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ORIGINAL ARTICLE

Changes in transcription during recovery from heat injury in *Salmonella typhimurium* and effects of BCAA on recovery

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Abstract Mechanisms of recovery from heat injury in *Salmonella typhimurium* were elucidated. Recovery of the heat-injured *S. typhimurium* cells in TSB resulted in full recovery after 3 h of incubation at 37°C. The DNA microarray analysis of 30- and 60-min recovering cells resulted in an increase in transcription of 89 and 141 genes, respectively. Among them, 15 genes, with known function, seemed to be somewhat involved in recovery. They encoded proteins involved in branched-chain amino acid (BCAA) transport (*livJ*, *livH*), cell envelope integrity (*ddg*), heat-shock response (*cpxP*, *rrmJ*), phage shock protein (*pspA*), ribosome modulation factor (*rmf*), virulence (*sseB*) transcriptional regulation (*rpoE*, *rpoH*, *rseA*, *rseB*, *rseC*) and ArcB signal transduction (*sixA*) and cytoplasmic membrane protein (*fxsA*). Among them, the effects of BCAA supplementation on recovery from heat injury were studied to confirm the importance of the BCAA transport *liv* genes during recovery. It was found that supplementation of TSB with 0.1% BCAA resulted in an enhanced recovery of injured cells in comparison to those recovered in TSB without BCAA. Supplementation of BCAA at 0.1% resulted in a cell count increase 4.4-fold greater than that of the control after 1 h incubation. It seems that BCAA promoted the recovery by promoting protein synthesis either directly through their use in translation or indirectly through stimulation of protein synthesis by activation of the Lrp protein.

Keywords *Salmonella* · Heat injury · Recovery · BCAA · DNA microarray

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Introduction

Heat treatment is one of the most commonly used sterilization methods in food processing. High temperature treatment has been correlated with the efficiency of sterilization, which however results in the denaturation of food products. Mild heat or insufficient heat treatment may only sublethally injure bacterial cells (Eijkman 1908), which may still pose a risk for food-borne illnesses such as salmonellosis. These injured cells are characterized by damaged permeability barriers and, in some cases, damaged components related to metabolic activities essential for maintenance of life (Wu 2008). Due to these detrimental effects, it is difficult to specifically detect certain bacteria since injured cells are more sensitive to the selective agents present in selective media (Ray 1979). As such, recovery of sublethally injured cells by successful enrichment is important in the detection of *Salmonella* in food products. Studies in the past have been done to improve techniques for the recovery of injured bacteria (Kang and Fung 2000; Chambliss et al. 2006; Wu 2008); however, the molecular biology of the mechanisms for recovery is still unknown. In order to design and develop media and culture conditions effective in the recovery of injured bacterial cells, the mechanisms of recovery need to be elucidated.

In a previous study on *Salmonella enteritidis*, the transcription levels of 42 heat-inducible genes and 46 oxidative

stress-inducible and DNA damage-inducible genes during recovery were studied by reverse transcription polymerase chain reaction (RT-PCR). It was found that 19 heat-inducible genes and 12 oxidative-stress and DNA damage-inducible genes were substantially transcribed during recovery (Kobayashi et al. 2004). In this study, we have expanded the spectrum of gene transcription analysis during recovery by the use of DNA microarray analysis. Many factors affect the recovery of injured cells; therefore, the activity of not only stress-induced genes but those involved in metabolism, transport, transcription and protein synthesis are thought to be important for recovery. Therefore, with the use of DNA microarray analysis, we observed the global changes in gene transcription during recovery. Although the most common cause of salmonellosis in Japan is *S. enteritidis*, at the time of the beginning of this study, DNA microarray of *S. enteritidis* is not available. Therefore, the authors have performed this study using *S. typhimurium*, which is also a common cause of food poisoning by *Salmonella*.

Through this study, it was found that transcriptional levels for the *liv* gene cluster encoding branched-chain amino acid (BCAA) transport increased during the recovery of injured cells. Therefore, in this study we investigated the effect of BCAA supplementation during recovery to confirm the importance of BCAA for the recovery of heat-injured cells.

Materials and methods

Bacterial strains and culture condition

Salmonella typhimurium NBRC 12529 was obtained from National Institute of Technology and Evaluation-Biological Resource Center (NBRC), Japan. The bacterium was cultured overnight in Tryptic Soy Broth (TSB, Becton Dickinson, Sparks, MD, USA) at 37°C with shaking at 130 rpm to obtain cells in stationary phase of growth.

