGAGGGCGTATTTATTA	GGATTGTTTAAGCCACTGCTGAA
invH	GCTCACCTTCTTCCAGGAACAT	ACTTCCGGGTTCAAGCAAAA
safC	ACTGCGGGATCTGTCGGTAA	GTCTGATACGGCACCACAAACA
safD	GATCCTCGCCATCACATTCAG	CGTTATCTGCAGCGGTTCTTAAA
stbA	CGCGATGGCTGTTTCTGATAA	CACGACGGGTTTAGCCTGATTA
flgB	CGTACCCGATCAGCCTTCTTT	GCCCCATCTGATATTTGAGACTGT
flgF	CAGTAACGTCAAGCCGGTTGA	TCTCATCTACGCTGGTGATAACCTT
flgH	CCGGCGGCAACTCTTTTAA	ATTGGCCAGAACCTGATCGA
fliF	CGCCGTGGTACATCTGGTTT	TGGATTGCGTGAGCAGATGA
fliL	AGCCGTCTGCTGTTGTTGTTTT	AGTGTCTCTTTAATGGCGGCAAT
lsrK	GCGGAGGATCTAAAGGCAAAT	TAGCGCGGTAGCTTCTTTGAC
lsrR	TGATGCCGCTTGTAGCGTAA	CACATCCCGCACGCTATTTT
lsrA	GAAATTATGCGCGGCTTGA	GACGCGTAAATAACCGATCAGTT
lsrC	GCACTGCTGGCAATAGTCTGTT	TTTGCGCGCTACTGAAAACC
lsrD	TGGCTTTCGGTCTGATTAATCC	GTTAGCGGCAAAGCGACAAT
lsrB	AAAGCGTATCCCGATCTGGAT	TCGCGAGATTATTGCGTTTAAG
lsrF	CCAAGACCAGAAAAACGGTCAT	AAGAGCGGCGCAATATTGAT
lsrG	TTTCACAAGACAACGCCACACT	AAGGCATCAAACCCATAAAAACTT
gyrB	GGCTACAGCAAAAAAGCCAAA	GGAGAATTTCGGATCCGGTACT

3. Results and discussion

3.1. Viability of Salmonella biofilm cells under superheated steam treatments

Our previous study showed that 10 sec SHS treatments at 200°C were sufficient to cause at least 4.76 Log_{10} reduction in the number of live *Salmonella* biofilm cells on stainless steel surfaces (Ban et al., 2014). Short processing times are advisable to provide cost-effectiveness and high quality food products. However, we were concerned about the circumstances wherein SHS may be applied unevenly on the surfaces of machinery or food material, due to cavities and curvature, and the SHS treatments may be executed insufficiently for shorter than 10 sec or at lower than 200°C. Therefore, the viability of *Salmonella* biofilm cells under improper SHS treatments was evaluated by exposing the bacterial biofilms on stainless steel coupons to SHS at 200°C for shorter periods. While SHS treatments of 3 sec or longer exhibited superior inactivation effects on *S.* Typhimurium, reducing live bacterial numbers 10^4-fold or more, SHS treatments of 1 sec only caused decreases of approximately 1.5 Log_{10} CFU/coupon, which were comparable with the effects of treatment with saturated steam (SS, 100°C) for 1 sec, and were insufficient for bacterial disinfection (Fig. 1). This result suggests that a large number of biofilm cells may withstand improper SHS treatments

