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Identification of Genes Differentially Expressed in RAW264.7 Cells Infected by *Salmonella typhimurium* Using PCR Method

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Salmonella typhimurium, **causing mouse typhoid, infects hosts such as macrophage cells, and proliferates in intracellular vacuoles causing infected cells to trigger numerous genes to respond against the infection. In this study, we tried to identify such genes in RAW264.7 cells by using the PCR screening method with degenerate primers. Fourteen genes were found to be differentially expressed after a 4 h infection in which the expression of 8 genes increased while expression of the others decreased. Most of the genes were involved in proinflammatory responses such as cytokines production and cell death. The mutation in *msbB* gene encoding the myristoyl transferase in lipid A of lipopolysaccharide (LPS) resulted in much lower toxicity to the inoculated animals. We compared the expression of the identified genes in wild-type and *msbB*-mutated *S. typhimurium* infections and found that *Lyzs* encoding lysozyme type M was differentially expressed. This gene is quite likely to be related to bacterial survival in the host cells.**

Keywords: *Salmonella typhimurium*, macrophage, infection, PCR, gene expression

Salmonella typhimurium is an enteropathogenic bacterium causing mouse typhoid. The bacteria are resistant to acidic conditions in the stomach and infect through the Peyer's patches in the small intestine (Lucas and Lee, 2000). As *S. typhimurium* systemically infects mice, this bacteria is employed as a model to study salmonellosis. After invasion of the cells such as macrophage and epithelial cells, the bacteria proliferate in the intracellular compartments called *Salmonella* containing vesicles (SCV), and finally kill their host cells (Sansonetti, 2002). At the onset of this infection, the host cells trigger numerous responses, such as the production of inflammatory cytokines, modification of signaling molecules, cytoskeletal rearrangement, apoptosis, and transcription regulation (Steele-Mortimer *et al.*, 2002).

Lipopolysaccharide (LPS) forms the outer leaflet of the outer membrane in Gram-negative bacteria and is a potent endotoxin causing strong immune responses such as septic shock (Khan *et al.*, 1998). LPS has three structural and functional domains: the lipid A, the core, and the O antigen. The biological and toxic effects associated with LPS are primarily caused by the lipid A (Somerville *et al.*, 1996). The mutations in lipid A biosynthesis genes result in reduced virulence of *S. typhimurium* (Jones *et al.*, 1997; Sunshine *et al.*, 1997; Khan *et al.*, 1998). For example, the *S. typhimurium msbB* mutant *strain,* which encodes the myristoyl transferase in lipid A (Karow and Georgopoulos, 1992) grows at the same rate as the wild-type bacteria in the murine livers and spleens following intravenous inoculation, but most of the mice were able to survive after recovering from the infection (Khan *et al.*, 1998). As *S. typhimurium* possessed tumor targeting and therapeutic abilities, and as *msbB* mutation has increased the LD_{50} of this bacteria 10,000 fold, the mutant bacteria were used as an antitumor agent in mice (Low *et al.*, 1999).

Previously reported microarray analysis demonstrated that several tens of host genes were highly expressed in the macrophage cells after the infection by wild-type and *phoP*-mutated *S. typhimurium* (Detweiler *et al.*, 2001; Jansen and Yu, 2006). However, the results obtained by microarrary analysis showed a change in expression of the identified genes but did not reflect a real quantitative difference. In this work, the differentially expressed genes in RAW264.7 cells after the *S. typhimurium* infection were newly identified by the polymerase chain reaction (PCR) method. The expression of these genes following the infection by the *msbB* mutant was analyzed.

Materials and Methods

Bacterial strains and host cell culture

Wild-type *Salmonella typhimurium* (14028s) and its *msbB*-mutated strain (SHJ2102) were grown in Luria-Bertani (LB) medium with vigorous shaking. A murine monocyte-macrophage RAW264.7 cell line used as a host was grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

Bacterial infection into host cell and RNA isolation

RAW264.7 cells (1×10^6/well) were seeded in 6-well culture plates and cultured for 1 day before use. Bacteria cultured overnight were diluted 1:40 in LB and cultured for another

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4 h at 37°C. Bacteria were centrifuged at 10,000×g for 5 min and resuspended in phosphate-buffered saline (PBS). The bacteria (MOI=100) was inoculated to RAW264.7 cells. After 30 min, the uninfected bacteria were removed by PBS. Then fresh DMEM containing 10% FBS and gentamycin sulfate (10 μg/ml) was added to the cells and incubated at 37°C and 5% CO_2. The RNAs from the infected host cells were extracted by Trizol solution (Invitrogen, USA) according to the manufacturer's instruction.