Heat treatment and enumeration of culturable cells

The overnight culture was suspended in 160 mL of sterile phosphate buffered saline (PBS, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, pH 7.4) in a conical flask (500 mL) to obtain bacterial concentrations of approximately 10^8 CFU/mL. The cell suspension was incubated in a water bath at 25°C for 10 min prior to heat treatment. Heat treatment was performed in a water bath at either, 45, 50, 55 or 60°C with gentle shaking at 70 rpm for 15 min. The cell suspension was then cooled for 10 min in a 25°C water bath, serially diluted with PBS, and 100 μL of each diluted suspension was spread over Tryptic Soy Agar (TSA, Becton Dickinson) and Deoxycholate-Hydrogen Sulfide-Lactose Agar (DHL, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). After incubation of plates at 37°C for 1 day, colonies were enumerated. Statistical significance was determined by the Student's *t* test.

Heat recovery assay

The 160 mL *S. typhimurium* cell suspension with 10^8 CFU/mL was prepared and subjected to the heat treatment at 55°C for 15 min and cooled as described. The cell suspension was then mixed with 160 mL of 2× TSB to provide nutrients for recovery. The cells were then incubated at 37°C for 6 h with shaking at 130 rpm. Colony counts were determined on TSA and DHL agar after heat treatment (0 h) and at time points 1, 2, 3, 4, 5 and 6 h. Colony counts from TSA were defined as total viable cell count, and colony counts from DHL were defined as intact cell count. Statistical significance was determined by the Student's *t* test.

Heat recovery assay with branched-chain amino acid supplementation

The 50 mL cell suspension with 10^8 CFU/mL was prepared and subjected to the heat treatment at 55°C for 15 min and cooled as described. Five mL of the suspension was dispensed into test tubes containing 4 mL of 2.5× TSB. A total volume of 1 mL of PBS and/or 5.0% BCAA mix had been added to attain final concentrations of 0.002, 0.01, 0.05, 0.1, and 0.167% to 4 mL 2.5× TSB prior to the addition of 5 mL of bacterial suspension for a final volume of 10 mL. A final concentration of 0.1% is equivalent to a final concentration of 0.1% L-Leu, 0.1% L-Ile and 0.1% L-Val (w/v). The cells were then incubated at 37°C for 3 h with shaking at 130 rpm. Colony counts were determined on TSA and DHL agar after heat treatment (0 h) and at time points 1, 2, and 3 h. Statistical significance was determined by the Student's *t* test.

DNA microarray profiling

Overnight cultures were heat-treated and allowed to recover as described previously. After recovery for 30 or 60 min, 200 mL of the cell suspension was harvested by centrifugation at 8,000 rpm for 10 min. The supernatant was discarded, and a mixture of 700 μL Dw-saturated phenol and 700 μL TES buffer, pre-warmed to 65°C, was added to dissolve the pellet. The cells were vortexed for 20 s, transferred to a 2.0 mL Eppendorf tube and incubated at 65°C for 30 min with occasional vortexing. The cells were then placed on ice for 2 min and centrifuged at

15,000 rpm for 10 min. The aqueous phase was transferred to a new tube, and 750 μL of TRIzol® LS was added. The mixture was then vortexed and placed at room temperature for 5 min. Two-hundred microliters of chloroform was added, and the mixture was vortexed and centrifuged at 15,000 rpm for 5 min. The aqueous phase was transferred to a new tube, and an additional 500 μL of chloroform was added, vortexed and then centrifuged at 15,000 rpm for 5 min. The aqueous phase was transferred to a new tube, and 500 μL of isopropanol was added to precipitate RNA. The mixture was vortexed and centrifuged (15,000 rpm, 5 min), and the pellet was dissolved in 200 μL of DEPC-treated deionized water. Six-hundred microliters of 99% ethanol and 20 μL of 3 M NaOAc (pH 5.2) were added, and the mixture was stored at −80°C for 60 min to precipitate the RNA. The pellet obtained by centrifugation (15,000 rpm, 5 min) was dried under a vacuum for 7 min and dissolved in 20 μL of DEPC-treated deionized water. Quality and quantity of total RNA was checked spectrophotometrically, and the samples were stored at −80°C until further use. The collected RNA samples were further purified and concentrated using the Qiagen RNeasy Mini Kit and treated with DNase according to the manufacturer's instructions. The quality of purified RNA was confirmed by formaldehyde agarose gel electrophoresis. The cDNA was synthesized and fluorescently labeled using the Superscript™ Plus Indirect cDNA Labeling System (Invitrogen) according to the manufacturer's instructions. Fifty micrograms of RNA was used for cDNA synthesis. Following cDNA synthesis, 25 μL of 100 mM EDTA and 10 μL of 1 M NaOH were added to the mixture and incubated at 60°C for 30 min in order to hydrolyze and degrade the original RNA. The reaction mixture was then neutralized by addition of 10 μL of 1 M HCl. The cDNA was purified using the Microcon YM-30 Centrifugal Filter Unit (Millipore) according to the manufacturer's instructions, eluted with 20 μL sterilized water, and pelleted by speedvac centrifugation. The pellet was resuspended in 10 μL of 50 mM $NaHCO_3$ and placed at room temperature for 3 min. Ten microliters of Alexa Fluor® Reactive Dye was added (Alexa Fluor® 647 for control cells and Alexa Fluor® 555 for cells recovering from heat injury), and the mixture was incubated in the dark at room temperature for 16 h to allow for coupling of the dye to cDNA. The fluorescently labeled cDNA was purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions and stored at −20°C until further use.