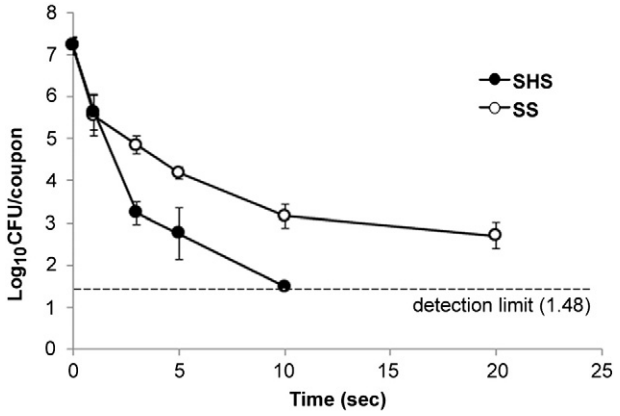

Fig. 1. Survival of *Salmonella* biofilm cells on the surface of stainless steel coupons after superheated steam (SHS) and saturated steam (SS) treatments. *S.* Typhimurium biofilm cells formed on stainless steel coupons (7.22 ± 0.21 Log_{10} CFU/coupon) were exposed to SHS (200°C; closed circle) or SS (100°C; open circle) for 1, 3, 5, 10, and 20 sec. The number of live bacteria after the heat treatments was enumerated and plotted. *Salmonella* cells exposed to SHS for 20 sec were inactivated below the detection limit of 1.48 Log_{10} CFU/coupon, and the result is not shown.

of 1 to 2 sec, and regrow after the SHS treatments cease. Sirsat et al. (2011) and Pin et al. (2012) showed that *Salmonella* previously exposed to heat stress conditions (42–45°C) changed their transcriptional patterns to become more resistant to heat stress. We therefore investigated the transcriptional changes occurring in *Salmonella* during SHS treatments.

3.2. Transcriptional surge of heat shock and stress resistance genes by superheated steam

When bacteria are exposed to heat stresses, they activate the expression of more than 20 genes encoding chaperones, proteases, and transcriptional regulators and stabilize heat-denatured proteins through the heat shock-associated proteins, improving the bacterial resistance to environmental challenges such as heat stresses (Chuang and Blattner, 1993). The bacterial heat shock response is implicated not only in heat shock stress, but also in a variety of unfavorable conditions including oxidative stress, high osmolarity, nutrients starvation, and hostile host environments (Groisman and Saier, 1990; Kusukawa and Yura, 1988; Morgan et al., 1986; Volker et al., 1992).

In order to understand the response of *Salmonella* to extreme heat stresses, transcription of 14 genes involved in heat shock response was compared between SHS treated and untreated bacteria by quantitative real-time PCR (qRT-PCR). The transcription of most heat shock stress-responsive genes was increased by 1 sec SHS treatment but declined with longer SHS treatments (Fig. 2). Two regulatory genes, *rpoE* and *rpoH*, which encode sigma factors σ^{24} and σ^{32}, respectively, showed similar transcriptional surges after 1 sec of SHS treatment, in parallel with general heat shock genes. Concurrent transcriptional induction of *rpoE* and *rpoH* following the temperature upshift agreed with the fact that RpoE is required for *rpoH* induction in response to high temperatures (Erickson et al., 1987; Wang and Kaguni, 1989). The transcription of *rpoS*, encoding a sigma factor involved in general stress responses including nutrient starvation, was not increased by short exposure to SHS (Fig. 2). The heat shock genes analyzed include *groEL*, *groES*, *dnaK*, and *dnaJ*, coding for major folding chaperones, *agsA*, *ibpA* and *ibpB*, encoding small heat shock proteins, *clpB* for a stabilizing chaperone, *hlsO* for oxidative stress-related Hsp33, *yrfH* for Hsp15 with DNA and RNA binding activity, and *htpG* belonging to the σ^{32} regulon (Jenkins et al., 1991). Interestingly, the treatment with saturated steam (SS) at 100°C did not cause immediate transcriptional increases of the heat shock genes in *Salmonella* biofilm cells (fold changes of 0.9415 ± 0.287 in 11 heat shock genes by 1 sec SS treatments); rather, longer SS treatments

