Construction of Several Delection Mutants for Genes Involved in Biofilm Formation and Recovery of Heat-injured Salmonella: Delta {agfA} and Delta {bcsA} Mutants of Salmonella Enteritidis; Delta {ahpC}, Delta {ahpF}, and Delta {katG} Mutants of S. Typhimurium; and Delta {rpoE}, Delta {rpoH}, and Delta {rpoS} Mutants of S. Enteritidis and S. Typhimurium

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Construction of Several Deletion Mutants for Genes Involved in Biofilm Formation and Recovery of Heat–injured *Salmonella*: *ΔagfA* and *ΔbcsA* Mutants of Salmonella Enteritidis; *ΔahpC*, *ΔahpF*, and *ΔkatG* Mutants of *S.* Typhimurium; and *ΔrpoE*, *ΔrpoH*, and *ΔrpoS* Mutants of *S.* Enteritidis and *S.* Typhimurium

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Salmonella is one of major food–poisoning bacteria. In food companies, controlling the bacterial growth is critically important to continue provision of good products to customers. At first, to study the attachment of the bacteria to food materials and final products, we focused on biofilm formation that is thought to be one of the reasons of the attachment. Thus, the genes related in formation of biofilm were also focused on, and *ΔagfA* and *ΔbcsA* mutants of *Salmonella* Enteritidis were constructed by using 2–step PCR and pKOBEGA helper plasmid. Secondary, we focused on heat–injured *Salmonella* after sublethal heat treatment as previously reported (Kobayashi *et al.*, 2005). The heat–injured *Salmonella* showed expression of several specific genes to recover the bacterial functional state as well as other bacteria. To clarify the mechanism of the recovery, several gene mutants (*ΔahpC*, *ΔahpF*, and *ΔkatG* mutants of *Salmonella* Typhimurium, and of *ΔrpoE*, *ΔrpoH*, and *ΔrpoS* mutants of *S.* Enteritidis and *S.* Typhimurium) were constructed.

INTRODUCTION

In food industry, controlling bacteria is seriously important for keeping good quality of food products. To overcome bacterial contamination, attachment of food–poisoning bacteria to food materials, survival of bacteria after food processing, and secondary contamination of bacteria in final products should be considered. Attachment of bacteria is often reported to linked to biofilm formation (Austin *et al.*, 1998; Solano *et al.*, 2002). Bacteria in biofilm are attached and semi–immobilized on the surface of foods. Thus, sensitivity of the attached bacteria to sterilizers or food processing steps such as mild heat treatment is reduced, leading their contamination in final products.

Biofilm is formed of mainly polysaccharides and proteins. Cellulose and curli fimbriae are suggested to be involved in the attachment of *Salmonella* and biofilm formation (Austin *et al.*, 1998; Gerstel and Römling, 2003; Solano *et al.*, 2002). The *bcs* (bacterial cellulose synthesis) operon plays an important role in the cellulose synthesis (Solano *et al.*, 2002) and the *agf* (aggregative fimbriae) operon plays an important role in the formation of curli fimbriae (Collinson *et al.*, 1996). In particular, *bcsA* encodes a catalytic subunit of cellulose synthetic enzyme and the enzyme synthesizes cellulose from a precursor UDP–glucose (Solano *et al.*, 2002).

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, however results in the denaturation of food products. Mild heat or insufficient heat treatment may only sublethally injure bacterial cells, which may still pose a risk for foodborne 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 (Chambliss *et al.*, 2006; Kand and Fung, 2000; Wu, 2008), however the molecular biology of the mechanisms for recovery is still unclear. 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 our previous study (Kobayashi *et al.*, 2005), factors involved in the recovery of *Salmonella* Enteritidis were examined. The levels of transcription of 86 stress–inducible genes were investigated by reverse transcription polymerase chain reaction (RT–PCR). Nineteen heat–inducible (*clpB, clpX, degP, dnaJ, fkpA, ftsJ, gapA, hflB, hslJ, hslU, hslV, htpG, htrA, lon, mopA,*

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mopB, mreB, rpoE, and *ppiD*), and 12 oxidative–stress and DNA damage–inducible (*ahpC, ahpF, fldB, fur, grxA, dinF, katG, mutM, recA, soxR, trxC*, and *zwf*) genes were transcribed extensively. Out of them, it was found that the *ahpC* gene encoding a small subunit of alkyl hydroperoxide reductase, the *ahpF* gene encoding a large subunit of the enzyme, the *katG* gene encoding catalase, and the *rpoE* gene encoding the extracytoplasmic heat stress sigma factor E (σ^E), were significantly up–regulated during recovery.

Heat treatment is well known to accompany generation of reactive oxygen species (ROS) leading to oxidative stress. Because the *ahpC*, *ahpF*, and *katG* genes are involved in scavenging ROS, the induction of three genes would be necessary to avoid extension of oxidative damages during heat treatment. The *rpoE*, *rpoH* and *rpoS* genes, which encode the RNA polymerase sigma factors E, H and S, respectively, are known to play significant roles in stress responses. Sigma factor E (σ^E) plays a major role in protection of bacterial cells against extracytoplasmic or cell envelope stress (Raivio and Silhavy, 2001), sigma factor H (σ^H) protects against cytoplasmic stress (Morita *et al*., 1999), while sigma factor S (σ^S) is associated with the general stress response in cells (Hengge–Aronis, 2002). These sigma factors promote the binding of RNA polymerase to specific promoters and thus aid in gene transcription. In particular, the heat shock promoters associated with the heat shock response are known to primarily encode chaperones and proteases that aid in coping with heat stress (Morita *et al*., 1999; Raivio and Silhavy, 2001).

To make clear functions of a gene, physiological studies using deletion mutants are often performed. In *Salmonella*, single gene deletion mutant of *Salmonella* have previously been constructed in using a lambda–Red mediated method. In this method, the bacteriophage λ's Red function genes are encoded on a temperature sensitive plasmid pKOBEGA (Solano *et al*., 2002). The Red function genes include γ, β, and *exo*, which replace the RecBCD functions to promote the recombination of linear DNA fragments with chromosomal DNA (Chaveroche *et al*., 2000).

In this study, in order to elucidate the mechanisms of biofilm formation of *Salmonella* and of recovery for heat–injured *Salmonella*, we tried to construct the deletion mutants for the *agfA, bcsA, ahpC, ahpF, katG, rpoE, rpoH*, and *rpoS* genes, respectively by using λ–Red mediated method.

MATERIALS AND METHODS

Bacterial strains and plasmids

Salmonella Typhimurium NBRC 12529 (ST) and *S*. Enteritidis IFO 3313 (SE) were obtained from National Institute of Technology and Evaluation–Biological Resource Center (NBRC). The pKOBEGA plasmid was kindly provided by Dr. J–M. Ghigo, Institute Pasteur, France. The pACYC177 plasmid was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Culture conditions

SE and ST cells were cultured overnight in TSB at 37 °C with shaking at 130 rpm to obtain cells in stationary phase of growth.

Genomic DNA preparation

DNA was extracted from 2 ml of overnight culture of SE or ST using DNeasy Tissue Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions.

Preparation of gene fragments using 3–step PCR

Amplification of gene fragment for homologous recombination was performed using 3–step PCR as described in Fig. 1. Ex Taq DNA polymerase (Takara), KOD Dash DNA polymerase (Toyobo, Osaka, Japan), or Phusion DNA polymerase (Finnzymes, Keilaranta, Finland) were used for following PCR equipped with a Takara PCR Thermal Cycle Dice (Takara).

