

Exploration of *Salmonella* effector mutant strains on MTR4 and RRP6 degradation

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SUMMARY *Salmonella enterica* serovar Typhimurium (*Salmonella*), a pathogenic bacterium, is a major cause of foodborne diseases worldwide. *Salmonella* injects multiple virulence factors, called effectors, into cells and causes multiple rearrangements of cellular biological reactions that are important for *Salmonella* proliferation and virulence. Previously, we reported that *Salmonella* infection causes loss of MTR4 and RRP6, which are nuclear RNA degradation factors, resulting in the stabilization and accumulation of unstable nuclear RNAs. This accumulation is important for the cellular defense for *Salmonella* infection. In this study, we examined a series of *Salmonella* mutant strains, most of which are strains with genes related to effectors translocated by T3SSs encoded on *Salmonella* pathogenic islands, SPI-1 and SPI-2, that have been depleted. Among 42 *Salmonella* mutants, 6 mutants' infections canceled loss of MTR4 and RRP6. Proliferation assay of *Salmonella* in the cell revealed that six mutants showed poor proliferation in the host cell, demonstrating that poor proliferation contributed to cancellation of MTR4 and RRP6 loss. This result indicates that certain events associated with *Salmonella* proliferation in host cells cause loss of MTR4 and RRP6.

Keywords T3SSs, SPI-1, SPI-2, Flagella, MTR4, RRP6

1. Introduction

Salmonella enterica serovar Typhimurium (*Salmonella*), a pathogenic bacterium, is a major cause of foodborne diseases worldwide. The *Salmonella* genome carries two particular regions involved in virulence, *Salmonella* pathogenicity islands named SPI-1 and SPI-2. T3SSs are nanosyringe-like organelles expressed by *Salmonella*, including T3SS-1 and T3SS-2, which are encoded on SPI-1 and SPI-2, respectively. T3SSs consist of a basal body and a needle-like complex through which *Salmonella* derived effector proteins are secreted into the cytoplasm of the host cell (1,2). T3SS-1 mainly facilitates the invasion of *Salmonella* into host cells, and T3SS-2 facilitates the pathogenesis of *Salmonella* and is necessary for the formation of the *Salmonella*-containing vacuole (SCV), the intracellular niche of replication (3).

Virulence genes located on SPI-1 and SPI-2 are required at different stages, specifically, the intestinal and the systemic phases of infection, respectively (4). Both pathogenicity islands contain many operons, the

expression of which is primarily governed by highly integrated transcriptional regulators. HilA, HilC and HilD, for instance, are regulators in SPI-1 (5,6). A series of operons, including *prg/org*, *inv/spa* and *sic/sip* in SPI-1 encode the components of T3SS machine and primary effector proteins (7). SsrA/B, the two-component regulatory system encoded in SPI-2, controls the expression of genes in SPI-2. By developing an *in vitro* system, Bustamante *et al.* revealed a cross-talk mechanism between SPI-1 and SPI-2 in which HilD encoded in SPI-1 differently regulates the regulons of SPI-1 and SPI-2 in the growth phase (8). In addition, Moest *et al.* pointed out that growing evidence suggests that the two T3SSs' regulation can be interdependent and the periods of secreting bacterium proteins overlap (2).

Salmonella has another T3SS, the flagellar system (9). The flagellar T3SS exports substrate subunits that assemble into a functional flagellum and regulatory factors that control the assembly process. Flagellar gene expression is under spatiotemporal control by a transcriptional hierarchy of three promoter classes.

flhDC, controlled by a class 1 promoter, encodes a flagellar master regulator. A FlhD₄C₂ complex activates class 2 promoter transcription. FliZ, controlled by the class 2 promoter, activates SPI1 gene regulation through HilD-posttranscriptional regulation (10). The SPI1 master regulator HilD activates *flhDC* gene expression (11). Furthermore, the SsaB protein encoded on SPI-2 is involved in flagella assembly by affecting the post-transcription expression of *flhDC* (12). Therefore, the cross-regulation network between SPI-1, SPI-2 and the flagellar system likely contributes to *Salmonella* virulence.

