Role of Phage Shock Protein in Recovery of Heat-injured Salmonella

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Sublethally heat-injured cells of Salmonella in food can recover under favorable conditions, leading to foodborne illness. To elucidate the molecular mechanism of recovery from heat injury, the global changes in gene transcription of Salmonella Typhimurium were investigated in previous study. In this study, the functions of genes involved in phage shock response (viz., phage shock protein (psp) genes), the transcription levels of which were found in previous study to be increased during recovery from heat injury, were investigated in recovering cells. The increase in pspABCDEFG transcription levels during the recovery process was confirmed by qRT-PCR. To understand the role of pspA genes in heat injury recovery, a pspA deletion mutant (ΔpspA) and a pspA-overexpressing strain (S. Typhimurium pBAD30/pspA(+) were constructed. ΔpspA showed slightly lower viable counts and membrane potential than those of the wild-type strain during recovery. On the other hand, there was no significant difference in the viable counts between S. Typhimurium pBAD30/pspA(+) and the control strains S. Typhimurium pBAD30/pspA(-) and S. Typhimurium pBAD30(+) during recovery. It would seem that a lack of PspA protein alone somewhat affects the recovery of S. Typhimurium from heat injury, but overexpression of PspA alone is not sufficient to overcome this effect.

Key words: Phage shock protein / Heat injury / Salmonella / Recovery / Membrane potential.

INTRODUCTION

Pathogens in food are commonly controlled by thermal and non-thermal methods, such as heating, freezing, drying, high hydrostatic pressure, and the addition of antimicrobials and chemicals (Jay, 2012; Wesche et al., 2009; Wu, 2008). Under these severe conditions, the population of bacterial cells in food can be divided into those that are killed and those that survive, with the latter group containing some cells that are sublethally injured (Russell, 1984; Wu et al., 2001). Sublethally injured cells generated after heat treatment still have the potential to recover from injury and become functionally normal as intact cells under a favorable environment (Wu, 2008). However, it is difficult to detect these sublethally injured cells owing to their long lag time of growth and susceptibility to the selective agents in selective media. Heat treatment is one of the most important methods used to eliminate bacterial pathogens in food. Mild heat treatment is suitable for the production of food with high quality in flavor, texture, and taste, but often results in an increased generation of sublethally injured but recoverable cells in food products.

Salmonella spp. are major foodborne pathogens that cause thousands of incidents of foodborne diseases through their consumption from many kinds of food, including eggs, milk, meat, vegetables, fruits, and their processed food products. Due to underestimation of the population caused by sublethally injured but recoverable cells in food, various improvements in the growth media have been attempted to facilitate the growth of the cells (Back et al., 2012; Gurtler and Kornacki, 2009; Taskila et al., 2011; Zheng et al., 2013).
However, not much investigation has been done on the molecular mechanism behind *Salmonella* recovery from heat injury, despite the important need to develop or improve methods facilitating the recovery of the sublethally injured cells for study so that intervention steps can be taken to effectively control the bacterium in food.

To elucidate the molecular mechanism behind *Salmonella*’s recovery from heat injury, we had previously investigated the global changes in gene transcription in *Salmonella enterica* serovar Typhimurium using DNA microarrays. According to the DNA microarray results, among the genes with increased transcription in the recovery process, those encoding sigma factor protein, phage shock proteins, and branched-chain amino acid (BCAA) transporter, and those involved in metal homeostasis, seemed to be important (Wen et al., 2012).

Phage shock protein (Psp) is a member of the Psp response system, which deals with extracytoplasmic stress, and was first described in *Escherichia coli* K-12, being produced during infection of the bacterium by filamentous phage f1 (Brissette et al., 1990). Psp and its homolog have also been discovered in *Salmonella enterica, Yersinia enterocolitica, and Burkholderia pseudomallei* (Darwin and Miller, 1999; Model et al., 1997; Southern et al., 2015). In *Salmonella enterica*, the protein of this system is encoded in the *pspABCDEFG* operon, the divergently transcribed *pspF* gene, and the separate *pspG* gene (Huvet et al., 2011; Weiner and Model, 1994). Psp was reported to facilitate maintenance of the proton motive force (PMF) and membrane integrity (Kleerebezem et al., 1996; Maxson and Darwin, 2004). Based on these reports on the functions of Psp and our DNA microarray results, Psp would seem to play an important role in recovery from the injury caused by heating.