In the first PCR, two fragments (the upstream and the downstream (or internal) regions flanking the target gene) were amplified by PCR. For the both reactions, sequences of genomic DNA of *S*. Typhimurium (Accession number, NC_003197; McClelland *et al*., 2001) were used for designing primers listed in Table 1. The reverse

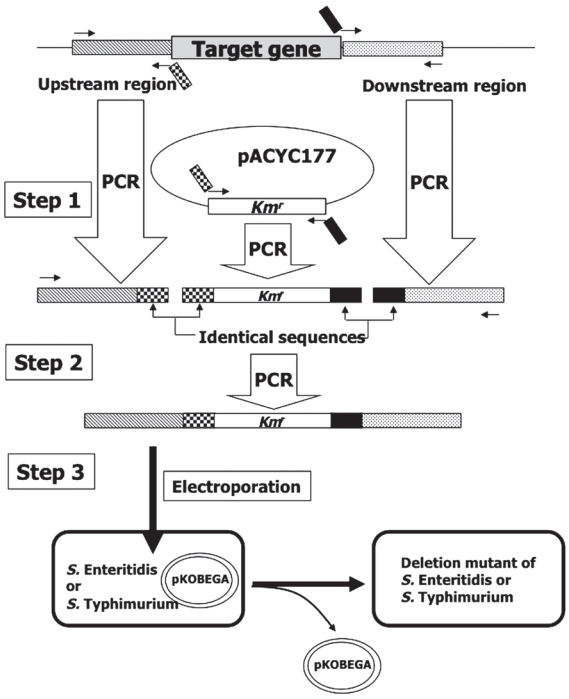

Fig. 1. Construction of *Salmonella* deletion mutant by 3 step–PCR method. The first step consists in amplifying independently the upstream and downstream regions of the target gene except for *bcsA* and resistance marker (*Km^r*). In the case of *bcsA*, internal region was used instead of downstream region. The two PCR products obtained in step 1 were mixed with the amplification product of *Km^r* at equimolar concentrations and submitted to a second round of PCR to generate a resistance marker cassette flanked by upstream and downstream regions homologous to the target gene. In the third step, large amount of the desired linear DNA are electroporated into competent cells of *S*. Enteritidis or *S*. Typhimurium carrying pKOBEGA.

Table 1. Primers used for amplification of upstream, internal, and downstream regions of target genes

Target gene	Strain	Amplified part	Primer name	Sequence	Gene ID used for primer design	Size of region for amplification (bp)	Site on genome of SE (NC_ 011294) or ST (NC_003197)
agfA	*S.* Enteritidis (SE)	Upstream region	agfA–u–F1	5'–AAT CGC ACA TCT GAC AGC TG–3'	GI: 1184712	1231	2008271–2008290
			agfA–u–M13R1	5'–CTG GCC GTC GTT TTA CAA CGT CGT GAG GTA AAA CCC CCA TCG GAT TGA TT–3'*			2007060–2007083
		Downstream region	agfA–d–M13F1	5'–CAT GGT CAT AGC TGT TTC CTG TGT GAA GCG TCT GCG CTA ATA AAA A–3'		829	2006581–2006600
			agfA–d–R2	5'–TAC TCA ATA TTC ATC ACC GCC AGG C–3'			2005772–2005796
bcsA	SE	Upstream region	bcsE–R1	5'–CGC ATC AGA TTT AGC ATG TGA ATC AAT–3'	GI: 6948684	925	3685423–3685449
			M13–EF1	5'–CAT GGT CAT AGC TGT TTC CTG TGT GAA TCG TCC TGT AAC TGA CTG CCA ATG C–3'			3684525–3684550
		Internal region	m13–AF4	5'–CTG GCC GTC GTT TTA CAA CGT CGT GAG AAC GGG TGC GCC AAA AGA CA–3'		1008	3683929–3683949
			bcsA–R2	5'–AGT ATG CGC ATC TTC CGT CAC–3'			3682942–3682962
ahpC	*S.*Typhimurium (ST)	Upstream region	ahpC–up–F	5'–AGC TAG CTA GGA ATT CGT CGC GTT AGC GCC AAG ATC GTC CAT TAG C–3'	GI: 1252128	1042	669358–669388
			ahpC –up–R	5'–CTG GCC GTC GTT TTA CAA CGT CGT GGC GTT AAC TAA CAA CCC GAT TCG GGC GAC A –3'			670332–670360
		Downstream region	ahpC–down–F	5'–GAC CAC TGT GGA TCC AAG CAG TAG CCAG CAC CAT GAC GCA AGT TGC ATT AAT GCA G–3'		976	671175–671205
			ahpC–down–R	5'–GAT CGA TCG AGA ATT CCG ATA ACG TCA ACG TCG TAA TCG CTG ACA TGC–3'			672078–
ahpF	ST	Upstream region	ahpF–up–F	5'–CAA TGG CTT TAA CCG GCG CAG GCA GTT CTT–3'	GI: 1252129	1267	669927–669956
			ahpF–up–R	5'–CTG GCC GTC GTT TTA CAA CGT CGT GAT GGG CGC GGG TGC GCC CAT GAC TGA AAC AA–3'			671135–671165
		Downstream region	ahpF–down–F	5'–GAC CAC TGT GGA TCC AAG CAG TAG CCC TGG GGA ACG CAC CGG GTA AAA CAC CTT C–3'		1045	672907–672936
			ahpF–down–R	5'–TGA CCC CGT CCT TTA CCC ATG TCT TGA GGC–3'			673897–673926
katG	ST	Upstream region	katG–up–F	5'–AGC TAG CTA GGA ATT CTT TTT TCA CGC CAA CCA GCG GGA AGC CCT G–3'	GI: 1255633	915	4318534–4318563
			katG–up–M13R	5'–CTG GCC GTC GTT TTA CAA CGT CGT GAG TAA TAA AGC TCC CGG CAG GGA GCT GGA GG–3'			4319419–4319448
		Downstream region	katG–down–M13F	5'–CAT GGT CAT AGC TGT TTC CTG TGT GAC AGG TTA ATT CGT CAG CGG CTG CTA TTC GG–3'		868	4321769–4321798
			katG–down–R	5'–GAT CGA TCG AGA ATT CGC CGA ATATCG ACA CAG GCA CCT TAT CTG G–3'			4322607–4322636
rpoE	ST and SE	Upstream region	rpoE–up–F	5'–CGG GTG CGT CCG ACA GAA AT–3'	GI: 1254163	1035	2778343–2778362(ST)
							2730614–2730633(SE)
			rpoE–up–M13R	5'–CTG GCC GTC GTT TTA CAA CGT CGT GCC GCT TTG ATG GAT GGC GAA–3'			2779358–2779377(ST)
							2731629–2731648(SE)
		Downstream region	rpoE–down–M13F	5'–CAT GGT CAT AGC TGT TTC CTG TGT GCC GAG GTA ATG TCT CCC CAA–3'		949	2780004–2780023(ST)
							2732275–2732294(SE)
			rpoE–down–R	5'–CCC CGG CAG ACC CAT TTT AT–3'			2780933–2780952(ST)
							2733205–2733224(SE)
rpoH	ST	Upstream region	rpoH–up–F	5'–CTG ACT CTC CGG CGA TGT TA–3'	GI: 1255091	1053	3734816–3734835
			rpoH–up–R–M13Hsu	5'–AGT TGG TCA GTT CGG TTC CCT TAG CTG GAT AAG GCG TTT ACG C–3'			3735849–3735868
		Downstream region	rpoH–down–F2	5'–CAT GGT CAT AGC TGT TTC CTG TGT GAA CCG TGC CAA AGC CGA TGA–3'		997	3736827–3736846
			rpoH–down–R2	5'–CCA GCG TCT GTT ACA TGG TC–3'			3737804–3737823
rpoH	SE	Upstream region	rpoH–up–F	5'–CTG ACT CTC CGG CGA TGT TA–3'	GI: 1255091	1053	3615668–3615687
			rpoH–up–R–Kmr	5'–CAG GAT TTT GAG ACA CAA CGT GGG CTG GAT AAG GCG TTT ACG C–3'**			3616703–3616717
		Downstream region	rpoH–down–F2	5'–CAT GGT CAT AGC TGT TTC CTG TGT GAA CCG TGC CAA AGC CGA TGA–3'		997	3617679–3617698
			rpoH–down–R2	5'–CCA GCG TCT GTT ACA TGG TC–3'			3618656–3618675
rpoS	ST	Upstream region	rpoS–up–F	5'–CCG ATC ATT ACC CTG ATG GG–3'	GI: 1254447	984	3064399–3064418
			rpoS–up–M13R	5'–CTG GCC GTC GTT TTA CAA CGT CGT GTG CGT CAT GCG AAA CGC TTG–3'			3065363–3065382
		Downstream region	rpoS–down–M13F	5'–CAT GGT CAT AGC TGT TTC CTG TGT GTC CTA CCC GTG ATC CCT TGA–3'		1031	3066495–3066514
			rpoS–down–R	5'–TCA CAC CGC CGC CAA AAA TG–3'			3067506–3067525
rpoS	SE	Upstream region	rpoS–up–F2	5'–CCG ATC ATT ACC CTG ATG GG–3'	GI: 1254447	984	2949158–2949177
			rpoS–up–R1–M13	5'–CTG GCC GTC GTT TTA CAA CGT CGT GAT GCG TCA TGC GAA ACG CTT G–3'			2950122–2950141
		Downstream region	rpoS–down–F8–M13	5'–CAT GGT CAT AGC TGT TTC CTG TGT GTT ATC GCT GCG GTA AAT AAC GCA GC–3'		1486	2951308–2951332
			rpoS–down–soto–R	5'–CCG CCG GAA ATT CCT ACC GCG CTC ATG GCA–3'			2952764–2952790