A large number of RNAs are continuously being produced in eukaryotic nuclei, and RNA degradation systems are recruited to keep the balance of these genomic outputs, such as by discarding the transcriptional byproducts and malformed transcripts (13). The RNA exosome, a 3'-5' ribonuclease complex, facilitates the degradation of some labile nuclear RNAs (14). The RNA exosome consists of nine core subunits and an essential catalytic subunit, RRP44 (15). Among them, six subunits surround a central channel and contain domains, which are homologous to the bacterial phosphorolytic ribonuclease RNase PH (16,17); three subunits, which are positioned on top of the RNase PH-like ring, harbor S1 or KH RNA-binding domains (18). RRP44 is believed to interact with the "bottom" of the PH-ring (16), and RRP6 is believed to be located next to the exosome entrance, on the opposite side of RRP44 (19). The active ribonucleases RRP6 and RRP44 (DIS3) in human nuclei facilitate the nine subunits' large and inert core of the RNA exosome to obtain its catalytic activity (20,21). In addition, RRP6 is involved in interactions with other cofactors such as RRP47 and MTR4 (22).

The NEXT complex, composed of MTR4, Zn-finger protein ZCCHC8, and RNA-binding factor RBM7, mainly targets early and unprocessed RNA by recruiting the nuclear RNA exosome complex (23). In addition, the PAXT complex, which also contains MTR4, mainly targets long and polyadenylated RNA by recruiting the nuclear RNA exosome complex (13). MTR4 is an RNA helicase that interacts with several protein adaptors and facilitates the RNA exosome recognizing its target (24). Thus, MTR4 and RRP6 are important components of the RNA exosome in the nuclear RNA degradation pathway. Recently, we revealed that the unstable nuclear ncRNAs are mainly degraded by the MTR4-mediated nuclear RNA decay pathway. In addition, RRP6 and MTR4 are dramatically decreased upon *Salmonella* infection, resulting in stabilizing the labile nuclear ncRNAs (25).

Because effectors of *Salmonella* are the main influence for cell physiology in *Salmonella* infection, we considered whether any effectors are involved in the degradation of MTR4 and RRP6. To test this idea, we constructed a series of *Salmonella* mutant strains and examined the effect of these mutants for loss of MTR4 and RRP6. Among the 42 *Salmonella* mutants

examined in this study, 6 canceled loss of MTR4 and RRP6. A proliferation assay of *Salmonella* in the cell revealed that 6 mutants showed poor proliferation in the host cell, demonstrating that poor proliferation attributed in cancellation of loss of MTR4 and RRP6. This result indicates that certain events associated with *Salmonella* proliferation in the host cell causes loss of MTR4 and RRP6. Thus, this is the first report of exploring *Salmonella* effectors that may be involved in degrading the components of the RNA exosome among many *Salmonella* mutant strains. Our study has the potential to lay a good foundation for future research on *Salmonella* effector and RNA exosome upon *Salmonella* infection.

2. Materials and Methods

2.1. Cell lines and culture

Hela TO cells, purchased from Clontech (Palo Alto, CA), were maintained in Dulbecco's modified Eagle's medium (DMEM) purchased from Wako (Tokyo, Japan), supplying with 10% fetal bovine serum (FBS) purchased from Life Technologies (Grand Island, NY). FBS was heat-inactivated at 56°C for 30 min. Hela TO cells were cultured in a humidified incubator (Thermo Fisher Scientific) with 5% CO₂ at 37°C.

2.2. Construction of *Salmonella* mutant strains

Salmonella enterica serovar Typhimurium (*Salmonella*) mutant strains were constructed based on wild type *Salmonella*. The detailed information about these mutants is shown in Tables 1 and 2.

2.3. *Salmonella* culture

Salmonella was cultured with 5 mL LB5 at 37°C overnight (around 16.5 h) in a shaking bath. A total of 50 µL of the full growth was inoculated with a fresh 5 mL LB5 at 37°C for 2 h. *Salmonella* was collected by centrifuge and resuspended with a corresponding volume of 1 × PBS before infection.

2.4. Heat-killed *Salmonella*

After resuspending the subcultured *Salmonella* with a corresponding volume of 1 × PBS, the *Salmonella* was incubated at 80°C for 1 h to heat-kill it.

2.5. *Salmonella* infection

A 12-well plate was used in this study, and 2 × 10⁵ Hela cells were plated in each well. Hela cells were infected with WT-*Salmonella*, *Salmonella* mutant strains or heat-killed *Salmonella* at 100 multiplicity of infection (moi). After infection with 100 moi *Salmonella* or 1 µg/mL LPS (WAKO, Japan), Hela cells were incubated at 37°C for 1