To confirm the results obtained by DNA microarray in our previous study (Wen et al., 2012), qRT-PCR was applied in the present work to quantify changes in the transcription of *pspABCDEFG* in heat-treated *S. Typhimurium* cells during recovery in rich medium. To investigate the role of the *psp* genes, a *pspA* deletion mutant (Δ*pspA*) and a *pspA*-overexpressing strain were constructed and their changes in viability and membrane potential were compared before and after heat treatment as well as during recovery.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture**

*Salmonella enterica* serovar Typhimurium NBRC 12529 was obtained from the Biological Resource Center, NITE (NBRC, Chiba, Japan). The bacterium was cultured overnight in tryptic soy broth (TSB; BD, Franklin Lakes, NJ, USA) at 37°C, with shaking at 130 rpm, to obtain cells in the stationary phase of growth. Tryptic soy agar (TSA; BD) and deoxycholate hydrogen sulfide lactose (DHL) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) were used for counting both intact and injured but recoverable cells and only intact cells, respectively. *Escherichia coli* DH5α (TaKaRa Bio, Shiga, Japan) was used as the host in the cloning of *psp* genes. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and the transformants were cultured overnight in Luria Broth (LB; BD) at 37°C. To avoid suppression of the expression of the arabinose operon of plasmid pBAD30 by glucose in TSB, LB was used in the overexpression experiment. Antibiotics were used at the following concentrations, as required: ampicillin (100 μg/ml) and kanamycin (25 μg/ml).

**Construction of pspA deletion mutant**

A 1-step inactivation method (Solano et al., 2002) was used for disrupting the *pspA* gene in *S. Typhimurium*, with some modifications. Amplification of the gene fragment for homologous recombination was performed using a 3-step PCR, as described by Honjoh et al.

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**Table 1. Bacterial strains and plasmids used in this study**

| Strain / Plasmid | Description | Source |
|------------------|-------------|--------|
| *S. Typhimurium* | *Salmonella enterica* serovar Typhimurium NBRC 12529, wild-type strain | NBRC |
| *S. Typhimurium* pKOBEGA | *S. Typhimurium* carrying helper plasmid pKOBEGA | This work |
| *S. Typhimurium ΔpspA::Km<sup>+</sup>* | *S. Typhimurium* ΔpspA::Km<sup>+</sup>, *pspA* deletion mutant | This work |
| *S. Typhimurium* pBAD30 | *S. Typhimurium* with pBAD30 | This work |
| *S. Typhimurium* pBAD/pspA | *S. Typhimurium* with pBAD30/pspA, PspA overexpression strain | This work |
| *Escherichia coli* DH5α | *E. coli* for cloning | TaKaRa |
| pBAD30 | Expression vector | Guzman et al. (1995) |
| pBAD30/pspA | pBAD30 with 698 bp *pspA* gene | This work |
| pGEM-T Easy Vector | Cloning vector | Promega |
sequences of the genomic DNA of S. Typhimurium were used for designing the primers listed in Table 2. Two pairs of primers (pspA-up-F/pspA-up-M13R and pspA-down-M13F/pspA-down-R) were used to amplify the up-stream and down-stream regions of the pspA gene from S. Typhimurium genomic DNA. Sequences of the genomic DNA of S. Typhimurium (Accession No. NC_003197; McClelland et al. 2001) were used for designing the primers listed in Table 2. The PCR mixture was prepared according to the manufacturer’s instructions. The primers kmr-M13F and kmr-M13R were used to amplify the kanamycin resistance gene from E. coli plasmid pACYC177. The PCR was carried out with Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA), under the following conditions: 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and elongation at 72°C for 1 min. For ligation of the upstream region of pspA, kanamycin resistance gene, and down-stream region of pspA, sequences of M13 were included in the primers pspA-up-M13R, pspA-down-M13F, kmr-M13F, and kmr-M13R. Phusion DNA Polymerase (ThermoFisher Scientific, Waltham, MA, USA) was used for the PCR, under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and elongation at 72°C for 3 min. The ligation fragment prepared was amplified by using the primers pspA-up-F and pspA-down-R and Takara ExTaq DNA polymerase, under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and elongation at 72°C for 3 min. The helper plasmid pKOBEGA carrying the resulting PCR product (including the upstream and downstream regions of pspA and the kanamycin resistance gene) was transformed into S. Typhimurium by electroporation. Cuvettes (2 mm gap) were also cooled on ice before use. A 2 µL aliquot of the PCR product was added to the competent cells and kept on ice for 2 min. The cells were then transferred to the cooled cuvette and electroporated using the ECM 630 (BTX, Division of Genetronics, Harvard Apparatus, Inc., Holliston, MA, USA) electro cell manipulator, which was operated at 1.9 kV, 150 µF, and 50 µF. The cells were then recovered in 1 ml of TSB and incubated at 30°C for 2-3 h to allow for homologous recombination. Thereafter, 100 µL cells were plated onto LB agar containing 25 µg/ml kanamycin (LBA/Kan) and incubated at 37°C. Colonies that grew on LBA/Kan plates following electroporation were picked and plated onto both LBA/Kan and LB agar containing 100 µg/ml ampicillin (LBA/Amp) to check for incorporation of the kanamycin resistance gene and the loss of pKOBEGA. The process was repeated until colonies grew only on LBA/Kan and not on LBA/Amp. The ΔpspA mutants were verified by PCR using three pairs of primers (pspA-Forward/pspA-Reverse, kmr-Forward/kmr-Reverse, and pspA-v-F/pspA-v-R), under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and elongation at 72°C for 3 min. The transcriptional levels of all genes of S. Typhimurium ΔpspA::Kmr were detected by qRT-PCR using the method described above.