Four micrograms of each fluorescently labeled cDNA was hybridized to the Filgen Array *Salmonella typhimurium* LT2 DNA microarray (Filgen, Combimatrix) according to the manufacturer's instructions, with hybridization settings at 50°C for 16 h. The microarray was scanned using the FLA-8000 Fluorescent Image Analyzer (Fujifilm), and the raw data were abstracted using ArrayVision™ (version 6.0).

The raw data were analyzed using TIGR MIDAS (version 2.19) for Lowess normalization of data when necessary. Normalized data were then Slice analyzed with a data keep range of greater than 2.0σ ($P < 0.05$). Cluster (version 2.11) and TreeView (version 1.60) software were used to group genes with similar expression patterns and for creating heat map representations of gene transcription. Transcription levels with an increase or decrease of greater than twofold were considered to be significant.

Results

Heat injury and recovery

The effects of heating temperature on culturability after heat treatment on *S. typhimurium* were shown in Fig. 1. Culturable counts on DHL were lower than those of TSA at all temperatures ($P < 0.05$). The difference between TSA and DHL culturable counts, or the number of injured cells, was found to be highest after heat treatment at 55°C ($P < 0.05$).

The changes of culturability after heat treatment and during recovery of *S. typhimurium* were shown in Fig. 2. Culturable counts decreased from 10^7 CFU/mL by plating on TSA and to 10^4 CFU/mL by plating on DHL agar immediately after heat injury at 55°C. This showed that 99.99% of *S. typhimurium* cells that formed colonies on TSA were injured but recoverable and is in agreement with findings for *S. enteritidis* (Kobayashi et al. 2004). The total culturable cell counts on TSA began to increase after 3 h recovery in TSB, whereas that for DHL increased within

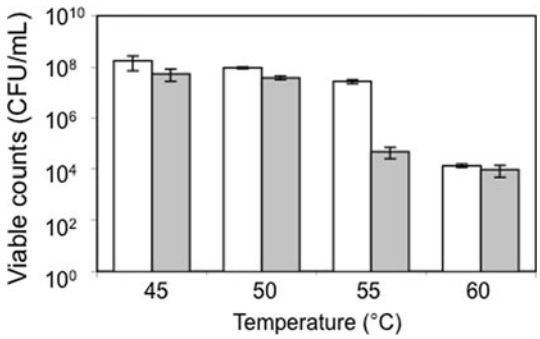

Fig. 1 Effects of heating temperature on culturability after heat treatment on *Salmonella typhimurium*. Bacterial cells were suspended in phosphate buffered saline (pH 7.4) at 10^8 CFU/mL and heated at 45, 50, 55, or 60°C for 15 min. Viable counts were determined by plating on TSA (*white bars*) and DHL agar (*gray bars*). Mean ± SD was calculated from the data for three separate experiments

1 h. The culturable counts on DHL reached a similar level to that of TSA after 3 h incubation.

Analysis of gene expression of heat-injured cells after 30- and 60-min recovery

Salmonella typhimurium cells were heat injured for 15 min and recovered in TSB for 30 or 60 min before RNA was extracted. Due to the low amount of RNA present in heat-injured cells, the RNA extraction procedure was repeated approximately 15 times to obtain sufficient RNA for microarray analysis. In contrast, sufficient RNA was obtained from the control cells after approximately two RNA extractions. cDNA was synthesized from the total RNA, coupled with Alexa Fluor® dyes, hybridized to the Combimatrix *Salmonella typhimurium* LT2 DNA microarray, scanned and analyzed.