Table 1. 42 *Salmonella* mutant strains

Strains	Relevant characteristics	References
<i>S. enterica</i> serovar Typhimurium		
χ 3306	Virulent strain, <i>gyrA1816</i> pStSR1001 ⁺	Gulig and Curtiss, 1987
χ 3337	Virulence plasmid-cured derivative of x3306, <i>gyrA1816</i> pStSR1001, <i>spv</i>	Gulig and Curtiss, 1987
χ 3306 <i>phoP</i>	<i>phoP::aph-ΔTer</i> in χ 3306, Δ <i>PhoP</i>	Matsui <i>et al.</i> , 2000
CS2007	<i>clpP::Cm</i> in χ 3306, Δ <i>ClpXP</i>	Microbiol. Immunol., 44(6), 447-454, 2000
CS2022	<i>Δlon::Cm</i> in χ 3306, Δ <i>Lon</i>	Yamamoto <i>et al.</i> IAI 60: 3164-74. 2001
CS2609	<i>flhD::Tn10</i> in χ 3306, Δ <i>FlhDΔFlhC</i>	Takaya <i>et al.</i> , IAI 71: 690-6. 2003
CS2725	<i>ΔhilD</i> in χ 3306, Δ <i>HilD</i>	Tomoyasu <i>et al.</i> MM 48: 443-52. 2003
CS2802	<i>ΔhilC ΔhilD</i> in χ 3306, Δ <i>HilCΔHilD</i>	Takaya <i>et al.</i> MM 55: 839-52. 2005
CS3752	<i>ΔsptP::Km</i> in χ 3306, Δ <i>SptP</i>	This study
CS3754	<i>ΔsopD2::Km</i> in χ 3306, Δ <i>SopD2</i>	This study
CS3794	<i>ΔavrA::Km</i> in χ 3306, Δ <i>AvrA</i>	This study
CS3802	<i>ΔpipA::FRT</i> in χ 3306, Δ <i>PipA</i>	This study
CS3803	<i>ΔpipB::FRT</i> in χ 3306, Δ <i>PipB</i>	This study
CS3804	<i>ΔgtgA::FRT</i> in χ 3306, Δ <i>GtgA</i>	This study
CS3809	<i>ΔpipC::Km</i> in χ 3306, Δ <i>PipC</i>	This study
CS3822	<i>ΔgogA::FRT</i> in χ 3306, Δ <i>GogA</i>	This study
CS4022	<i>ΔprgI::FRT</i> in χ 3306, Δ <i>PrgI</i>	This study
CS4037	<i>ΔsspH2::Km</i> in χ 3306, Δ <i>SspH2</i>	This study
CS4844	<i>ΔgogB::Cm</i> in χ 3306, Δ <i>GogB</i>	This study
CS4845	<i>ΔsseK1::Km</i> in χ 3306, Δ <i>SseK1</i>	This study
CS4846	<i>ΔsseI::Km</i> in χ 3306, Δ <i>SseI</i>	This study
CS4848	<i>ΔsseL::Km</i> in χ 3306, Δ <i>SseL</i>	This study
CS4850	<i>ΔsseK2::Cm</i> in χ 3306, Δ <i>SseK2</i>	This study
CS4852	<i>ΔsifA::Cm</i> in χ 3306, Δ <i>SifA</i>	This study
CS4853	<i>ΔsseJ::Km</i> in χ 3306, Δ <i>SseJ</i>	This study
CS4854	<i>ΔsteC::Km</i> in χ 3306, Δ <i>SteC</i>	This study
CS4856	<i>ΔpipB2::Cm</i> in χ 3306, Δ <i>PipB2</i>	This study
CS4857	<i>ΔsifB::Cm</i> in χ 3306, Δ <i>SifB</i>	This study
CS4862	<i>ΔssaB::FRT</i> in χ 3306, Δ <i>SsaB</i>	This study
CS4863	<i>ΔgtgE::FRT</i> in χ 3306, Δ <i>GtgE</i>	This study
CS4864	<i>ΔsseFG::FRT</i> in χ 3306, Δ <i>SseFG</i>	This study
CS10004	<i>ΔaroA::FRT</i> in χ 3306, Δ <i>AroA</i>	This study
CS10135	<i>ΔssaG::FRT</i> in χ 3306, Δ <i>SsaG</i>	Takaya <i>et al.</i> , JBC (2019)
CS10216	<i>ΔsrfJ::FRT</i> in χ 3306, Δ <i>SrfJ</i>	This study
CS10218	<i>ΔsteD::Cm</i> in χ 3306, Δ <i>SteD</i>	This study
CS10221	<i>ΔsteA::FRT</i> in χ 3306, Δ <i>SteA</i>	This study
CS10222	<i>ΔsteB::FRT</i> in χ 3306, Δ <i>SteB</i>	This study
CS10223	<i>ΔsteD::FRT</i> in χ 3306, Δ <i>SteD</i>	This study
CS10224	<i>ΔsseK3::FRT</i> in χ 3306, Δ <i>SseK3</i>	This study
CS10225	<i>ΔsteE::Cm</i> in χ 3306, Δ <i>SteE</i>	This study
CS10226	<i>ΔsteE::FRT</i> in χ 3306, Δ <i>SteE</i>	This study
CS10227	<i>ΔslrP::FRT</i> in χ 3306, Δ <i>SlrP</i>	This study
CS10228	<i>ΔsarA::Cm</i> in χ 3306, Δ <i>SarA</i>	This study