**Construction of pspA-overexpressing strain of S. Typhimurium**

Genomic DNA of overnight cultured S. Typhimurium was prepared by using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The DNA concentration was

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**Table 2. Primers used for real-time PCR**

| Primer              | Primer sequence (5′-3′)                          |
|---------------------|--------------------------------------------------|
| pspA-Forward        | ATCCCGCTGTAGTTGAACAGG                             |
| pspA-Reverse        | TCAGCTGACAGCGAGCATC                              |
| PspA+EcoRI Forward | GAATTCATGAGGATGTTATATGG                          |
| PspA-XbaI Reverse   | TCTAGAATTGATATCTTCTTCA                             |
| M13 Forward         | TGGAAAACGAGCGGAGCAG                               |
| M13 Reverse         | CAGGAAGACGCTATGACCA                               |
| pBAD30-seq-up       | CTGTTTCTCATACCCGTGG                               |
| pBAD30-seq-down     | GGCTGAAAATCTTCTCT                                 |
| pBAD30-v-up         | TGCTACTCGTCAAGGCGGCT                             |
| pBAD30-v-down       | GGTTCCACAGCAGCAAGCGG                             |
| pspA-up-F           | GCACAAACGACGGATGCTATG                             |
| pspA-up-M13R        | CATGGTCATAGGCTTTTCTGT                              |
| pspA-down-M13F      | GGCATAGGGCGGCGCGTCTAT                             |
| pspA-down-R         | CATGGTCATAGGCTTTTCTGT                             |
| kmr-M13F            | AGACGGCATGCTGTGTTTACATACATCGTCTCAAAATCGTCTG      |
| kmr-M13R            | AGACGGCATGCTGTGTTTACATACATCGTCTCAAAATCGTCTG      |
| pspA-Forward        | ATCCCGCTGTAGTTGAACAGG                             |
| pspA-Reverse        | TCAGCTGACAGCGAGCATC                              |
| kmr-Forward         | GGTTGATTGATGCTATGTT                              |
| kmr-Reverse         | ATGGCAAGATCCTGTGTTAC                             |
| pspA-v-F            | CAAACTTTTTTGCTACATG                              |
| pspA-v-R            | AGTTTAAACGCTAGTGCTG                              |