*Underling part indicates M13 vector–derived sequence.

**Double–underlining part indicates *Km*^{r} gene–derived sequence.

primer of the upstream fragment and the forward primer of the downstream fragment included an overhang of the phage M13 vector–derived or kanamycin resistance (*Km*^{r}) gene sequences (Table 2).

The pACYC177 vector contains *Km*^{r} gene and then was used as a template for PCR amplification of a *Km*^{r} cartridge. Both the forward and reverse primers of the *Km*^{r} gene fragment also included an overhang of the phage M13 vector–derived sequence. The sizes of M13 vector–derived sequences were 23–25 bp and the regions were used to be homologous to one another so that the three DNA fragments (upstream, *Km*^{r} cartridge, downstream or internal) could be fused and amplified in the second PCR.

PCR mixtures were prepared according to the manufacturer's instructions and other PCR conditions were listed in Table 3.

Table 2. Primers used for amplification of *Km*^{r}cartridge for corresponding genes

Primer name	Sequence	Target gene replaced by *Km*^{r} cartridge
Kmr–M13F	5'–CAC GAC GTT GTA AAA CGA CGG CCA GAG CCA CGT TGT GTC TCA AAA TCT CTG ATG TT–3' *	*agfA, bcsA, ahpC, ahpF, katG, rpoE*(ST,SE), *rpoS*(ST, SE)
Kmr–M13R	5'–CAC ACA GGA AAC AGC TAT GAC CAT GAC CGT CCC GTC AAG TCA GCG TAA TGC TCT GC–3'	*agfA, bcsA, katG, ahpC, ahpF, rpoE*(ST,SE), *rpoH*(SE), *rpoS*(SE, ST)
Kmr–F–M13Hsu	5'–TAA GGG AAC CGA ACT GAC CAA CTC CA CGT TGT GTC TCA AAA TCT CTG–3'	*rpoH*(ST)
Kmr–R–M13Hsu	5'–CAC ACA GGA AAC AGC TAT GAC CAT GC CGT CCC GTC AAG TCA GCG–3'	*rpoH*(ST)
Kmr–F–rpoH	5'–GGC AGC GTA AAC GCC TTA TCC AGC CCA CGT TGT GTC TCA AAA TCT CTG ATG TTA–3'**	*rpoH*(SE)

*Underling part indicates M13 vector–derived sequence.
**Double–underlining part indicates *rpoH* gene–derived sequence.

Table 3. PCR conditions for amplification of respective regions of target genes, and of *Km*^{r} cartridge

Target gene	Amplified part	Enzyme used for PCR	Condition*			
			Pre–heating	Denaturation	Annealing	Extenstion
agfA	Upstream, downstream	KOD Dash	95°C, 3 min	94°C, 30 s	60°C, 10 s	74°C, 1 min
	Umstream–*Km*^{r}–downstream	Ex Taq	94°C, 1 min	98°C, 5 s	–	68°C, 5 min
bcsA	Upstream	Ex Taq	95°C, 3 min	98°C, 10 s	55°C, 30 s	72°C, 1 min
	Internal	Ex Taq	95°C, 3 min	98°C, 10 s	60°C, 30 s	72°C, 1 min
	Umstream–*Km*^{r}–internal	KOD Dash	95°C, 3 min	94°C, 30 s	60°C, 15 s	74°C, 1 min
ahpC	Upstream, downstream	Ex Taq	95°C, 3 min	95°C, 10 s	65°C, 30 s	74°C, 1 min
	Umstream–*Km*^{r}–downstream	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 3 min
ahpF	Upstream, downstream	Ex Taq	94°C, 3 min	94°C, 10 s	60°C, 30 s	74°C, 1 min
	Umstream–*Km*^{r}–downstream	Ex Taq	95°C, 3 min	95°C, 10 s	60°C, 30 s	74°C, 3 min
katG	Upstream, downstream	Ex Taq	94°C, 3 min	95°C, 10 s	56°C, 30 s	72°C, 1 min
	Umstream–*Km*^{r}–downstream	Ex Taq	95°C, 3 min	95°C, 10 s	56°C, 30 s	72°C, 3 min
rpoE	Upstream, downstream	Ex Taq	95°C, 3 min	98°C, 10 s	50°C, 30 s	72°C, 1 min
	Umstream–*Km*^{r}–downstream	Ex Taq	95°C, 3 min	95°C, 10 s	56°C, 30 s	72°C, 3 min
rpoH for ST	Upstream, downstream	Phusion	98°C, 30 s	98°C, 10 s	57°C, 30 s	72°C, 40 s
	Umstream–*Km*^{r}–downstream	Phusion	98°C, 30 s	98°C, 10 s	57°C, 30 s	72°C, 3 min
rpoH for SE	Upstream, downstream	Ex Taq	95°C, 3 min	95°C, 10 s	58°C, 10 s	74°C, 1 min
	Umstream–*Km*^{r}–downstream	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min 30 s
rpoS for ST	Upstream, downstream	Ex Taq	95°C, 3 min	95°C, 10 s	50°C, 30 s	72°C, 1 min
	Umstream–*Km*^{r}–downstream	Ex Taq	95°C, 3 min	95°C, 10 s	52°C, 30 s	72°C, 3 min
rpoS for SE	Upstream	Ex Taq	95°C, 3 min	95°C, 10 s	58°C, 10 s	74°C, 1 min
	Downstream	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min 30 s
	Umstream–*Km*^{r}–downstream	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min 30 s
Km^r cartridge		Ex Taq	95°C, 3 min	95°C, 10 s	56°C, 30 s	72°C, 1 min

*All PCR conditions were one cycle of pre–heating, followed by 35 cycles of denaturation, annealing, and extension.