Km: Kanamycin-resistant gene, 25 μg/mL; Cm: Chloramphenicol-resistant gene, 20 μg/mL; FRT: Flp recognition target.

h, followed by two washings with 1 × PBS. Then, 1-mL/well DMEM supplied with 10% heat-inactivated FBS and 100 μg/mL gentamicin was added into the well. The infected Hela cells were continually incubated at 37°C in the humidified incubator with 5% CO₂ for another 16 h.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

SYBR Premix Ex Taq II (Takara) was employed to amplify the genomic DNA. A Thermal Cycler Dice Real Time System (Takara) was used to conduct qPCR analysis.

2.7. Western blot (WB)

Cells were collected with 80 μL 2 × SDS loading buffer,

followed by ultrasonication, centrifugation at 4°C, and boiling at 98°C for 3 min. Lysates were resolved by 10% SDS-PAGE and a semi-dry blotter (Bio-Rad Laboratories, Hercules, CA) was used to transfer to polyvinylidene difluoride (PVDF) membranes (Millipore). After being blocked with 3% BSA for 1 h at room temperature, the PVDF membranes were incubated with the indicated primary antibodies (anti-MTR4 antibody was generated during a previous study (25), anti-RRP6 was purchased from abcam in the UK) for 1 h at room temperature, followed by incubating with the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) (Millipore, USA) for 1 h at room temperature. The chemiluminescence signals were detected with a Luminescent Image Analyzer (LAS-4000, Fujifilm) after addition of HRP substrate (Millipore).

Table 2. Oligonucleotides used for construction of mutant strains and plasmids in this study: Construction of *Salmonella* mutants