Identification of differentially expressed host genes using PCR

Total RNAs from the uninfected and 4 h-infected cells were used for cDNA synthesis. Differentially expressed host genes were screened by ACP-based PCR method using the GeneFishing™ DEG kits (Seegene, Korea) (Kim *et al.*, 2004). Briefly, the uninfected and 4 h-infected RAW264.7 cells first-strand cDNAs were synthesized at 42°C in a final reaction volume of 20 μl containing 3 μg of the total RNAs of the host cells, 4 μl of 5× reaction buffer (Promega, USA), 5 μl of 2 mM dNTP, 2 μl of 10 μM dT-ACP1 primer (Seegene, Korea), 0.5 μl of RNasin® (40 U/μl; Promega, USA), and 1 μl of MMLV reverse transcriptase (200 U/μl; Promega, USA). After 1.5 h incubation, the mixture was heated at 94°C for 2 min and diluted with 80 μl of water. Second-strand cDNA synthesis and amplification were done in a final reaction volume of 20 μl containing 3-5 μl (about 50 ng) of diluted first-strand cDNA, 1 μl of 10 μM dT-ACP2, 1 μl of 10 μM arbitrary ACP, and 10 μl of 2× SeeAmp™ ACP™ Master Mix (Seegene, Korea). The PCR condition was as follows: one cycle of 94°C for 1 min, 50°C for 3 min and 72°C for 1 min, followed by 40 cycles of 94°C for 40 sec, 65°C for 40 sec, and 72°C for 40 sec. The amplified PCR products were separated in 2% agarose gel. We used a total of 120 arbitrary ACP primers.

The differentially amplified PCR fragments in 4 h-infected cells were extracted from the gel using the GENCLEAN® II Kit (Q-BIO gene, USA), and directly cloned into a TOPO TA cloning vector (Invitrogen, USA) according to the manufacturer's instructions. The cloned fragments were sequenced with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA).

Northern blot analysis

The gene fragments in TOPO TA cloning vectors were digested with *Eco*RI, purified, and used as probes. The probes were radiolabeled by Rediprime™II random prime labeling system (Amersham, UK) according to manufacturer's

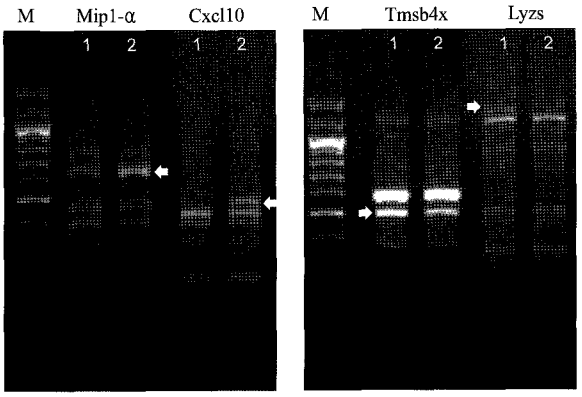

Fig. 1. The identification of genes induced or repressed in RAW 264.7 cells after the infection of wild-type *S. typhimurium*. After 4 h infection, messengers from the infected host cells were amplified by PCR using the GeneFishing™ DEG kits. 1, non-infected cells; 2, the infected cells.

Table 1. The relative expression of the induced or repressed genes in RAW264.7 cells by the infection of wild-type or *msbB*-mutated *S. typhimurium*. The mRNA levels after the bacterial infection were relatively calculated against those in non-infected cells with northern blotting

Gene names	Genbank Acc. No.	mRNA evels in each infection time (h)				Functions
		Wild type		*msbB* mutant		
		1 h	4 h	1 h	4 h	
Oasl1	NM_145309.2	4.2	10.3	4	12	Cell death
Mip-1α	NM_011337	9.7	9.9	8.3	10.4	Cytokine
Cxcl10	BC030067	12.2	3.8	15.8	11	Cytokine
Odc	M20617	1.6	3	1.7	3.9	Cell death
Pdcd1	NM_008798	2.3	2.8	3.2	3.8	Cell death
Mum1	BC075617	1.6	3.2	2.3	2.4	Unknown
Actg1	NM_009609	2.8	4.3	2.1	2	Cytoskeleton
Hypothetical	AF525300	0.9	1.1	0.7	0.9	Unknown
Tmsb4x	BC018286	1	0.9	1.2	1	Cytoskeleton
Psmd3	NM_009439	1.1	1	1.2	1	Protein degradation
Copz1	BC085314	1.1	0.8	1	0.9	Protein transport
SR-B1	BC004656	1.1	0.6	0.9	0.6	Cholesterol transport
Lyzs	NM_017372	0.9	0.5	1.1	1	Vacuole
Mcm2	NM_008564	1.2	0.5	1.7	0.9	Cell cycle

instruction.