As a result of 30-min recovery from heat injury, it was found that transcription levels for 89 genes increased and that for 17 genes decreased. Genes with increased transcription were divided into four broad categories: putative proteins (40 genes), stress-induced (14 genes), energy generation and metabolism (16 genes) and miscellaneous (19 genes) (Table 1).

Sixty-minute recovery of heat-injured cells resulted in increased transcription of 141 genes and decreased transcription of 64 genes. Genes with increased transcription were divided into four broad categories: putative proteins (67 genes), stress-induced (17 genes), energy generation and metabolism (14 genes), and miscellaneous (43 genes) (Table 2).

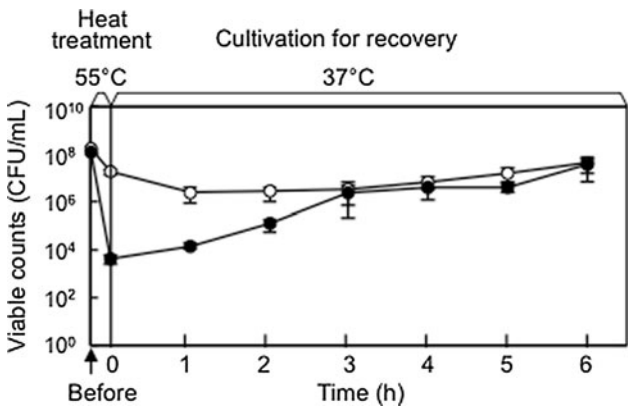

Fig. 2 Changes in culturability of *Salmonella typhimurium* after heat injury and during recovery. Bacterial cells were suspended in phosphate buffered saline (pH 7.4) at 10^8 cfu/mL and heated at 55°C for 15 min. An equal volume of 2× TSB was added to the heat-treated suspension and incubated for 6 h. Viable counts were determined by plating on TSA (*open circles*, CFU/mL) and DHL (*filled circles*, CFU/mL) agar. Mean ± SD was calculated from the data for three separate experiments

Cluster and TreeView analysis of DNA microarray results

The results of DNA microarray were grouped based on gene expression patterns using Cluster (version 2.11) and visualized using TreeView (version 1.60). Genes with increased transcription for the *S. typhimurium* recovering cells were considered to be important in recovery. This analysis resulted in the identification of 15 genes, with known function, seemed to be somewhat involved in recovery. They encoded proteins involved in branched-chain amino acid (BCAA) transport (*livJ*, *livH*), cell envelope integrity (*ddg*), heat-shock response (*cpxP*, *rrmJ*), phage shock protein (*pspA*), ribosome modulation factor (*rmf*), virulence (*sseB*) transcriptional regulation (*rpoE*, *rpoH*, *rseA*, *rseB*, *rseC*) and ArcB signal transduction (*sixA*) and cytoplasmic membrane protein (*fxsA*). In addition, genes encoding putative proteins (*stm1250*, *stm1251, stm1252, stm2954.1N, stm3651, stm4031*) and a hypothetical protein (*ybeD*, *ydiV*) were also found to be important for recovery.

Effects of BCAA addition on recovery from heat injury of *S. typhimurium*

The heat recovery assay with branched-chain amino acids (BCAA) supplementation was performed on heat-injured *S. typhimurium*. It was found that supplementation of TSB with 0.1% and 0.05% BCAA resulted in an enhanced recovery of injured cells in comparison to those recovered in TSB without BCAA (Fig. 3). The injured cells supplemented with 0.1% and 0.05% BCAA were found have comparable TSA and DHL counts after 2 h incubation ($P < 0.05$). It was found that after 1 h incubation, the cell count relative to time zero increased by 12.8-, 19.0-, 57.3-, 23.8-, 16.9- and 8.6-fold for control, 0.167, 0.1, 0.05, 0.01, and 0.002% BCAA supplementation, respectively (Fig. 4). In particular, 0.1% BCAA supplementation resulted in a cell count increase 4.4-fold greater than that of the control after 1 h incubation.