Primer	Sequence
AroA-P1-F	tcctgacgttacaaccatcgcgcgggtcgtggccagtgtaggctggagctgcttc
AroA-P2-R	ggfccaggatcgttaactggcgtatcggacagtgccaccagcatatgaatatcctcttag
AroA-check-F	gtgttggcgggtatgccc
AroA-check-R	gtcgaactggcgaacagaag
GogB-P1-F	agccatattgcaatatgcataacaagtaacgagggcagacagtgtaggctggagctgcttc
GogB-P2-R	gatcatcatgctgattccgataataccatcttagctcatgcatatgaatatcctcttag
GogB-check-F	ttgctgaatggctaacagc
GogB-check-R	catgtagcttagagttagg
GtgE-P1-F	taggcagcgtttacagaagtaatacagcaactcctcaggggtgtaggctggagctgcttc
GtgE-P2-R	aactatcataaaatgggtacaccagctttccaggaggggcatatgaatatcctcttag
GtgE-check-F	tagccacctccccaaaatcc
GtgE-check-R	ttaccccatagcttccccg
PipB2-P1-F	tgataaattttatcatgcaactgtgtctctctggaggtgtaggctggagctgcttc
PipB2-P2-R	tgttgtgtcctgtagcattgtggcgtctcagtagccatgaatatcctcttag
PipB2-check-F	gcagcacatgcaactgaag
PipB2-check-R	ctcagctactattcagtagc
SifA-P1-F	gtgaaatccttcaactccccaaagaaacgaaagcgtgtaggctggagctgcttc
SifA-P2-R	aacagccgctttgtttctgagcgaacgtgtagcgtggtcatatgaatatcctcttag
SifA-check-F	cgcagttgagataaaaagg
SifA-check-R	ggaagtacgtgagtaaaccc
SifB-P1-F	aaagagtgagatgtttctcaagtgtatctcaacgcgtgtaggctggagctgcttc
SifB-P2-R	atactattatggtgatcaactcgtgtagcagcctcacatgaatatcctcttag
SifB-check-F	tcaggtgtttaccgataag
SifB-check-R	cgaagcaattcgtccatag
SrfJ-P1-F	ccggaacttccatgaaaggcagactcatcttccgatcgggtgtaggctggagctgcttc
SrfJ-P2-R	catagcaactgtagcgcctgacggcggcagcgttaacgcatatgaatatcctcttag
SrfJ-check-F	atcgtctgaaccgagattg
SrfJ-check-R	tccgccacgttccctatc
SsaB-P1-F	ctcccatttattgctgaggaggattcatgctggcagttgtgtaggctggagctgcttc
SsaB-P2-R	tgtgtataaaccgtttaaaccatccccatccctgtgcatatgaatatcctcttag
SseF-P1-F	gcggcaagtaatafatgctgatgtaatagtcctctccgggtgtaggctggagctgcttc
SseF-check-F	gttatgcggatgcctcatgg
SseG-P2-R	tccggcgcacgtttctggcgttacctgagccagaaaccatgaatatcctcttag
SseI-P1-F	catattggaagcggatgtctcccctcatcagtaaccgtgtaggctggagctgcttc
SseI-P2-R	gttctgacagacgtcctccacggtgcttacatttaccatgaatatcctcttag
SseI-check-F	gaaaitaaggccaggaag
SseI-check-R	ctgtcatctgtgatagtgctc
SseJ-P1-F	gcgtgttaataaagtaaggaggacactatgccattgagtggtgtaggctggagctgcttc
SseJ-P2-R	tgctcaaggcgtaccgacggatggaactttatcagtgcatatgaatatcctcttag
SseJ-check-F	atgtaccaggcaataacctc
SseJ-check-R	cgggtggcattttatgactc
SseK1-P1-F	ttatgatccaccatataatgataatgtcccgccttctgtgtaggctggagctgcttc
SseK1-P2-R	ccatttccgctactgacatgcctcggccatgaaacttggccatgaatatcctcttag
SseK1-check-F	tagctgacagcgaattgcaac
SseK1-check-R	atatctccgttctgaacagc
SseK2-P1-F	aaagtaactcaaacatcgcacctacgctcagtcacctgtgtaggctggagctgcttc
SseK2-P2-R	ggctatcatgattacctccaagaactggcagttaaactgccatgaatatcctcttag
SseK2-check-F	cgcttaggtttagagacctc
SseK2-check-R	tggctctcaacttctcactc
SseK3-P1-F	gcaactccagctattactctgcctcatcaggtagtgcaaacgtgtaggctggagctgcttc
SseK3-P2-R	gccttagcccaccgacagaccatcaatgtatggatcggccatgaatatcctcttag
SseL-P1-F	aagaggtgagcagtagggccttaccattgtgtttagcgcgtgtaggctggagctgcttc
SseL-P2-R	tactggagactgattcatatatttcccgccgggttggcatalgaatatcctcttag
SseL-check-F	gtatcgcagcgtttaccagc
SseL-check-R	gtggtggaatcattgacggc
SteA-P1-F	gttgattgacatagtcataatgagagaggtaggacgtgtaggctggagctgcttc
SteA-P2-R	agttatgtagcagctttatgtcggcccccattgcccataatgaatatcctcttag
SteA-check-F	cggcagtgattcgtgtgc
SteA-check-R	ctgaggcggatcgtgct
SteB-P1-F	atctcaaccctgtgtcttccaggcttagtcaatgtggacgtgtaggctggagctgcttc
SteB-P2-R	ctgtggaatagcaatgccgggaagacatggcatgacactcatatgaatatcctcttag
SteB-check-F	gcagatgtcagttcttgaag
SteB-check-R	gaccagaagatgggcaactc
SteC-P1-F	gcgagatgaagactgtacacgatggcggcccttcttggagttaggctggagctgcttc
SteC-P2-R	ataccttagccacaagatcctctcctccggcggcgtgcatatgaatatcctcttag
SteC-check-F	cagaggatgagacatatgccg

3. Results

3.1. Live *Salmonella*, but neither heat-killed *Salmonella* nor LPS, induces loss of MTR4 and RRP6

Our previous study showed that *Salmonella* infection induces loss of MTR4 and RRP6, which are important components of the RNA exosome for RNA degradation in the nucleus, thus stabilizing the labile lncRNAs (25).