The electrophoresis and blotting were done as described (Sambrook *et al.*, 1989). Briefly, the same amount of total host RNAs (20 μg) were separated in 1% denaturing agarose gel electrophoresis for 2 h at 100 V. After running, the RNAs were transferred to a nylon membrane overnight. The following day, the membrane was pre-incubated in Rapid-hyb prehybridization solution (Amersham, UK) for 3 h at 65°C. After the prehybridization, the membrane was hybridized with a radiolabeled probe at 65°C overnight. And the membrane was twice washed with 0.5x SSC containing 0.1% SDS and 0.1x SSC containing 0.1% SDS at 65°C. The radiolabeled bands on the membrane were analyzed by the FLA3000 system (Fuji, Japan).

Results and Discussion

To identify the host genes which were differentially expressed after *S. typhimurium* infection, RAW264.7 cells were infected by the bacteria for 4 h. The genes were amplified by an ACP-based PCR method using 120 independent arbitrary primers. The PCR bands were compared to those using cDNA from the uninfected cells. In 0.7% agarose gel electrophoresis, 14 bands showed changes in their expression after a 4 h infection. Expression of both Mip1-α and Cxcl10 was higher in *S. typhimurium* infected macrophages than in non-infected macrophages (Fig. 1A). In contrast Tmsb4x and Lyzs was lower in *S. typhimurium* infected macrophages than in non-infected macrophages (Fig. 1B). The band intensities were increased in 8 bands but decreased in the others. The PCR fragments were cloned in plasmid and sequenced (Table. 1). The identified genes were related to various cellular functions such as inflammatory cytokine, cell death, protein degradation, vacuole formation, and so on.

The mRNA levels of the genes after infection was quantitatively analyzed by northern blotting. To do this, RAW 264.7 cells were infected by wild-type and *msbB*-mutated *S. typhimurium* for 1 and 4 h. The results were closely correlated to those by PCR screening. Most genes with the exception of *Lyzs* were not changed in the *msbB* mutant infection (Table 1 and Fig. 2). However, in the case of *Lyzs*, the gene was repressed 0.5 fold after a 4 h infection with wild type strain, but not changed in the *msbB* mutant infection (Fig. 3). It indicated that the expression of this gene would be affected by LPS. Indeed, lipopolysaccharide induces the release of inflammatory cytokines and cytosolic protease (Durum *et al.*, 1985; Beutler and Cerami, 1986). Moreover, lipopolysaccharide activated macrophages become microbicidal and tumoricidal (Beutler and Cerami, 1986).

Tens of genes in U937 cells were affected after in the *S. typhimurium* infection by microarray analysis (Detweiler *et al.*, 2001). The affected genes were mainly related to proinflammatory responses such as cytokines or cell death. Although microarray is a powerful tool in studying whole gene expression, it does not always correctly reflect the quantitative change. Therefore, we assumed that some affected genes were not analyzed in microarray. In this study using the PCR screening method, no genes except for *Mip1α* were detected in the microarray. The newly found genes in this study were also mainly related to proinflammatory responses. Most genes, except *Lyzs*, were similarly expressed in host cells during wild-type and *msbB*-mutant bacteria infections.

The expression patterns of *Lyzs* genes were different in *msbB*-mutant infections. The *Lyzs* expression was decreased 0.5 fold in a 4 h infection by wild-type bacteria. However, it was not changed in the *msbB* mutant infection. Lysozyme is known to be an antimicrobial protein that plays an