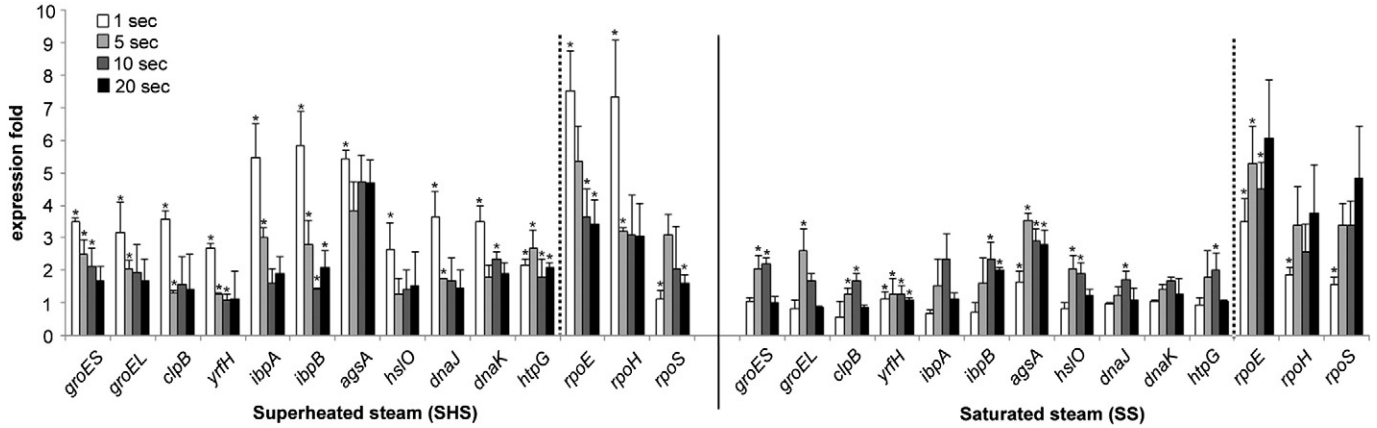

Fig. 2. Expression of heat shock-responsive genes after SHS and SS treatments. *Salmonella* biofilms formed on stainless steel coupons were treated with SHS or SS for 1, 5, 10, and 20 sec, and the total RNA was isolated to compare the transcriptional levels of heat shock-responsive genes. Expressions of 11 heat shock and 3 sigma factor genes after SHS and SS treatments were relatively quantified in comparison to the untreated biofilm control cells using qRT-PCR. Values were normalized to the *gyrB* mRNA levels, and represent the average results from three independent biological samples. Genes showing significant differences in mRNA levels between the heat treatments and the untreated control were denoted by asterisks ($P < 0.05$).

tended to result in more transcription of the heat shock stress-responsive genes (Fig. 2). The prompt transcriptional changes upon exposure to SHS were observed in other genes as well, as described below.

3.3. Vulnerability of SHS-treated Salmonella to subsequent heat stress

Salmonella is a mesophile and shows comparable growth rates between 30 and 42°C (Sirsat et al., 2011). Sublethal stresses leading to the induction of stress-responsive genes enable *Salmonella* to resist incoming stressors (Kwon et al., 2000). Pin et al. (2012) demonstrated that genes coding for products involved in heat shock response were induced by sublethal heat stress at 45°C, with induction maintained for 30 min after the stress ceased, increasing the *Salmonella* resistance to subsequent thermal stress at 50°C. The increased transcription of heat shock-responsive genes by SHS treatments might affect *Salmonella* resistance and enable *Salmonella* to resist subsequent heat stress. In order to examine the possibility, cells from the *Salmonella* biofilms pretreated with SHS or SS for 1 and 3 sec were exposed to heat stress at 45°C for 5 min in succession, and the viable cells were enumerated (Fig. 3). Cells from control biofilms which were not subjected to SHS or SS treatments antecedently showed 1.3 ± 0.04 Log_{10} CFU/coupon decreases in survival under the thermal stress at 45°C for 5 min. However, the biofilm cells that survived 1 sec SHS treatments exhibited more significant reductions of 2.6 ± 1.02 Log_{10} CFU/coupon in survival by additional heat treatment at 45°C for 5 min. The different resistance to heat stress between SHS-pretreated and untreated biofilm cells indicates that *Salmonella* biofilm cells pretreated with SHS were more sensitive to mild heat stresses than naïve cells which were not exposed to heat before. Furthermore, the decreased survival expressions after additional heat treatments were comparable between the 1 sec SHS- and 1 sec SS-pretreated biofilm cells, showing decreases of 2.6 ± 1.02 and 1.9 ± 0.32 Log_{10} CFU/coupon, respectively. This result was not accordant with the qRT-PCR data, which indicated that SHS induced transcriptional surges in the heat shock and stress resistance genes within 1 sec. The short interval of 5 min prior to subsequent thermal stress may not have been long enough for *Salmonella* to produce sufficient products needed for protein stabilization and DNA repair against the mild heat stresses. However, in terms of bacterial disinfection, this result suggests that short SHS treatments of 1 to 2 sec, complemented with sequential mild heat treatments, can be an effective alternative to long SHS treatments of 10 sec or more, which may lead to the deterioration of food quality when SHS is applied to food products for sterilization. The vulnerability of the SHS-pretreated cells to subsequent heat stresses did not agree with the increased heat-resistance of *Salmonella* by sublethal heat pretreatments at 45°C (Pin et al., 2012). This difference might be attributable to the different heat pretreatment conditions leading to lethal (200 or 100°C in this study) or sublethal (42 or 45°C in others) injuries. The former kills bacteria, whereas the latter adapts bacteria to hostile thermal stresses.