Preparation of electrocompetent *Salmonella* containing pKOBEGA

Cells were cultured overnight in 5 ml of TSB at 37 °C. The following day, 1.5 ml of the culture was transferred to a fresh culture of 150 ml TSB and allowed to grow at 37 °C to an OD_{610} of 0.5–1.0. The culture was then centrifuged at 7,500 × *g* for 10 min at 4 °C, and the pellet was washed twice with 150 ml cold sterilized water and centrifuged. The pellet was resuspended in 5 ml of cold sterilized water and centrifuged. Finally, the pellet was resuspended in 500 μl of 10% glycerol and 60 μl were distributed into 1.5 ml eppendorf tubes. The cells were frozen at –80 °C until further use.

The pKOBEGA plasmid (Solano *et al.*, 2002) was introduced into the electrocompetent cells by electroporation in a cuvette (2 mm gap) using the ECM 630 (BTX

Table 4. Primers used for verification of deletion mutants

Target gene	Primer name	Sequence	Reaction No.
agfA(SE)	agfA–u–F3	5'–GCA TTA ACC TGG ACA GCA CA–3'	1, 2
	agfA–d–R3	5'–ATA ACG CCG CCC TGT ACC GTT T–3'	1, 3
	Kmr–M13F	shown in Table 2	2
	Kmr–M13R	shown in Table 2	3
bcsA(SE)	bcsE–R1	shown in Table 2	1, 2
	bcsA–R2	shown in Table 2	1, 3
	Kmr–ORF–F	5'–GCC ATC CTA TGG AAC TGC CTC GGT GAG TTT–3'	2
	Kmr–ORF–R	5'–CGC GGC CTC GAG CAA GAC GTT TCC CGT TGA–3'	3
ahpC(ST)	ahpC–up–v–F	5'–CAGAGGTTGGCCCGACAAACGTAGCG–3'	1, 2
	ahpC–down–v–R	5'–CGCCTTTGGTGCGATACTGATCCTCGC–3'	1, 3
	Kmr–M13–F	shown in Table 2	3
	Kmr–ahpC–R	shown in Table 2	2
ahpF(ST)	ahpF–up–v–F3	5'–GGC GCC TCG AAG GAT TTC AGG AT–3'	1, 2
	ahpF–down–v–R3	5'–GCG AAC GTC CTG CGT CAT GGG ATA–3'	1, 3
	Kmr–ORF–F	shown above	3
	Kmr–ORF–R	shown above	2
katG(ST)	katG–up–v–F	5'–GCG CGT TCA GTG TCG CGG CGT GAC ATG AAG–3'	1, 2
	katG–down–v–R	5'–GCC TGG CGG CGC AAC GCC GGA TGA AGT GTA–3'	1, 3
	Kmr–ORF–F	shown above	3
	Kmr–ORF–R	shown above	2
rpoE(ST,SE)	rpoE–up–v–F	5'–ACG CGC ATC GGC AGT TTG GTG TCC ATG TCC–3'	1, 2
	rpoE–down–v–R	5'–ATA CGG TTA GCG CCG TGC AGG CCG GTG TAA–3'	1, 3
	Kmr–ORF–F	shown above	3
	Kmr–ORF–R	shown above	2
rpoH(SE)	rpoH–up–v–F	5'–CGT CTG AAC GAC GGC GAC GGC GGA TTC TAT–3'	1, 2
	ropH–down–v–R	5'–CCG ATA GGC CAG GGT CAG ACG ATT TCG CAG–3'	1, 3
	Kmr–F1	5'–CCG TAC TCC TGA TGA TGC ATG ATG CAT GGT TAC TCA C–3'	3
	Kmr–R1	5'–GAG AAA TCA CCA TGA GTG ACG ACT GAA TCC–3'	2
rpoH(ST)	rpoH–up–v–F	5'–TTC AGA TCG CCT TTC TCA TCC CAC GAC AGC–3'	1, 2
	rpoH–down–v–R	5'–GTT TAA CCG CGT AGG GGT GAC GGT ACT GAT–3'	1, 3
	Kmr–F–M13Hsu	5'–TAA GGG AAC CGA ACT GAC CAA CT CCA CGT TGT GTC TCA AAA TCT CTG–3'	3
	Kmr–R–M13Hsu	5'–CAC ACA GGA AAC AGC TAT GAC CAT GC CGT CCC GTC AAG TCA GCG–3'	2
rpoS(SE)	rpoS–up–soto–F	5'–TTC AGA TCG CCT TTC TCA TCC CAC GAC AGC–3'	1, 2
	rpoS–down–v–R2	5'–GGA TTC AGT CGT CAC TCA TGG TGA TTT CTC–3'	1, 3
	Kmr–F1	5'–CCG TAC TCC TGA TGA TGC ATG ATG CAT GGT TAC TCA C–3'	3
	Kmr–R1	5'–GAG AAA TCA CCA TGA GTG ACG ACT GAA TCC–3'	2
rpoS(ST)	rpoS–up–v–F	5'–CGT CTG AAC GAC GGC GAC GGC GGA TTC TAT–3'	1, 2
	rpoS–down–v–R	5'–CCG CCG GAA ATT CCT ACC GCG CTC ATG GCA–3'	1, 3
	Kmr–ORF–F	shown above	3
	Kmr–ORF–R	shown above	2

Table 3. PCR conditions for verification of deletion mutants

Target gene	Reaction No.	Enzyme used for PCR	Condition*			
			Pre–heating	Denaturation	Annealing	Extenstion
agfA	1, 2, 3	Phusion	98°C, 30 s	98°C, 10 s	–	74°C, 2 min
bcsA	1	KOD Dash	95°C, 3 min	94°C, 30 s	65°C, 10 s	74°C, 1 min 30 s
	2, 3	KOD Dash	95°C, 3 min	94°C, 30 s	65°C, 10 s	74°C, 40 s
ahpC	1	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 3 min
	2, 3	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min
ahpF	1	KOD plus	94°C, 3 min	94°C, 15 s	58°C, 30 s	68°C, 4 min
	2, 3	KOD plus	94°C, 3 min	94°C, 15 s	58°C, 30 s	68°C, 2 min
katG	1	KOD plus	94°C, 2 min	94°C, 15 s	58°C, 30 s	68°C, 5 min
	2, 3	Ex Taq	95°C, 3 min	95°C, 10 s	58°C, 30 s	72°C, 1 min 30 s
rpoE	1	KOD Dash	94°C, 2 min	94°C, 15 s	58°C, 30 s	72°C, 5 min
for ST	2, 3	Taq	95°C, 3 min	95°C, 1 min	60°C, 1 min	72°C, 1 min
for SE	2, 3	KOD Dash	94°C, 2 min	94°C, 30 s	60°C, 30 s	74°C, 1 min
rpoH for ST	1	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min 45 s
	2, 3	Phusion	98°C, 30 s	98°C, 10 s	69°C, 30 s	72°C, 1 min 15 s
rpoH for SE	1	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min 30 s
	2, 3	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min
rpoS for ST	1	KOD Dash	94°C, 2 min	94°C, 15 s	58°C, 30 s	72°C, 5 min
	2, 3	Taq	95°C, 3 min	95°C, 1 min	60°C, 1 min	72°C, 1 min
rpoS for SE	1	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min 30 s
	2, 3	Phusion	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 1 min
Km^r cartridge		Ex Taq	95°C, 3 min	95°C, 10 s	56°C, 30 s	72°C, 1 min

*All PCR conditions were one cycle of pre–heating, followed by 35 cycles of denaturation, annealing, and extension.