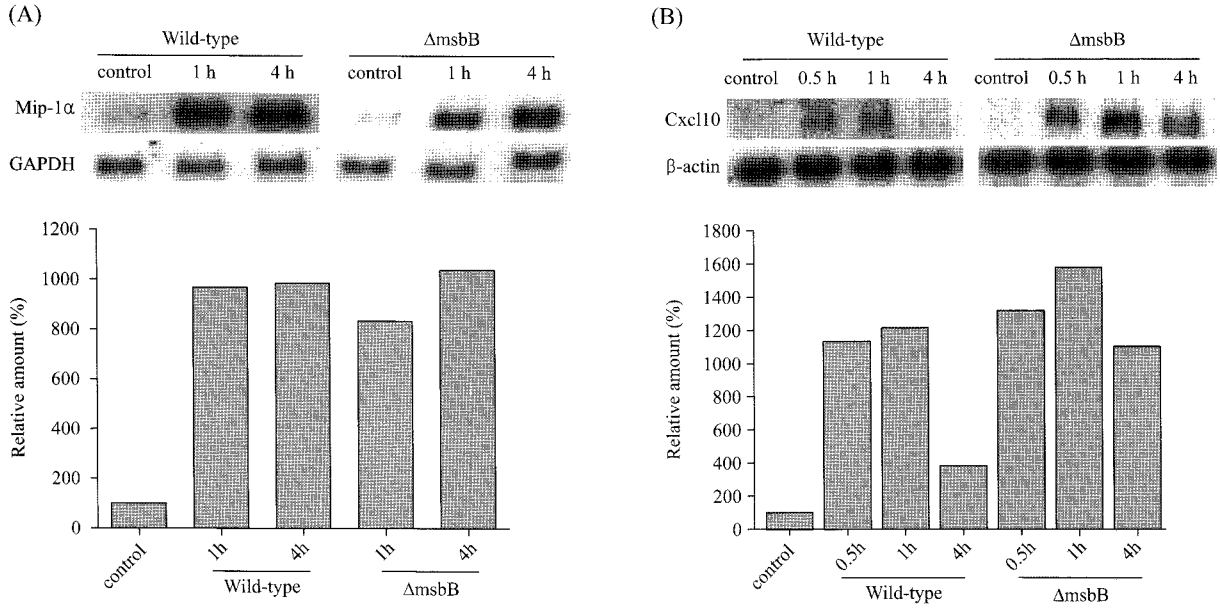

Fig. 2. The northern blotting analysis of the induced genes after the infection of RAW264.7 cells by wild-type and *msbB* mutant *S. typhimurium* for 1 h and 4 h. The same membranes were reprobed by GAPDH cDNAs. The relative mRNA amounts of infected cells compared to those of non-infected cells were depicted in the bottom tables. (A), *Mip-1α*; (B), *Cxcl10*.

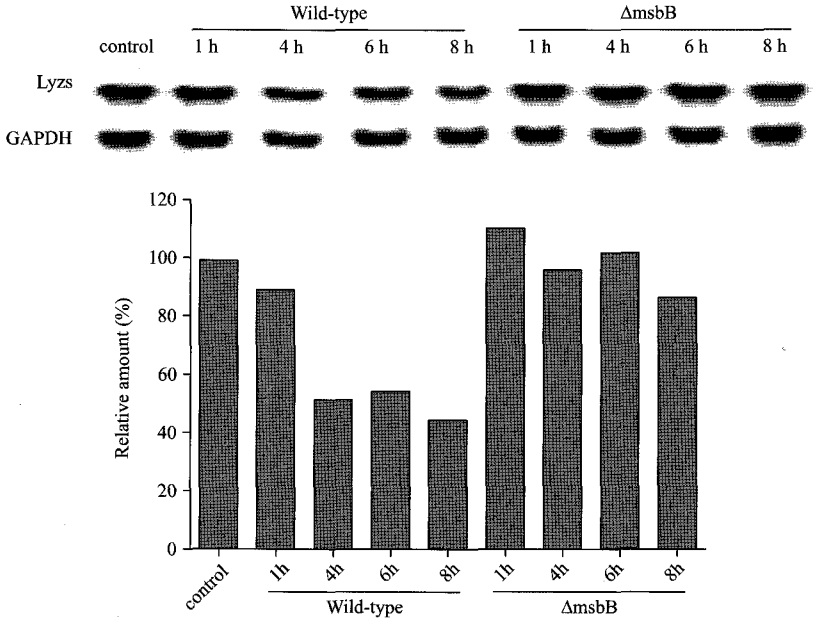

Fig. 3. The northern blotting analysis of the repressed genes after the infection of RAW264.7 cells by wild-type and *msbB*-mutated *S. typhimurium* for the indicated times. The same membranes were reprobed by GAPDH cDNA. The relative mRNA amounts of infected cells compared to those of non-infected cells were depicted in the bottom.

important role in non-immune host defense (Ganz, 2004). Moreover, deficiency of this enzyme may increase susceptibility to infection. In macrophages, professional phagocytic cells, large particles including bacteria enter the endocytic pathway by the process of phagocytosis. However, Salmonella avoid degradation by blocking assembly of a functional hydrolytic phagolysosome, although there are certainly multiple interactions between the SCV (*Salmonella* Containing Vacuole) and the endocytic pathway (Pizarro-Cerda *et al.*, 1997; Ernst *et al.*, 1999). In previous findings (Salzman *et al.*, 2003), lysozyme showed low levels of expression in Paneth cells of the intestine by *Salmonella* infection but the SPI1 mutant with host invasion defect did not decrease the *Lyzs* expression. Similarly to the SPI1 mutant, the *msbB* mutant possessed the defective invasion activity against RAW264.7 cells (unpublished data). Therefore, the decreased expression of *Lyzs* encoding lysozyme type M in wild-type *Salmonella,* but not in the *msbB* mutant, at the least contributes to invasion ability. Most likely, the changed structure of LPS in the *msbB* mutant would cause such phenomenon because it disturbs some signaling pathway including p38 (Salzman *et al.*, 2003).

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