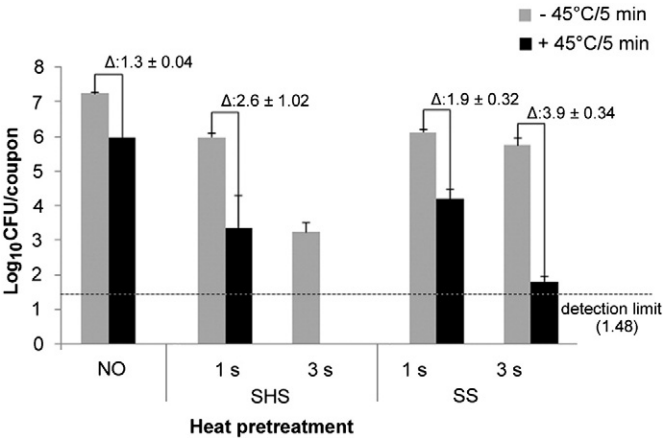

Fig. 3. Resistance of SHS-pretreated biofilms against subsequent heat stress. *Salmonella* biofilms treated with SHS or SS for 1 and 3 sec were subsequently exposed to heat stress at 45°C for 5 min (black bar), or untreated (grey bar). The sensitivity to additional thermal stresses was calculated by difference in viability (Δ) between before and after the subsequent heat treatments. Biofilm cells not pretreated with either SHS or SS showed 1.3 ± 0.04 Log_{10} CFU/coupon decreases by the mild heat stress, and the difference was compared with those of the SHS- or SS-pretreated biofilm cells. *Salmonella* biofilm cells pretreated with SHS for 3 sec were inactivated below the detection limit (1.48 Log_{10} CFU/coupon) by the subsequent heat treatments. All of the biofilm cells showed significant decreases in survival by the heat treatments at 45°C for 5 min ($P < 0.05$).

3.4. Distinct transcriptional responses to SHS between Salmonella pathogenicity islands

Salmonella possesses specialized gene clusters called *Salmonella* pathogenicity islands (SPIs) for proficient virulence control. SPI-1, a 40-kb island located at centisome 63 of the genome, is composed of more than 39 genes whose products are required for *Salmonella* to invade host cells and provoke proinflammatory responses (Ellermeier and Slauch, 2007). SPI-2, another 40-kb locus at centisome 30 of the genome, harbors 38 genes whose products play important roles in *Salmonella* survival inside host cells and systemic infection (Fass and Groisman, 2009). SPI-1 and SPI-2 expression is controlled by hierarchical regulatory networks composed of cognate transcriptional regulators within the islands and global regulators responding to a variety of environmental stimuli, such as thermal stress. The regulation of SPI genes by