Division of Genetronics, Harvard Apparatus, Inc., Holliston, MA, USA) electro cell manipulator with conditions: 2.5 kV, 200 Ω and 25 μF. The cells were recovered in a mixture of 980 μl of SOB and 20 μl of 1 M glucose. Recovered cells were incubated at 30 °C for 1 h with shaking (238 rpm), plated onto LB agar containing ampicilin, and grown at 37 °C overnight. Colonies showing ampicillin resistance were then picked into a culture of 5 ml LB containing 10 μg/ml ampicillin and incubated overnight. The following day, 2.5 ml of the preculture was added to 200 ml of LB and allowed to grow at 30 °C to an OD_{660} of 0.15–0.18. Ten percent L–(+)–arabinose (filter–sterilized) solution was added to the culture to obtain a final concentration of 0.08% and the culture was allowed to grow until an OD_{660} of 0.65. The cells were then centrifuged at 7,500 × *g* for 10 min at 4 °C, resuspended in an equal volume of cold 10% glycerol and centrifuged at 7,500 × *g* for 10 min at 4 °C again. This wash step was repeated twice. The final pellet was resuspended in 40 ml of 10% glycerol and stored at –80 °C until further use.

Introduction of gene fragment

Electrocompetent *Salmonella* cells containing the pKOBEGA plasmid were thawed on ice before use. Cuvettes (2 mm gap) were also cooled on ice before use. Two μl of the PCR product were 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) electro cell manipulator with conditions: 1.9 kV, 150 Ω and 50 μF. The cells were then recovered in 1 ml TSB and incubated at 30 °C for 2–3 h to allow for homologous recombination. One hundred μl and the remaining volume of cells were plated onto LB agar containing kanamycin at 25 μg/ml and incubated at 37 °C.

PCR verification of the mutants

To confirm the disruption of *ΔagfA*, *ΔbcsA*, *ΔahpC*, *ΔahpF*, *ΔkatG*, *ΔrpoE*, *ΔrpoH*, and *ΔrpoS* in SE or ST mutant strains, the primers listed in Table 4 were used for PCR. Genomic DNA was used as a template. Verification was carried out by PCR with ExTaq DNA polymerase, KOD Dash DNA polymerase, KOD plus DNA polymerase (Toyobo), Phusion DNA polymerase, or Taq DNA polymerase (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions and under the conditions listed in Table 5. PCR products were visualized by agarose gel electrophoresis.

RESULTS AND DISCUSSION

PCR amplification of Kmr cartridges flanked by homologous regions to target genes

To clarify the mechanism for biofilm formation of *S.* Enteritidis, we tried to construct two kinds of deletion mutants (*ΔagfA* and *ΔbcsA*). To clarify the mechanism for recovery of heat–injured *S.* Enteritidis, which is one

of the most causes of food–poisoning in Japan, we constructed three kinds of deletion mutants (*ΔrpoE*, *ΔrpoH*, and *ΔrpoS*) of *S*. Enteritidis for stress–inducible genes involving in recovery after heat–treatment. As complete genome DNA sequence of *S*. Typhimurium has been determined (McClelland *et al*., 2001), six kinds of deletion mutants (*ΔrpoE*, *ΔrpoH*, *ΔrpoS*, *ΔahpC*, *ΔahpF*, and *ΔkatG*) of *S*. Typhimurium were also constructed. However, genome DNA sequence of *S*. Enteritidis (Accession number, NC_011294) has been recently released (Thomson *et al*., 2008). Thus, locus of target gene on the genome was also shown in Table 1.

To construct deletion mutants, upstream region of target gene, a kanamycin cartridge, and downstream of the target gene were amplified by the 1st step PCR. For the amplification of the parts of the upstream and downstream regions for target genes, primers listed in Table 1 were used. The expected fragments were successfully amplified (data not shown).

Genomic DNA of *S*. Enteritidis was used as a template for amplification of *agfA*, *bcsA*, *rpoE*, *rpoH*, and *rpoS*. At first, the size of the amplified upstream region of *agfA* with agfA–u–F1 and agfA–u–M13R1 primers was 1231 bp, and that of the amplified downstream region of *agfA* with *agfA*–d–M13R1 and agfA–d–R2 primers was 829 bp.

The size of the amplified upstream region of *bcsA* with bcsE–R1 and M13–EF1 primers was 925 bp, and that of the amplified internal region of *bcsA* with m13–AF4 and bcsA–R2 primers was 1008 bp.

The size of the amplified upstream region of *rpoE* with rpoE–up–F and rpoE–up–M13R primers was 1035 bp, and that of the amplified downstream region of *rpoE* with rpoE–down–M13F and rpoE–down–R primers was 949 bp.

The size of the amplified upstream region of *rpoH* with rpoH–up–F and rpoH–up–R–Kmr primers was 1053 bp, and that of the amplified downstream region of *rpoH* with rpoH–down–F2 and rpoH–down–R2 primers was 997 bp.

The size of the amplified upstream region of *rpoS* with rpoS–up–F2 and rpoS–u–R1–M13 primers was 984 bp, and that of the amplified downstream of *rpoS* with rpoS–down–F8–M13 and rpoS–down–R–soto primers was 1486 bp.

Genomic DNA of *S*. Typhimurium was used as a template for amplification of *ahpC*, *ahpF*, *katG*, *rpoE*, *rpoH*, and *rpoS*. The size of the amplified upstream region of *ahpC* with ahpC–up–F and ahpC–up–R primers was 1042 bp, and that of the amplified downstream region of *ahpC* with ahpC–down–F and ahpC–down–R primers was 976 bp.

The size of the amplified upstream region of *ahpF* with ahpF–up–F and ahpF–up–R primers was 1267 bp, and that of the amplified downstream region of *ahpF* with ahpF–down–F and ahpF–down–R primers was 1045 bp.

The size of the amplified upstream region of *katG* with katG–up–F and katG–up–M13R primers was 915 bp, and that of the amplified downstream of *katG* with katG–down–M13F and katG–down–R primers was 868 bp.

The size of the amplified upstream region of *rpoE* with rpoE–up–F and rpoE–up–M13R primers was 1035 bp, and that of the amplified downstream of *rpoE* with rpoE–down–M13F and rpoE–down–R primers was 949 bp.

The size of the amplified upstream region of *rpoH* with rpoH–up–F and rpoH–up–R–M13Hsu primers was 1053 bp, and that of the amplified downstream of *rpoH* with rpoH–down–F2 and rpoH–down–R2 primers was 997 bp.

The size of the amplified upstream region of *rpoS* with rpoS–up–F and rpoS–up–R1–M13 primers was 984 bp, and that of the amplified downstream region of *rpoS* with rpoS–down–M13F and rpoS–down–R primers was 1031 bp.

The size of Km^r cartridge amplified with Kmr–M13F and Kmr–M13R primers was 1032 bp. The size of Km^r cartridge with Kmr–F–M13Hsu and Km–R–M13Hsu primers was 1029 bp. The size of Km^r cartridge amplified with Kmr–F–rpoH or Kmr–M13R primers was 1031 bp.

Finally, the three amplified fragments (upstream, Km^r cartridge, and downstream (or internal) regions) were used for the 2nd step PCR. During the PCR, upstream– Km^r –downstream (or internal) fragment was successfully fused during the PCR amplification (data not shown).