Discussion

Sublethally injured cells are characterized by injured permeability barriers and/or decreased metabolic activities, which render them to be more susceptible to selective agents (Wu 2008). Injured cells are able to form colonies on non-selective media such as TSA but not on selective media such as DHL. Therefore, the colony counts on TSA can be defined as the total viable cell count and that on DHL can be defined as the intact cell count. The difference

Table 1 DNA microarray analysis of *S. typhimurium* gene transcription after 30-min recovery

Gene group	Genes with increased transcription during recovery from heat injury
Putative proteins (40 total)	
Cytoplasmic proteins (15 total)	*stm1250* (8.6), *stm1252* (7.6), *stm4031* (7.2), *ymdF* (6.3), *stm1513* (5.1), *stm1267* (5.0), *yfiP* (4.8), *yciE* (4.1), *yliH* (3.8), *stm3411* (3.5), *stm4030.S* (3.5), *yciG* (2.9), *stm3777* (2.6), *stm1665* (2.4), *yecE* (2.2)
Cell envelope protein (10 total)	*yfeK* (4.4), *cdaR* (4.0), *yggM* (3.8), *stm3030* (3.7), *stcD* (3.4), *ychH* (3.2), *envF* (3.2), *stm0212* (3.0), *stcC* (2.2), *stm0345* (2.1)
Other (15 total)	*stm1251* (10.2), *ydiV* (8.0), *ybgS* (4.7), *stm1255* (3.8), *fixX* (3.6), *yhjW* (3.4), *stm4508.1N* (3.3), *stm0867* (3.1), *stm0581* (2.7), *stm0347* (2.4), *allP* (2.4), *ygaU* (2.3), *stm0014* (2.3), *stm1548.s* (2.1), *stm2954.1N* (2.1)
Stress induced (14 total)	
Cytoplasmic heat shock protein (1 total)	*ibpA* (2.5)
Cell envelope heat shock protein (4 total)	*cpxP* (8.9), *fkpA* (5.0), *htrA* (2.9), *spy* (2.7)
Phage shock protein response (5 total)	*pspA* (6.3), *pspC* (6.0), *pspG* (5.8), *pspB* (5.5), *pspE* (2.1)
Other (4 total)	*rmf* (8.1), *trxC* (6.8), *bcfB* (4.5), *mutM* (3.3),
Energy generation and metabolism (16 total)	
Fatty acid metabolism (4 total)	*fadA* (6.9), *fadB* (5.7), *fadE* (4.8), *fadL* (2.7)
Pyruvate metabolism (1 total)	*aceF* (5.2)
Glyoxylate bypass (2 total)	*aceB* (6.2), *aceA* (5.5)
Amino acid metabolism and transport (8 total)	*livJ* (8.8), *livH* (7.1), *pphB* (5.6), *livF* (5.4), *livK* (5.3), *livG* (4.1), *ansB* (3.2), *gabT* (2.6)
Vitamin metabolism (1 total)	*thiH* (2.0)
Miscellaneous (19 total)	
Transcriptional regulation (7 total)	*rseA* (13.4), *rpoE* (12.3), *rseB* (11.5), *rpoH* (11.0), *rseC* (9.2), *envR* (3.9), *nhaR* (3.9)
Virulence genes (3 total)	*sseB* (5.1), *bcfA* (4.1), *ssaM* (2.8)
Flagellar assembly (1 total)	*fliC* (2.7)
Metal homeostasis (2 total)	*cutC* (5.9), *znuC* (2.9)
Membrane integrity (2 total)	*ddg* (10.6), *smpA* (2.4)
Other (4 total)	*sixA* (7.0), *stm2636* (4.3), *stm3558* (3.6), *rpsV* (2.2)

Total RNA was extracted from *Salmonella typhimurium* cells after 30-min recovery, used for cDNA synthesis, and hybridized to *Salmonella typhimurium* LT2 DNA microarrays. Genes with increased transcription were classified based on function. Numbers in parentheses represent the fold increase in transcription

in number of colony counts on TSA and DHL can thus be defined as the injured cell count (Kobayashi et al. 2004).

Heat-injury experiments at 45, 50, 55, and 60°C showed that the greatest number of injured cells was obtained with heat treatment at 55°C. At 60°C heat treatment, the total viable cell count decreased drastically for *Salmonella*, suggesting that the heat stress at 60°C is too much for cells to recover from. Therefore, the recovery experiments were done with a heat treatment temperature of 55°C to maximize the number of injured cells. Heat treatment at 55°C resulted in injured cell counts of approximately 10^7 cfu/mL.

Recovery from heat injury for *S. typhimurium* was found to be similar to that done in a previous study by Kobayashi et al. (2004) using *S. enteritidis*. *S. typhimurium* cells were able to fully recover and form colonies on DHL after 3 h of recovery in TSB. The DNA microarray analysis of 30- and 60-min recovering *S. typhimurium* cells resulted in an increase in transcription of 89 and 141 genes, respectively. However, approximately half of the genes were found to encode putative proteins. Although the *S. typhimurium* genome has been sequenced, the translational products of many of the genes are still undetermined. Therefore, we have limited the analysis of DNA microarray results to genes encoding proteins of known functions.