Underlines indicate M13 vector-derived sequences.
determined by using a NanoDrop ND-1000 spectrophotometer. The pspA gene was amplified from the genomic DNA using the pspA+EcoRI Forward and pspA+XbaI Reverse primers. The PCR product was purified by using the QiAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and then ligated into the pGEM-T Easy Vector (Promega, USA) according to the manufacturer’s protocol to construct pGEM-pspA. The transformation of pGEM-pspA into E. coli DH5α competent cells was carried out using the ECM 630 electro cell manipulator (BTX) under the conditions of 2500 V, 200 Ω, and 25 µF. Ampicillin-resistant transformants were selected and verified by PCR using the M13 Forward and M13 Reverse primers. After its extraction from the E. coli transformants, pGEM-pspA was digested with XbaI and EcoRI, and a 698-bp fragment of pspA was inserted into the corresponding cloning sites on the vector pBAD30 (ThermoFisher Scientific) digested with the same enzymes. The constructed plasmid pBAD30/pspA was verified by PCR with the pBAD30-seq-up, pBAD30-seq-down, pBAD30-v-up, and pBAD30-v-down primers, and was then transformed into competent cells of S. Typhimurium NBRC 12529 to produce S. Typhimurium pBAD30/pspA by the method described above. The pspA expression of S. Typhimurium pBAD30/pspA was induced by L-arabinose (ST pBAD30/pspA(+)) and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel. In addition, S. Typhimurium pBAD30 was constructed. S. Typhimurium pBAD30/pspA without L-arabinose addition (ST pBAD30/pspA(-)) and S. Typhimurium pBAD30 supplemented with L-arabinose (ST pBAD30(+)) were set as controls.

Heat treatment and recovery

The S. Typhimurium wild-type and ΔpspA::Kmr’ strains were cultured in TSB, and then diluted to an OD600 value of 0.127 in 160 ml of sterile PBS in a conical flask (500 ml) to obtain bacterial concentrations of approximately 108 CFU/ml. The heat treatment and heat recovery assay were performed according to our previous experimental procedures, with some modifications (Wen et al., 2012). The cell suspension was incubated in a 25°C water bath for 10 min prior to heat treatment, and then heated in a 55°C water bath with gentle shaking at 80 rpm for 15 min. The cell suspension was then cooled for 10 min in a 25°C water bath. The cell suspension was then mixed with same volume of 2× TSB and 2× LB for recovery of pspA deletion mutant and overexpression strain, respectively.

Measurement of viability

Bacterial cell viability was estimated from the colony counts determined by the plating method. Cells were serially diluted with PBS, and 100 µL of each diluted suspension was spread over TSA and DHL agar. After incubation of the plates at 37°C for 1 d, the colonies were enumerated. DHL is a selective medium for enteric bacteria including Salmonella and the selective agents are inhibitory to the injured cells characterized by damaged permeability barriers and damaged proteins involved in metabolic activities essential for viability. Only intact cells grow on DHL. While on TSA, non-selective medium, injured cells can recover and grow as well as intact cells. Colony counts on TSA represent the number of both intact cells and injured but recoverable cells. On the other hand, the counts on DHL agar represent the number of intact cells only. Therefore, the difference between the counts represents the number of heat-injured cells.

qRT-PCR

To validate the microarray results, the changes in transcription of psp genes (papA, B, C, D, E, F, and G) were confirmed by qRT-PCR. After the heat treatment as described above, S. Typhimurium cells were incubated in TSB for recovery. The total RNA was extracted from the cells incubated in TSB after 1 h recovery and from cells before heat treatment, as described previously (Wen et al., 2012). The RNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the RNA quality was verified by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA synthesis was done by using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The primers were designed using the Primer3 software (http://frodo.wi.mit.edu/), based on the DNA sequences of the target genes from the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/kegg2.html). Real-time PCR was performed with the Mx3000P Real-time PCR System (Stratagene, La Jolla, CA, USA) in a final volume of 25 µL containing 1 µL of 5 µM each primer, 0.1 µL of cDNA, 0.05 µL of 50× ROX Reference Dye II (TaKaRa Bio), 12.5 µL of 2× SYBR Premix Ex Taq (TaKaRa Bio), and sterile water. The PCR consisted of an initial denaturation at 95°C for 3 min for 1 cycle, and 40 cycles of 95°C for 30 ss, 60°C for 30 s, and 72°C for 30 s. To determine the relative gene expression, the 16S rRNA gene was used as reference, and data were analyzed using MxPro QPCR software version 2.0. Relative transcriptional levels of the genes in the cells at 1 h of recovery were calculated on the basis of the levels in cells before heat treatment.
Measurement of the membrane potential