Construction of deletion mutants

For construction of deletion mutants, *S*. Enteritidis or *S*. Typhimurium were preliminary transformed with a λ–Red helper plasmid, pKOBEGA (Solano *et al*., 2002). Electrocompetent cells were prepared from the resultant strains carrying pKOBEGA. The fused fragment including Km^r gene was introduced into the electrocompetent cells and the cells were recovered at 30 ºC to allow for the λ phage red functions to promote homologous recombination. Cells were subsequently plated on LB/Kan and incubated at 37 ºC. The pKOBEGA plasmid is lost when cells are incubated at 37 ºC, and the kanamycin plates were used to screen for strains positive for carrying the introduced Km^r gene (Solano *et al*., 2002). The deletion strain was confirmed by PCR as shown in Fig. 2–12.

We constructed *ΔbcsA* mutant of *S*. Enteritidis and confirmed of introduction of Km^r gene into the locus of *bcsA* (Fig. 2). The *bcsA* encodes a catalytic subunit of a cellulose synthesis enzyme, which consists of BcsA, BcsB, BcsC, and BcsZ (Zogal *et al*., 2001). The cellulose synthesis enzyme is thought to be involved in biofilm formation of *S*. Typhimurium (Zogal *et al*., 2001). This construction of *ΔbcsA* mutant of *S*. Enteritidis will lead to clarification of molecular mechanism for biofilm formation.

Construction of *ΔagfA* mutant of *S*. Enteritidis was also confirmed by PCR (Fig. 3). In *S*. Enteritidis, thin, aggregative fimbriae, which are composed of polymerized AgfA fimbrin proteins, are produced and suggested to be involved in biofilm formation (Collinson *et al*., 1996). The construction of *ΔagfA* mutant of *S*. Enteritidis will also help clarification of molecular mechanism for bio-

film formation.

We had focused an attention on *katG* gene because the transcription level of the gene was highly up–regulated during recovery of heat–injured *S*. Typhimurium (Kobayashi *et al*., 2005). As Fig. 4 shows, we successfully constructed *ΔkatG* mutant of *S*. Typhimurium. However, recoverability of heat–injured *ΔkatG* mutant of *S*. Typimurium was almost same as that of wild type (unpublished data). Thus, the reason of this phenomenon was due to complementary effects of *ahpC* and *ahpF* whose gene products also scavenge hydrogen peroxide as well as *katG*. Real time reverse transcription PCR analysis showed that *ahpC* and *ahpF* were highly up–regulated during recovery of heat–injured *ΔkatG* mutant (unpublished data). Thus, *ΔahpC* or *ΔahpF* mutants were constructed in this study (Figs. 5 and 6). Alkaline hydroperoxide reductase (Ahp) is composed of subunits of AhpC and AhpF. It is well known that AhpF functions as an electron donor to AhpC (Rocha and Smith, 1999; Storz *et al*., 1989). Furthermore, *ahpC* and *ahpF* are controlled under OxyR regulation as well as *katG*, and OxyR are activated by hydrogen peroxide (Farr and Kogoma, 1991).

In our previous study, *rpoE* encoding σ^E was signicficantly up–regulated during recovery of heat–injured *S*. Enteritidis (Kobayashi *et al*., 2005). Bacterial σ factors interact with RNA polymerase core enzyme to initiate transcription. There are several types of sigma factors involving in the expression of most housekeeping genes or of stress responsive genes. Furthermore, oxidative stress susceptibility of double or triple deletion *S*. Typhimurium mutants of *rpoE*, *rpoH*, and/or *rpoS* were studied (Bang *et al*., 2005). In the present study, we

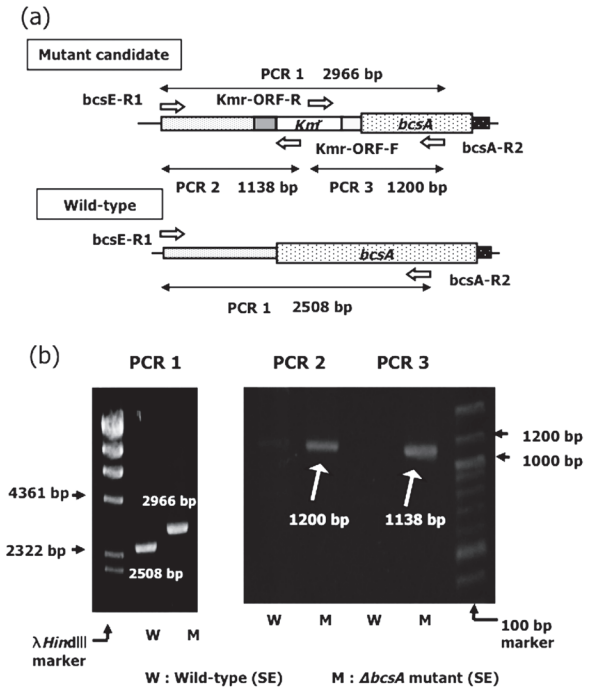

Fig. 2. Method and results for verification of the location of the gene replacement in *bcsA* deletion mutant of *S*. Enteritidis by PCR. (a) Location of primers for PCR 1, 2, and 3, and the size of PCR products expected for deletion mutant and wild type. (b) Agarose gel electrophoresis profiles of PCR products in wild type and deletion mutant of SE.

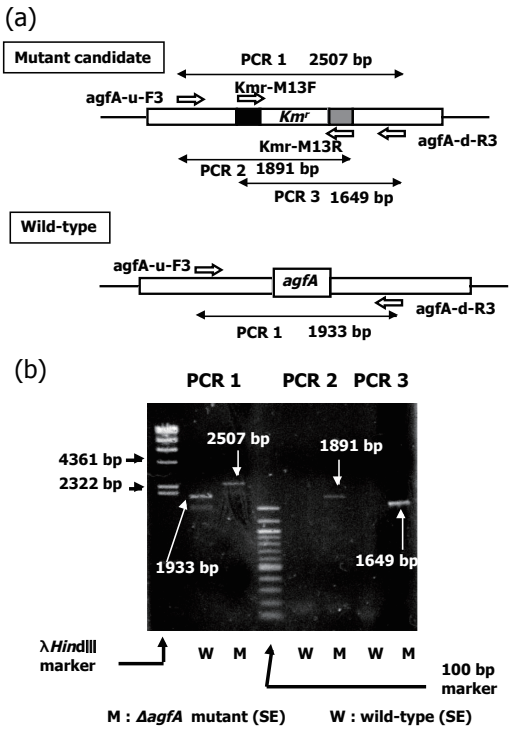

Fig. 3. Method and results for verification of the location of the gene replacement in *agfA* deletion mutant of SE by PCR. For other details, see legend to Fig. 2.