It was found that transcription levels of both the *rpoE* and *rpoH* gene increased after 30- and 60-min recoveries. However, the transcription levels for *rpoS* were found to be unchanged, suggesting that translational regulation by σ^E and σ^H are important during recovery from heat injury. The analysis using Cluster (version 2.11) resulted in the identification of 15 genes, with known function, seemed to be somewhat involved in recovery from heat injury detected as the difference in plate counts between TSA and DHL

Table 2 DNA microarray analysis of *S. typhimurium* gene transcription after 60-min recovery

Gene group	Genes with increased transcription during recovery from heat injury
Putative proteins (67 total)	
Cytoplasmic proteins (18 total)	*stm1252* (8.1), *stm1250* (7.9), *stm4031* (7.3), *yfiP (6.0), stm3411* (5.3), *stm4030.S* (4.6), *stm3906* (4.5), *stm4239* (3.7), *yhdN* (3.3), *stm1410* (3.3), *yciG* (3.1), *stm4157* (3.1), *stm1665* (3.0), *stm3652* (3.0), *yhhV* (2.6), *stm3907* (2.3), *yaiB* (2.2), *yciE* (2.1)
Cell envelope protein (20 total)	*yggM* (4.9), *envF* (4.5), *stcD* (4.4), *stm0345* (4.2), *stm4155* (4.0), *stm3030* (4.0), *stm4261* (3.8), *stm0342* (3.5), *stm0437* (3.5), *stcC* (3.2), *ycfR* (3.1), *yehE* (2.9), *stm4552* (2.5), *yjcB* (2.3), *stm0839* (2.2), *stm1994* (2.1), *yfiM* (2.3), *yggN* (2.4), *yfeK* (2.1), *yebF* (2.0)
Other (29 total)	*stm1251* (9.7), *ybeD* (7.9), *stm2954.1N* (7.7), *ydiV* (7.2), *stm3651* (7.0), *stm0581* (5.6), *stm0014* (5.6), *stm0347* (5.5), *stm4508.1N* (5.3), *mntH* (5.0), *stm1255* (4.3), *stm2552* (3.9), *ybgS* (3.8), *ycjX* (3.7), *yciM* (3.6), *stm3020* (3.5), *stm0015* (3.2), *ydiU* (2.5), *stm1551.1N* (2.9), *stm2620* (2.9), *stm0084* (2.8), *yheL* (2.5), *stm3133* (2.5), *stm1548.S* (2.5), *stm0030* (2.4), *stm1019* (2.4), *ygiM* (2.4),*ybaO* (2.2), *stm1014* (2.4)
Stress induced (17 total)	
Cytoplasmic heat shock protein (6 total)	*rrmJ* (7.4),*dnaJ* (5.4), *groES* (3.0), *ibpB* (2.4), *dnaK* (2.3), *clpX* (2.1),
Cell envelope heat shock protein (4 total)	*cpxP* (8.0), *fkpA* (6.9), *htrA* (2.6), *spy* (3.3),
Phage shock protein response (4 total)	*pspA* (7.0), *pspG* (5.4), *pspB* (3.3), *pspC* (3.3),
Other (3 total)	*bcfB* (5.2), *trxC* (3.8), *msrA* (2.7)
Energy generation and metabolism (14 total)	
Fatty acid metabolism (2 total)	*fadB* (4.9), *fadE* (4.0)
Pyruvate metabolism (1 total)	*dcoB* (3.5)
Glyoxylate bypass (1 total)	*aceA* (2.5)
Amino acid metabolism and transport (7 total)	*livJ* (8.3), *pphB* (6.2), *livH* (4.6), *livK* (3.4), *livF* (3.3), *gltI* (3.1), *livM* (2.4)
Vitamin metabolism (2 total)	*celF* (2.8), *mglA* (2.7)
Oxidative phosphorylation (1 total)	*dsbD* (4.5)
Miscellaneous (43 total)	
Transcriptional regulation (8 total)	*rpoE* (11.0), *rseA* (11.2), *rseB* (9.3), *rpoH* (8.1), *rseC* (4.7), *greA* (4.6), *nhaR* (4.4), *envR* (3.1)
Virulence genes (18 total)	*sseB* (8.6), *hilC* (6.4), *sseC* (5.9), *ssaG* (5.8), *sifA* (4.7), *bcfA* (4.5), *ssaL* (4.5), *pipB2* (3.8), *ssaM* (3.5), *ssaH* (3.3), *sseE* (3.3), *ssaP* (3.2), *spaS* (3.0), *ssaI* (2.6), *ssaO* (2.6), *invB* (2.5), *ssaK* (2.4), *msgA* (2.1)
Metal homeostasis (4 total)	*cutC* (4.9), *znuC* (3.2), *iscA* (3.1), *bfd* (2.9)
Membrane integrity (1 total)	*ddg* (10.1)
Ribosomal protein (2 total)	*rpmE2* (5.9), *rpmJ2* (3.0)
DNA replication (1 total)	*dnaG* (2.6)
Other (9 total)	*fxsA* (7.3), *stm3558* (5.3), *stm2636* (3.9), *sixA* (3.1), *stm1287* (2.9), *kdpB* (2.7), *stm0438* (2.5), *stm2616* (2.5), *nrdH* (2.4)