alternative sigma factors has been studied under stressful conditions (Matsui et al., 2008; Osborne and Coombes, 2009). We investigated the transcriptional changes in 8 SPI-1 and 7 SPI-2 genes after SHS and SS treatments in order to assess bacterial virulence after extreme thermal treatments. Both the SHS- and SS-treated cells showed decreased transcriptional levels of SPI-1 genes, whereas those of the SPI-2 genes increased in comparison to the untreated biofilm control cells (Fig. 4). Immediate transcriptional responses were again observed in the SHS-treated biofilm cells, and SHS caused more significant transcription changes than SS. The opposite transcriptional responses between SPI-1 and SPI-2 genes after thermal stress were also observed in planktonic *Salmonella* cells under sublethal stress at 42°C (Sirsat et al., 2011). However, the substantial consequence of the distinct transcriptional changes between SPI-1 and SPI-2 genes under thermal stresses has not yet been determined in the context of *Salmonella* resistance and virulence.

3.5. Antagonistic transcriptional relationship between flagellar and fimbrial genes in response to SHS treatments

Bacterial pathogenicity requires motility and adhesion to various host cell surfaces as well, so the transcriptional changes in genes involved in motility and adhesion were also examined in the SHS-treated biofilm cells. Two putative fimbrial subunit genes, *safD* and *stbA*, along with one putative fimbrial usher gene, *safC*, were upregulated by SHS and SS treatments, while five genes encoding structural components of flagella were downregulated by the extreme thermal shocks (Fig. 5). SHS treatments decreased the expression of flagellar genes more significantly and promptly than SS treatments. The reciprocal response between the upregulated fimbrial genes and downregulated flagellar genes in response to thermal stress was also observed at alleviated thermal treatments of 42°C (Sirsat et al., 2011). Depending on the environmental conditions, bacteria determine their lifestyle, between the motile planktonic state and adhesive sedentary state, and the transition between the two states is finely controlled by an antagonistically coordinated regulatory network including flagella master regulator FlhDC and general stress-responsive σ^s, which inversely control the signal molecule bis-(3′–5′)-cyclic-diguanosine monophosphate (Cotter and Stibitz, 2007; Pesavento et al., 2008). Fimbriae are implicated in cell–cell aggregation for biofilm formation, as well as surface adhesion (Barnhart and Chapman, 2006). The increased transcription of fimbrial genes by SHS and SS treatments suggests that exposure to SHS and SS may stimulate *Salmonella* to strengthen its biofilm matrices through additional fimbriae attachment, which would make it harder to be removed afterward.

3.6. Effect of SHS stress on quorum sensing genes

Bacteria utilize quorum sensing (QS) signals for interspecies communication and control of diverse cellular activities such as virulence and bioluminescence. Implication of QS in biofilm formation has been elucidated in many pathogenic bacteria. *P. aeruginosa* and *V. cholera* lacking QS systems produce thinner biofilms that are less resistant to detergents (Davies et al., 1998; Hammer and Bassler, 2003). *Salmonella* possesses three QS systems, termed SdiA/incomplete autoinducer-1 (AI-1), LuxS-Lsr/AI-2, and QseBC/AI-3 (Bearson and Bearson, 2008; Soni et al., 2008; Walters and Sperandio, 2006). The AI-2 QS circuit has been explored extensively in the context of biofilm and virulence regulation. However, the roles of the AI-2 QS system in controlling biofilm development and virulence in *Salmonella* are debatable. Varying biofilm phenotypes, from impaired to normal, were observed for *luxS* mutant strains constructed in diverse manners, suggesting the possibility that MicA, a small RNA encoded in the *luxS* adjacent genomic region, is responsible for controlling biofilm formation (Jesudhasan et al., 2010; Kint et al., 2010; Yoon and Sofos, 2008). AI-2 QS-mediated SPI-1 regulation was also controversial, but Choi et al. (Choi et al., 2012) recently revealed that LsrR, a transcriptional regulator involved in AI-2 transport and its catabolic processing, negatively controls SPI-1 and flagella gene expression. We examined the influences of SHS and SS treatments on the expression of the *lsr* operon encoding AI-2 transport components (LsrA, LsrB, LsrC, and LsrD), AI-2 kinase (LsrK), catabolic enzymes (LsrF and LsrG), and the transcriptional regulator (LsrR). In contrast to the genes described earlier, *lsr* genes showed different expression patterns between the SHS and SS treatments; moderate downregulation of *lsr* genes was accomplished by SHS treatment, while upregulation of most *lsr* genes was observed after SS treatment (Fig. 6). Although SHS resulted in more dramatic transcriptional changes in the genes concerning heat shock response, virulence, motility and adhesion than SS, the expression of *lsr* AI-2 QS genes was not significantly influenced by SHS. It remains to be determined how *Salmonella* biofilm cells take advantage of the different transcriptional responses of the AI-2 QS system in order to cope with the extreme thermal stress.