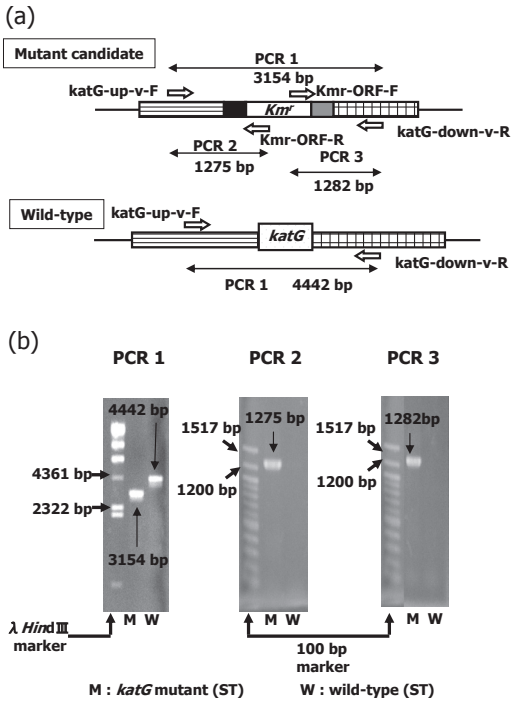

Fig. 4. Method and results for verification of the location of the gene replacement in *katG* deletion mutant of *S*. Typhimurium (ST) by PCR. (a) Location of primers for PCR 1, 2, and 3, and the size of PCR products expected deletion mutant and wild type. (b) Agarose gel electrophoresis profiles of PCR products in wild type and deletion mutant of ST.

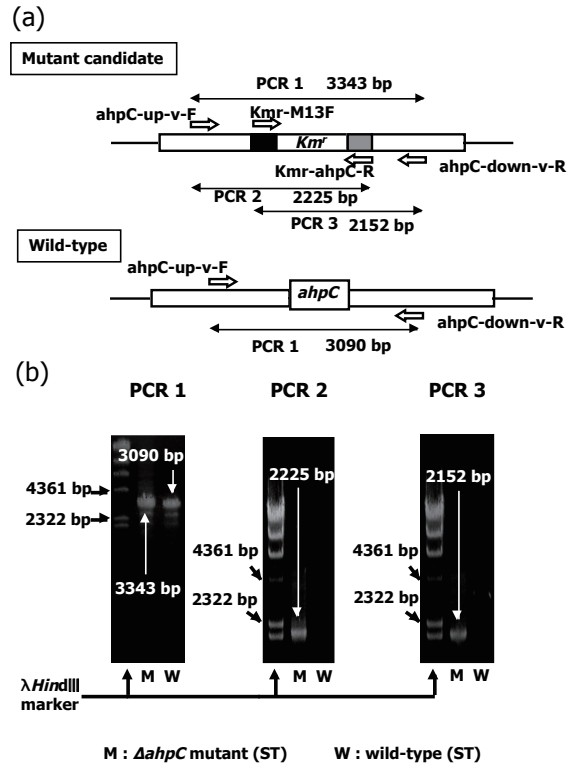

Fig. 5. Method and results for verification of the location of the gene replacement in *ahpC* deletion mutant of ST by PCR. For other details, see legend to Fig. 4.

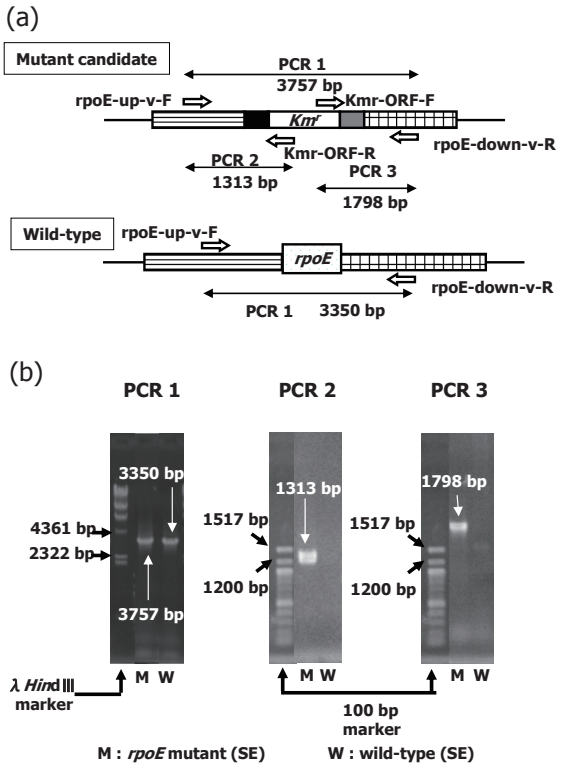

Fig. 7. Method and results for verification of the location of the gene replacement in *rpoE* deletion mutant of SE by PCR. For other details, see legend to Fig. 2.

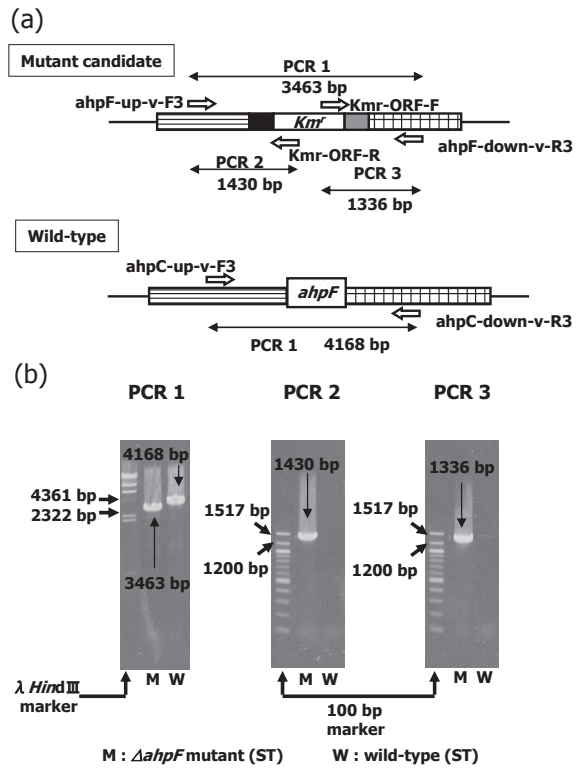

Fig. 6. Method and results for verification of the location of the gene replacement in *ahpF* deletion mutant of ST by PCR. For other details, see legend to Fig. 4.

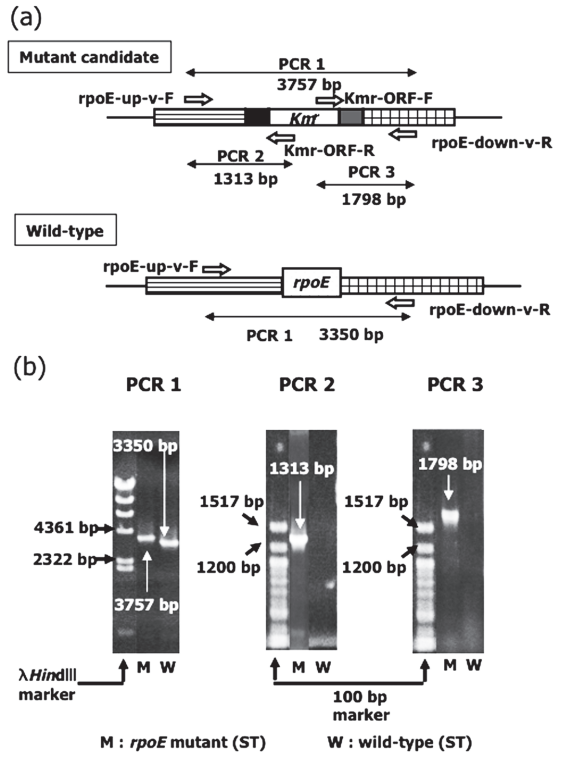

Fig. 8. Method and results for verification of the location of the gene replacement in *rpoE* deletion mutant of ST by PCR. For other details, see legend to Fig. 4.