The membrane potential was estimated by using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyldicyanine iodide (JC-1; Invitrogen) as described previously (Becker et al. 2005). Cells were harvested by centrifugation at 8000 g for 10 min and resuspended in permeabilization buffer. After a 5 min incubation with JC-1 dye, the cells were observed by fluorescence microscopy (Eclipse E600; Nikon, Tokyo, Japan) under excitation at 485 nm and emission above 520 nm. Red fluorescence (590 nm) indicates a high membrane potential whereas green fluorescence (530 nm) indicates a low value. ImageJ/Fuji software (National Institutes of Health) was used to measure the green and red emission fluorescence of individual cells loaded with JC-1 dye, and the red/green (590 nm/530 nm) ratio was calculated. At least 100 single cells were chosen for analysis and measured in each experiment, and measurements were taken for three times for each strain.

Statistical analysis

The experiment was replicated three times. The student’s t-test and Tukey’s Honestly Significant Difference (HSD) test were performed using Microsoft Office Excel software and RStudio software (version 1.0.136). A p value of <0.05 was considered as statistically significant.

RESULTS

Transcription of psp genes confirmed by qRT-PCR during recovery

To confirm the changes in transcription of genes pspABCDEFG during recovery from heat injury reported in our previous study (Wen et al., 2012), qRT-PCR was done. Transcription of all the psp genes increased significantly during recovery from heat injury compared with those of the genes before heat treatment. Gene pspA showed the highest increase, with 3567-fold change approximately. Genes pspB, C, and D, which are in the same operon as pspA and E, showed similar increased levels, but less than that of pspA and more than that of pspE. This result suggests that the transcription of psp genes was indeed induced by heat treatment.

Effects of pspA deletion on heat injury and recovery in S. Typhimurium

To understand the role of the psp genes and their product in the recovery from heat injury in S. Typhimurium, a pspA deletion mutant was constructed. Transcription of pspA was largely decreased, indicating that the pspA deletion mutant (S. Typhimurium ΔpspA::Km) was successfully constructed by homologous recombination using pKOBEGA. The transcription levels of the pspB, C, D, E, and G genes were moderately decreased, but not that of the pspF gene. Although the pspG gene is independent of the pspABCDE operon, its transcription was not significantly different from those of pspBCD. The same phenomenon was observed in the wild-type strain, irrespective of the heat treatment.

To investigate the effect of pspA gene deletion on heat injury and recovery, cell viability was measured by the plating methods using both TSA and DHL agar after the heat treatment and subsequent recovery culture in TSB. The changes of viable counts of the S. Typhimurium ΔpspA and wild-type strain are shown in Fig. 1 (a). After heat treatment, intact cell counts of the wild-type and ΔpspA strains determined on DHL agar had decreased significantly, from 7.5 to 4.4 log CFU/ml and from 7.4 to 4.2 log CFU/ml, respectively. The viable counts on TSA, which show the total number of intact cells and sub-lethally injured cells, were 6.9 and 6.7 log CFU/ml for the wild-type and ΔpspA strains, respectively, after heat treatment, indicating that more than 99.9% of the cells were injured but recoverable. There was no significant difference in viable counts on TSA between the wild-type and ΔpspA strains before and after heat treatment and during recovery in TSB. On the other hand, the viable counts of ΔpspA on DHL agar were significantly lower than that of the wild-type strain (n = 3, p < 0.05) at 3 h of recovery cultivation. In addition, viable counts on DHL reached a similar level to that on TSA after recovery cultivation for 3 and 4 h in the wild-type and ΔpspA strains (Fig. 1 (a)). There was only a slight difference between these two strains in their recovery from heat injury, consistent with the membrane potential results. The ratio of red fluorescent cells to green fluorescent cells was significantly higher in the wild-type strain than in ΔpspA after cultivation for 3 h (Fig. 1 (c)). These results suggest that pspA is somewhat involved in the maintenance of the PMF, but is not essential for recovery from injury caused by heating.

Effects of pspA overexpression on injury by heating and recovery in S. Typhimurium

After its transformation in S. Typhimurium, the presence of plasmid pBAD30/pspA in the transformed cells was confirmed by PCR with primers unique to pBAD30. The expression of pspA was induced by the addition of L-arabinose, and production of recombinant PspA was confirmed by SDS-PAGE (data not shown). The PspA protein was successfully overproduced in the presence of L-arabinose in S. Typhimurium pBAD30/pspA.