Table 2. Oligonucleotides used for construction of mutant strains and plasmids in this study: Construction of *Salmonella* mutants (continued)

Primer	Sequence
SteC-check-R	atctgtagcgaatgtgcccc
SteD-P1-F	atgaatgtcaacttcaggcgtggaatcgcaaacgccattgctgtaggctggagctgcttc
SteD-P2-R	ctatgacttgctgtgtttgctcattatggccaggctggccatagaatatcctccttag
SteD-check-F	gtgcagctcgacgtgcatgaagagggttatatg
SteD-check-R	ggctcttgaatacataacacc
SteE-P1-F	gcgcgttaacgcaggcgccacgttggtggtgattaccagtgtaggctggagctgcttc
SteE-P2-R	atgcaggccgcgctgtgaataacgcctgtcttttagccacatagaatatcctccttag
SteE-check-F	gaaaccgatgctgatgg
SteE-check-R	agcgccgaatcgcaatcc
sarA-P1-F	taatagtactaacagggtggcgagcacaatcgctccatagcatgtggtgtaggctggagctgcttc
sarA-P2-R	gatataaccggacgtgggttatgactggctgggtagtgcaactggcatagaatatcctccttag
pipA-P1	gctccggtcacctacagattaactcaaaagcggagtagtgtaggctggagctgcttc
pipA-P2	agatgtagaccattctgggaggtgaagatgcccatctccatagaatatcctccttag
pipA-check-F	cgctaacaatgctccggtgtaa
pipA-check-R	ggtcaatgtgccgctatttc
gtgA-P1	gtgtcttctgtaataccttatctctggaccaggaggaatggtgtaggctggagctgcttc
gtgA-P2	cgtaggcgattctgtggtgatgtgaccatctctttcatagaatatcctccttag
gtgA-check-F	aaatggtgggtgcaagggt
gtgA-check-R	gaaactaccagagcgggtgt
gogA-P1	ggattatccaatcctcatgacagaaggatttccagaccgtgtaggctggagctgcttc
gogA-P2	ctagattcgtagcgcattctgtggtgatgtgaccacatagaatatcctccttag
gogA-check-F	atctggggccacgcaatttt
gogA-check-R	ttactacaccacggcgtaa
avrA -P1	tggtacctggctcaatcattgaggcatatcttgcagcgtgtaggctggagctgcttc
avrA-P2	agtcttatggcgtggaaggattcctctggcaggcaaccatagaatatcctccttag
avrA-check-F	gccacagcccaaaaagaaa
avrA-check-R	atcctgtttgggatagct
sptP-P1	attgctaaggaaaactgataaggcatatgttgcacctggtgtaggctggagctgcttc
sptP-P2	cagcttgccctgcataagcaactggccttgcttcatagaatatcctccttag
sptP-check-F	taatggtgaactgctgcca
sptP-check-R	tgtgggcccctccattttat
pipB-P1	gagttctatcattgtaatccggagtgaggatgggtaggtgtaggctggagctgcttc
pipB-P2	tgcatgcggcggtagaccggtacgaaagaagcaatgaaagcatagaatatcctccttag
pipB-check-F	ggtttttacgccatctacgc
pipB-check-R	aatatcggggaaaacaggtg
pipC-P1	tacgtatcgcgttttatctcattaagaaagtatgtgacgggtgtaggctggagctgcttc
pipC-P2	cgfttatgacgcgttagcctgatgcccagaagatgcatagaatatcctccttag
pipC-check-F	agatcgtacagggatgatgg
pipC-check-R	tgagtgggtgctgcatct
sopD2-P1	ggggccttttaatgacttttatataagcatttgcgacgtgtaggctggagctgcttc
sopD2-P2	cggctagcccgtttgatgctgataaagaagcggccatagaatatcctccttag
sopD2-check-F	ctggttatgaccgctctt
sopD2-check-R	gcaggtctgatggatggtta
prgI-P1	ccaggccattggtatctcccaagcccacttaatttaacgggtgtaggctggagctgcttc
prgI-P2	ggacaatagtgcaatcgacataatccacctataactgacatagaatatcctccttag
prgI check-F	caagaaaagcgtcgagggt
prgI check-R	gcaagggtcattaccagcag
sspH2-P1	tggaagcggatgctctccgccaccatcagtaatcgccgctgtaggctggagctgcttc
sspH2-P2	ctaaggaggatattcatatgcaggtgaatgaggtcggtcgacaagaatattccggac
sspH2-check-F	cagcagagtatgatgctgc
sspH2-check-R	gattgtatctgtaaccggc
slrP-P1	gcatcaaaattagcaatgaggcctcaacagaggctgctgtaggctggagctgcttc
slrP-P2	ctaaggaggatattcatatgcggtgtaaacaggctctgataagcgcagcgtcgtcgta
slrP-check-F	ccctgatgcccaacagtaac
slrP-check-R	gaaggacctcaacctacaag

First, we considered whether only live *Salmonella* induces loss of MTR4 and RRP6. MTR4 and RRP6 were not decreased upon heat-killed *Salmonella* infection (Figure 1). In addition, LPS did not decrease MTR4 and RRP6. These show that live *Salmonella*, but not dead *Salmonella*, induces MTR4 and RRP6 degradation.