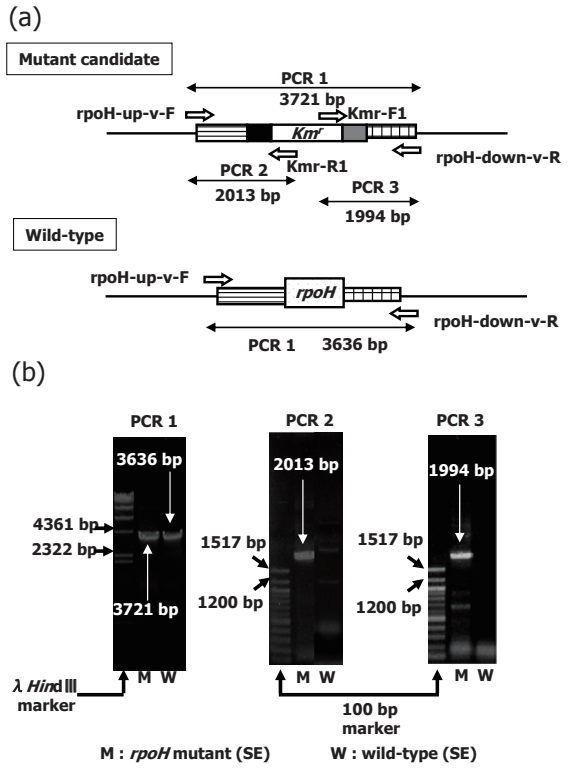

Fig. 9. Method and results for verification of the location of the gene replacement in *rpoH* deletion mutant of SE by PCR. For other details, see legend to Fig. 2.

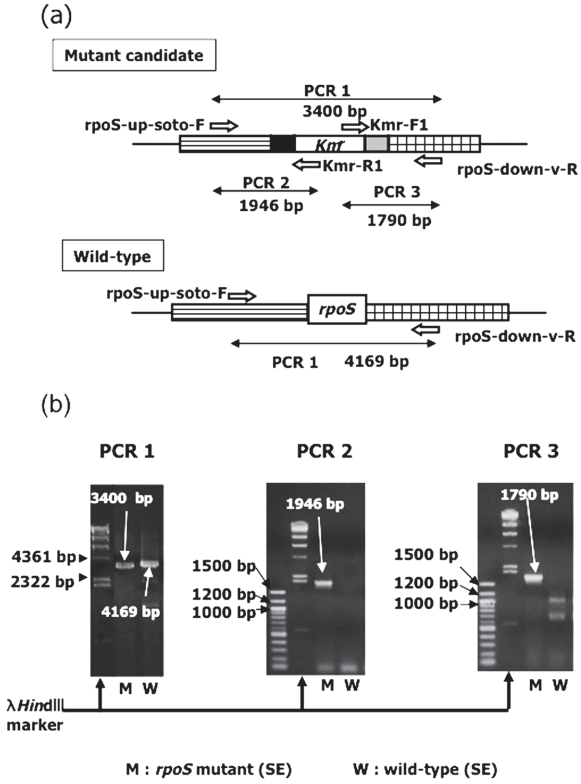

Fig. 11. Method and results for verification of the location of the gene replacement in *rpoS* deletion mutant of SE by PCR. For other details, see legend to Fig. 2.

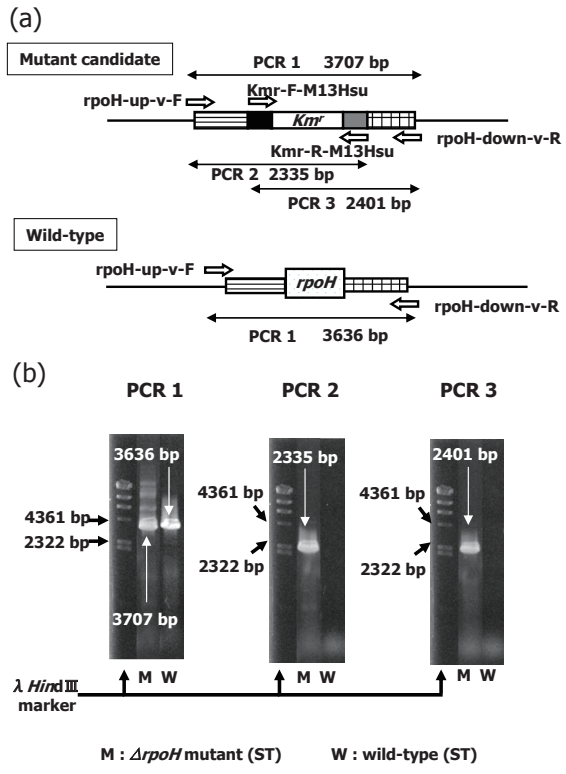

Fig. 10. Method and results for verification of the location of the gene replacement in *rpoH* deletion mutant of ST by PCR. For other details, see legend to Fig. 4.

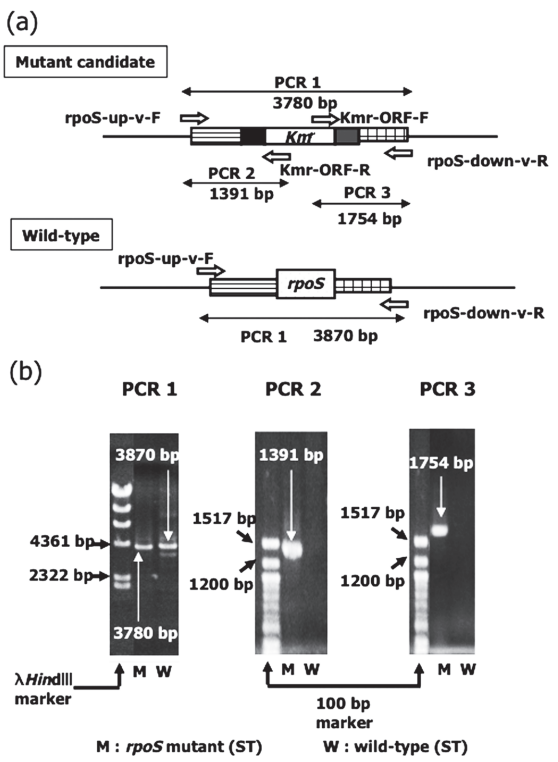

Fig. 12. Method and results for verification of the location of the gene replacement in *rpoS* deletion mutant of ST by PCR. For other details, see legend to Fig. 4.

successfully constructed *ΔrpoE*, *ΔrpoH*, and *ΔrpoS* mutants of *S*. Eneteritidis and *S*. Typhimurium (Figs. 7–12). In the stationary phase of *Salmonella*, σ^E is sequestered by a membrane–bound anti–sigma factor RseA in unstressed cells and is released into cytoplasm allowing following a signaling sigma factor cascade. The signal from σ^E seems to be transmitted via σ^H and an RNA–binding protein Hfq to σ^S. In the cascade, every one. of the sigma factors has their respective stress response. The σ^E, σ^H, and σ^S response to periplasmic stress, to heat shock, and to starvation, respectively. The final accumulation of σ^S seems to lead to increased expression of σ^S–regulated genes and antioxidant defences. Construction of the deletion mutants for the three sigma factors (*rpoE*, *rpoH*, and *rpoS*) will facilitate clarification of recovery mechanism of heat–injured *Salmonella* through investigation of expression of stress–response genes regulated by each sigma factor.

In particular, σ^S encoded by *rpoS* is suggested to be involved in biofilm formation of *E. coli* and *S*. Enteritidis (Solano *et al*., 2002). As Fig. 11 shows, *ΔrpoS* mutant of *S*. Enteritidis was also constructed. The construction of *ΔrpoS* mutant of *S*. Enteritidis will facilitate not only the clarification of mechanism of heat–injured *Salmonella* but also the clarification of biofilm formation.

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