Total RNA was extracted from *Salmonella typhimurium* cells after 60-min recovery, used for cDNA synthesis, and hybridized to *Salmonella typhimurium* LT2 DNA microarrays. Genes with increased transcription were classified based on function. Numbers in parentheses represent the fold increase in transcription

agar. The *liv* genes are known to encode proteins involved in the high-affinity transport of branched-chain amino acids (BCAA) across the inner membrane of Gram-negative bacteria. The *livJ* gene encodes the leucine-, isoleucine-, valine-binding protein (LIV-BP), the *livK* gene encodes the leucine-specific binding protein (LS-BP), and the *livFGHM* genes encode an ABC transporter. LivJFGHM proteins make up the LIV-I system, which is able to transport all BCAA, whereas the LivKFGHM proteins make up the LS-system, which transport leucine specifically (Adams et al. 1990). The branched-chain amino acids (BCAA) are known to be a quantitatively important group of amino acids, making up 20% of the total protein amino acids in Gram-negative bacteria (Garault et al. 2000). Heat injury is known to cause structural damage to cells, which results in the denaturation of proteins and loss of cellular materials through leakages in the permeability barrier (Busta 1978; Wu 2008). As a result, cell wall and protein synthesis are important in the repair process (Busta 1976). Therefore, amino acids, which are the building blocks of proteins, are

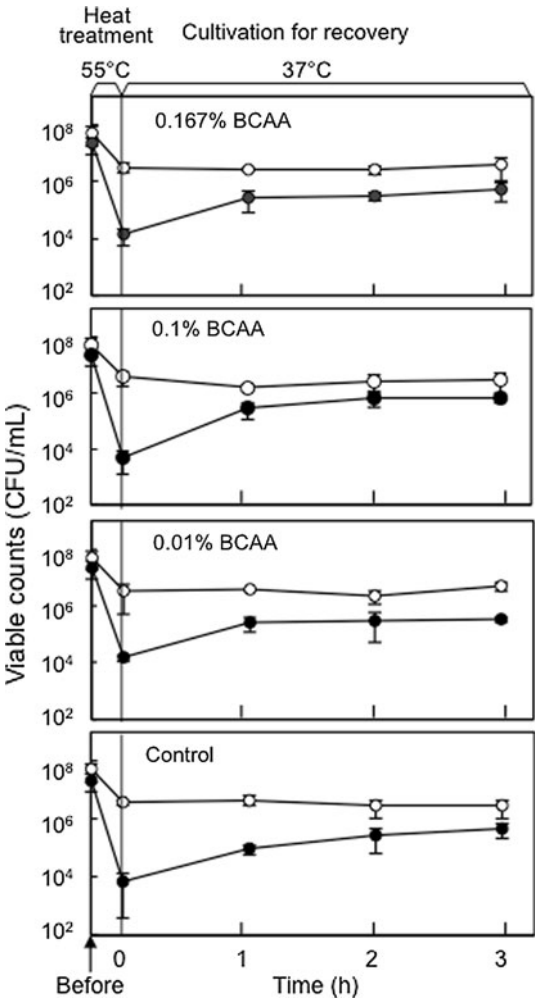

Fig. 3 Change in culturability of *Salmonella typhimurium* recovered in BCAA supplemented TSB. Bacterial cells were suspended in phosphate buffered saline (pH 7.4) at 10^8 CFU/mL and heated at 55°C for 15 min. An equal volume of 2× TSB supplemented with BCAA was added to the heat-treated suspension and incubated for 3 h. Final concentration of BCAA in the mixture was 0, 0.01, 0.1 or 0.167%. Viable counts were determined by plating on TSA (*open circles*, CFU/mL) and DHL (*filled circles*, CFU/mL) agar. Mean ± SD was calculated from the data for three separate experiments