Changes in viability were measured on the S. Typhimurium pBAD30/pspA(+), pBAD30/pspA(-), and pBAD30(+)- strains after heat treatment and subsequent recovery culture in LB (Fig.2(a)). After heat treat-
There was no significant difference in viable counts on both TSA and DHL agar between the strains, before and after heat treatment and during recovery in LB. As shown in Fig. 2(c), the changes in the membrane potential in all three strains showed no significant difference, intact cell counts of S. Typhimurium pBAD30/pspA(+), pBAD30/pspA(-), and pBAD30(+) on DHL agar were 4.1, 4.2, and 4.6 log CFU/ml, respectively, whereas the counts on TSA were 8.0, 7.9, and 7.8 log CFU/ml, respectively. The results indicate that more than 99.9% of the cells were injured but recoverable after the heat treatment.
significant differences after cultivation for 3 h.

**DISCUSSION**

PspA is the critical protein for *S. Typhimurium*, facilitating intracellular survival in mammalian cells by maintaining the proton motive force (PMF). The Psp system was found in gram-negative bacteria, Eubacteria, and even plant chloroplasts (Joly et al., 2010). Among the *psp* genes, only *pspA* is evolutionarily conserved. In *E. coli* and other enterobacteria, including *Salmonella*, PspA released from the PspA-PspF complex functions...
as an effector under stress, binding to the C-terminal portion of PspC or combining with the inner membrane directly to maintain the PMF (Flores-Kim and Darwin, 2016). Therefore, to investigate the role of psp during recovery from heat injury, a pspA deletion mutant and a PspA overexpression strain were constructed.

PspF, activator for the transcription of psp genes, is released by PspA under stress condition (Darwin, 2005). However, the transcription of pspA, B, C, D, E, and G was decreased in the pspA deletion mutant of S. Typhimurium. Our results suggest that PspA is somewhat involved in the positive regulation of pspBCDEG gene transcription. In this study, a pspA deletion mutant was used to investigate the roles of psp genes and their products on the recovery of heat-injured S. Typhimurium.

After heat treatment and during recovery culture, the viability and membrane potential were slightly lower in S. Typhimurium ΔpspA than that in the wild-type strain. It seems that the deletion of pspA alone somewhat affects the recovery from injury caused by heating. Becker et al. (2005) reported that the pspA null mutant of S. Typhimurium lacking ropE showed decreased viability at alkaline pH, but the S. Typhimurium pspA deletion mutant did not. They concluded that pspA seems important for compensating for the function of ropE in promoting stationary phase survival. Karlinsey et al. (2010) reported that the survival of a pspA deletion mutant in macrophages was similar to that of the S. Typhimurium wild-type strain. It has also been reported that a Psp-like system has evolved in mycobacteria (Datta and Ravi, 2015). These facts suggest that a system other than the Psp system compensated for the role of pspA to maintain the membrane potential, leading to the survival of the ΔpspA mutant after heat treatment and its recovery from the injury.

The PspA overexpression strain of S. Typhimurium did not promote the recovery from injury caused by heating (Fig.2). PspA is reported to preserve membrane integrity during stress by interacting with the PspB-PspC complex and with the inner membrane. Kobayashi et al. (2007) reported that PspA oligomers suppressed proton leakage from the membrane by binding to membrane phospholipids in E. coli, whereas PspA monomers did not. To clarify the role of PspA in the recovery of heat-injured S. Typhimurium, the formation of PspA oligomers in the overexpressing strain should be confirmed. Since the interaction of PspA with the PspB-PspC complex is important for preserving membrane integrity, it would seem that the amount of PspB-PspC complex on the inner membrane is not sufficient to bind overexpressed PspA.

The Psp system is associated with many important phenotypes, such as antibiotic resistance, biofilm formation, and virulence (Flores-Kim and Darwin, 2016). In this study, we have investigated the role of pspA in Salmonella recovery from heat injury, since the transcription of psp genes was largely increased during the recovery from the injury caused by heating (Wen et al., 2012). Even though pspA is the key component of the Psp system, the changes of pspA expression alone were not enough to change the recovery dramatically. To clarify the role of psp genes on recovery, the transcription of all the psp genes should be reduced by using siRNAs or multiple deletion mutants.

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