3.2. MTR4 and RRP6 decrement upon *Salmonella* infection

Both MTR4 and RRP6 are important components of

the RNA exosome in the mammalian nucleus. Upon wild type *Salmonella* infection, both MTR4 and RRP6 decreased dramatically (25). We hypothesized that MTR4 and RRP6 are not degraded by infection if important effector(s) involved in the degradation of these proteins are mutated. As shown in Table 1, 42 *Salmonella* mutant strains were constructed. WB analysis was performed to examine the degradation of MTR4 and RRP6 upon infection of these mutant strains. As shown in Figure 2, all 36 strains induced loss of MTR4 and RRP6, except Δ HilC Δ HilD, Δ HilD, Δ PrgI, Δ FlhD Δ FlhC, Δ ClpXP and Δ AroA.

3.3. Examination of proliferation of *Salmonella* mutant strains

Considering the growth condition of these mutant strains, next, we examined the proliferation of mutant strains in HeLa cells by monitoring the amount of the 16S ribosomal RNA gene (16S rRNA gene). Among these mutants, six mutant strains, Δ HilD, Δ HilC Δ HilD, Δ PrgI, Δ FlhD Δ FlhC, Δ ClpXP, and Δ AroA, did not grow well in the cells. HilC and HilD, transcriptional regulators encoded in SPI-1, are co-regulated and directly activate the expression of HilA (26), the central player of T3SS-1 regulation. In addition, HilD is necessary for activating regulons of both SPI-1 and SPI-2 (8). PrgI constitutes the needle of the T3SSs and is of great importance to effector translocation (27). T3SSs derive from flagella and still share regulatory mechanisms with them (28-30), after mutating the gene of the flagellum, the mutant strain Δ FlhD Δ FlhC also showed a poor proliferation (shown in Figure 3). The ClpXP protease, a member of the ATP-dependent protease family, is reported to regulate flagellum synthesis and SPI-1 expression negatively through FlhD₄C₂ degradation (10,31,32). As an auxotrophic mutation, deletion of *aroA* is commonly studied for attenuation without losing the ability of

immunostimulation. Felgner *et al.* found that deletion of *aroA* affects flagellin phase variation and the expression of virulence-associated the *arnT* and *ansB* genes (33). These genes, which show a poor proliferation, may greatly contribute to *Salmonella* invasion and/or proliferation in host cells.

4. Discussion

Salmonella infection induces an immune response in the host cells by invading and replicating inside the host cells. Lundberg *et al.* found that the expression of several invasion genes are growth phase regulated and correlate

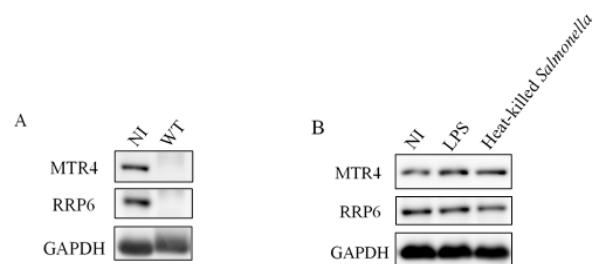


Figure 1. Alive *Salmonella*, but not heat-killed *Salmonella* or LPS, induced loss of MTR4 and RRP6. (A) MTR4 and RRP6 degradation upon *Salmonella* infection. NI: no infection; WT: wild type *Salmonella*. (B) LPS and heat-killed *Salmonella* did not induce loss of MTR4 and RRP6.