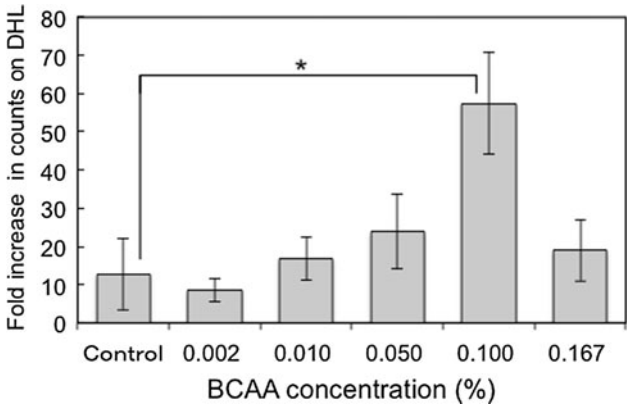

Fig. 4 Intact cell count increase, relative to time zero, of *Salmonella typhimurium* after 1 h recovery in TSB supplemented with BCAA. Mean ± SD was calculated from the data for three separate experiments of Fig. 3. *$P < 0.05$

necessary for recovery. Due to the increase in transcription of the *liv* gene cluster during recovery, it was hypothesized that BCAA are important for the recovery of injured cells. Therefore, in this study, we focused on function of BCAA in the recovery from heat injury. We investigated the effect of BCAA supplementation to the recovery broth during recovery.

It was found that BCAA supplementation during recovery did in fact improve recovery. In particular, injured cells incubated with 0.1% or 0.05% BCAA showed comparable TSA and DHL counts after 2 h recovery (Fig. 3). The colony count increase on DHL after 1 h recovery peaked at 0.1% BCAA supplementation. Higher and lower BCAA concentrations resulted in a decrease in colony count increase (Fig. 4). It has been shown that high concentrations of leucine (Quay et al. 1977) and valine (Lawther et al. 1981) can be toxic to cells, which could result in an upper limit of beneficial effects from BCAA supplementation. Further experiments are required to determine the optimal concentration of BCAA for recovery of heat-injured cells.

The effect of BCAA supplementation in humans has been studied extensively. In past studies it has been reported that BCAA supplementation can decrease exercise-induced muscle damage and promote muscle protein synthesis (Blomstrand et al. 2006; Negro et al. 2008). Several studies have shown that leucine in particular acts as a signal to stimulate protein synthesis (Buse and Reid 1975; Lynch et al. 2003). Similar roles for leucine have been observed in prokaryotes, where it interacts with the leucine-responsive regulatory (Lrp) protein (Haney et al. 1992; McFarland and Dorman 2008). Lrp is a global regulator of gene expression whose activity can be modulated by the presence of L-leucine. Operons regulated by Lrp range in function, including those for amino acid biosynthesis, amino acid degradation, transport and pili formation (Calvo and Matthews 1994). In particular, the operons for amino acid biosynthesis and transport are positively regulated by Lrp, while those for amino acid catabolism are negatively regulated, which would promote synthesis of proteins. A recent microarray study has shown that Lrp affects the transcription of 10% of genes in *E. coli* and 70% of stationary phase stress-induced genes (Tani et al. 2002); therefore, the full scope of Lrp regulation has yet to be fully understood. It is possible that the supplementation of BCAA may improve recovery via a mechanism involved with global regulation by Lrp, which would promote protein synthesis to replace those damaged from heat injury.

The heat injured *S. typhimurium* cells have damage in permeability barriers (surface structures and the cytoplasmic membrane) and are not able to form colonies on selective DHL agar since sodium deoxycholate, a selective reagent in the medium, has a strong solvent action (Leifson 1935). It seems that the addition of BCAA contributed to the recovery from the injury in metabolic pathways and in permeability barriers detected by the differences in plate counts between TSA and DHL agar.

The results of the DNA microarray analysis have aided in the identification of several genes hypothesized to be important for recovery from heat injury. However, the limitation of DNA microarray analysis is its inability to account for post-transcriptional regulation, and thus, it is unknown how much of the synthesized RNA is actually translated to protein. Therefore, the results of this study are intended to serve only as a basis for elucidating the molecular biology of recovery of heat-injured cells. In further studies, we intend to clarify the function of the genes seemed to be somewhat involved in recovery from heat injury.

Conflict of interest The authors declare no conflict of interest.

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