While the underlying defense or survival mechanism induced by the immediate transcriptional changes has not yet been defined in SHS-treated *Salmonella* biofilms, our results would be helpful to understand the transcriptional responses of *Salmonella* biofilm cells during the superheated steam-mediated sterilization processes, and to prevent potential hygiene problems caused by improper heat sterilization.

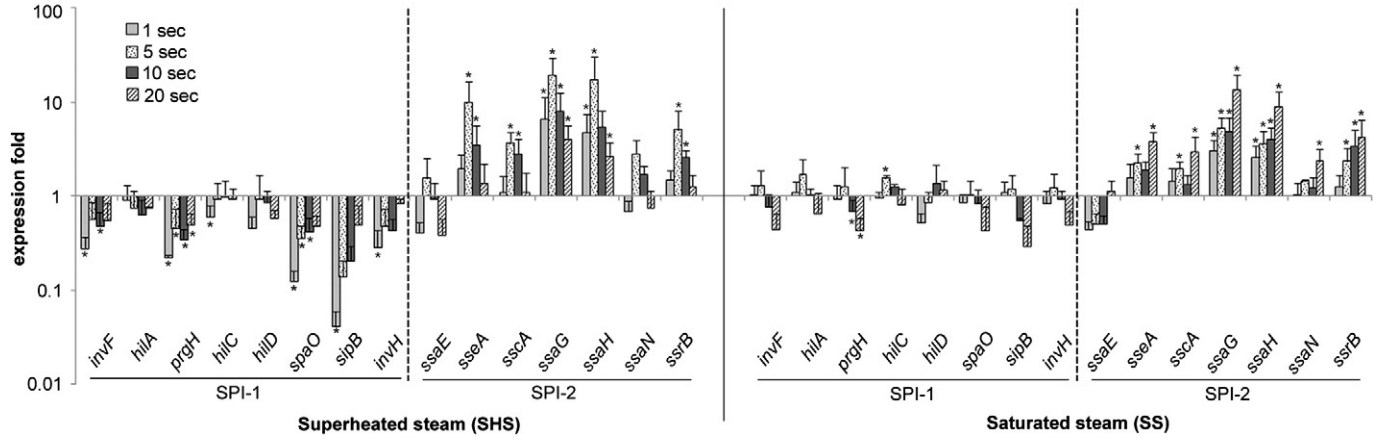

Fig. 4. Expression of *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2 genes after SHS and SS treatments. Stainless steel coupons covered with *Salmonella* biofilms were exposed to SHS or SS for 1, 5, 10, and 20 sec, and the total RNA was isolated and subjected to qRT-PCR to compare the transcriptional levels of SPI-1 and SPI-2 genes with those of the untreated controls. All mRNA levels were normalized to the *gyrB* mRNA, and the expression ratios between SHS- or SS-treated samples and the untreated control were presented as the average from three independently isolated RNA samples, and plotted on a logarithmic scale. Genes showing significant differences in mRNA levels between the heat-treated and untreated samples were denoted by asterisks ($P < 0.05$).