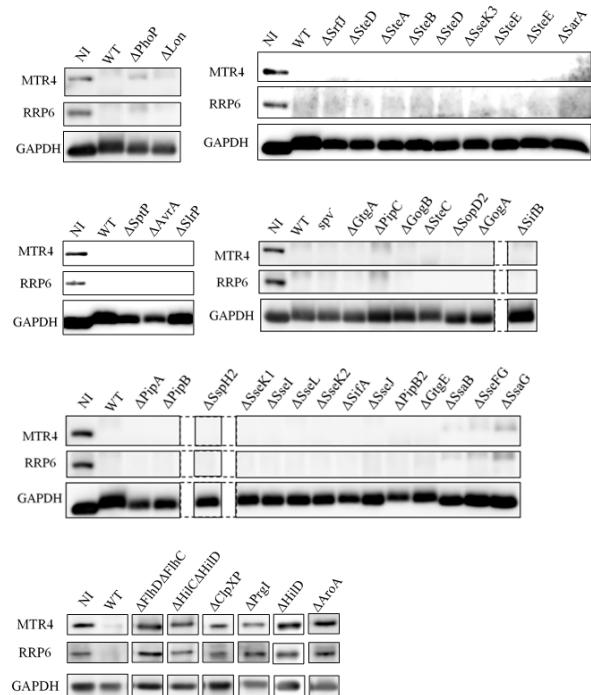


Figure 2. Investigation of loss of MTR4 and RRP6 in response to infection of *Salmonella* mutant strains. MTR4 and RRP6 were determined by WB.

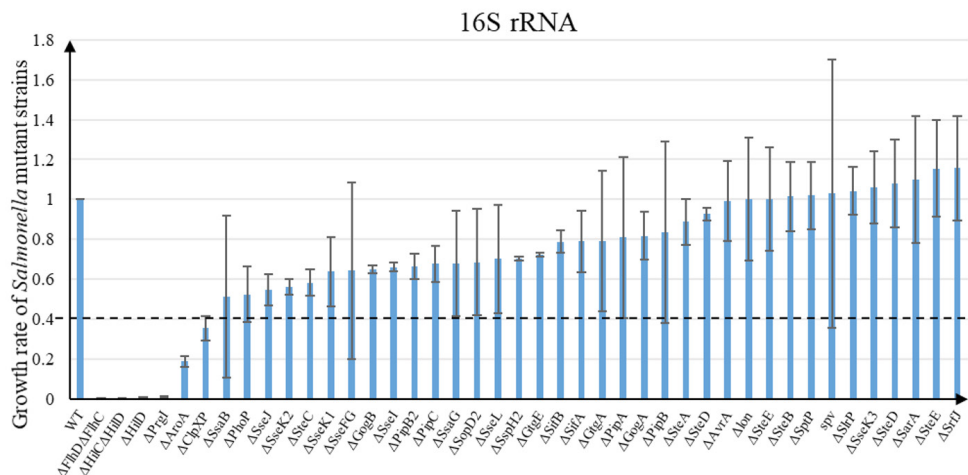


Figure 3. Proliferation of the 42 *Salmonella* mutant strains. To examine proliferation of *Salmonella* mutant strains, increment of 16S rRNA gene was measured by genomic PCR. 6 bars below the dashed line indicate the 6 mutant strains which did not grow well (< 40%). Data are shown as mean \pm SD ($n = 3$).

with apoptosis induction (34). Together with a series of effectors translocated by T3SSs, several regulators were also examined in our study. Our results showed that *Salmonella* mutant strains Δ ClpXP, Δ HilD, Δ HilC Δ HilD, Δ PrgI, and Δ AroA show poor proliferation, suggesting that *clpP*, *hilD*, *hilC*, *prgI*, and *aroA* are important factors for invasion and/or proliferation in host cells. Flagella are essential structures of bacteria. They provide the motility of *Salmonella* and increase adhesion to the host cells, thus facilitating the invasion process during host cell infection and triggering of the host immune system (35). Thus, the *Salmonella* mutant strain Δ FlhD Δ FlhC showed a poor proliferation in host cells after the flagellum gene (*flhD*) mutated. The poor proliferation may have been caused by attenuate adhesion or invasion abilities after the *flhD* mutated. In addition, ClpXP and AroA were reported to be involved in flagellum synthesis or flagellin phase variation (31,33).

In this study, although we mainly explored the effectors contributing to the degradation of MTR4 and RRP6, none of the well grown mutant strains canceled the degradation of MTR4 and RRP6. Several possibilities may contribute to this result. First, there may be no such effector for inducing loss of MTR4 and RRP6; instead, the loss might be the result of a complex immune response rather than a specific gene. In addition, a previous study showed that killed *Salmonella* or its LPS cannot induce lncRNA or eRNA, which may indicate that only those *Salmonellae* that are alive and able to invade the host cells can induce loss of MTR4 and RRP6 (25). Our study indicates that certain events associated with *Salmonella* proliferation in the host cell causes loss of MTR4 and RRP6, resulting in nuclear RNA stabilization. Because limited mutants were examined here, we cannot exclude the possibility that there might be such genes, but they are not included in the mutants that we constructed.

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