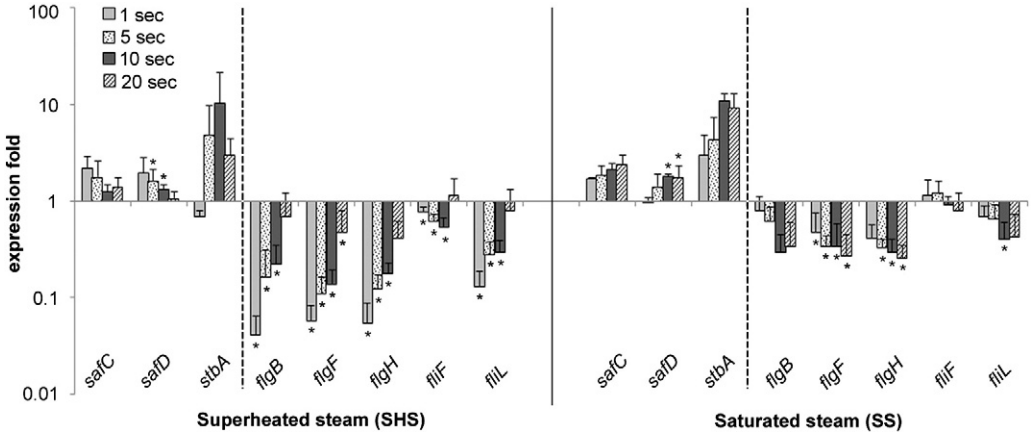

Fig. 5. Transcription of flagella and fimbriae related genes in response to SHS and SS treatments. *Salmonella* biofilm cells formed on stainless steel coupons were subjected to SHS or SS treatments for 1, 5, 10, and 20 sec, and immediately submerged in a solution containing PBS and RNAprotect Bacteria Reagent to quench RNA degradation, as described in the materials and methods. RNA purified from the biofilms was used in qRT-PCR with primers specific to 5 flagellar and 3 fimbrial genes. The mRNA levels of the 11 tested genes were normalized with that of *gyrB* for each condition, and the transcriptional ratios between heat-treated and untreated samples were averaged and plotted. At least three replicates were processed for statistical analyses, and mRNA levels showing significant increases or decreases due to heat treatments were marked with asterisks ($P < 0.05$).

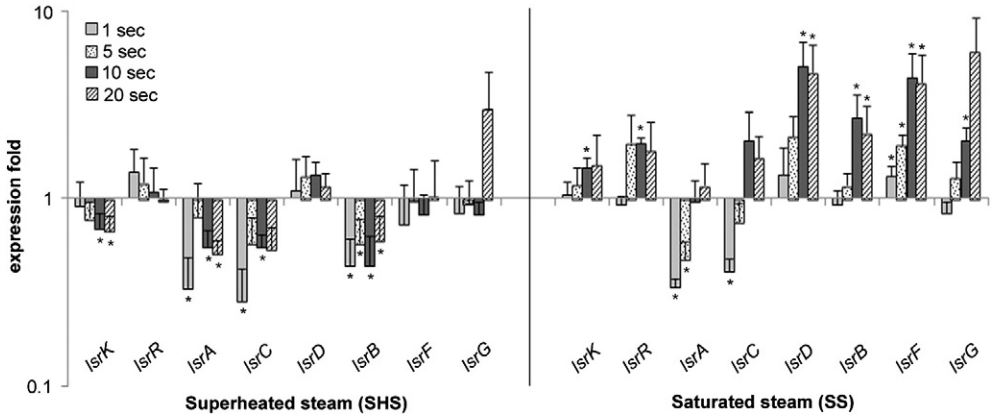

Fig. 6. Transcriptional response of *lsr* operons to SHS and SS treatments. Total RNA was isolated from *Salmonella* biofilms, following SHS or SS treatment for 1, 5, 10, and 20 sec, and processed to assess the transcriptional changes of the *lsr* genes in response to SHS and SS using qRT-PCR. The mRNA levels of the *lsr* genes were standardized with that of *gyrB* for each condition. The expression of the *lsr* genes after SHS or SS treatments was compared with that of the *lsr* genes in the untreated control, and the folds of expression were averaged from three independently isolated RNA samples. Genes showing significant transcriptional changes due to SHS or SS treatments were marked with asterisks ($P < 0.05$).

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