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Characterization of the HOCl-specific Transcription Factor HypT in *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar Typhimurium 에서 HOCl 특이적 전사인자 HypT 의 특성 규명

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석사학위논문

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이 논문을 석사학위논문으로 제출함
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서울대학교 대학원
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노태훈의 석사학위논문을 인준함
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ABSTRACT

Salmonella enterica serovar Typhimurium is one of the most common causes of food-borne illnesses. Despite many studies, no effective vaccine has been developed, and antibiotic therapy is also difficult that the alternative treatment is in great need. Hypochlorous acid (HOCl) is produced by immune system to kill invaded bacteria. HypT, a HOCl-specific transcription factor, is known to regulate several genes associated with HOCl-derived damage in *Escherichia coli*, but the detailed mechanism of HypT-mediated defense is not yet studied. This study investigated the mechanism of HypT-mediated defense in *Salmonella enterica* serovar Typhimurium SL1344. Based on the previous studies in *E. coli*, candidate genes were selected and transcription levels were compared. Expression of the genes related with iron acquisition was higher in the absence of *hypT*, indicating HypT represses the genes related with iron acquisition. HypT also repressed the genes related with iron acquisition in the absence of HOCl, and the repressive function was enhanced in the presence of HOCl. The difference in the expression of the genes related with iron acquisition was consistent to the phenotypes, and a higher level of intracellular iron content was detected in the absence of *hypT*. In addition, HypT was found to bind directly to the upstream of the promoter region of the iron acquisition

genes to regulate the transcription level. The expression of the *hypT* was not influenced by HOCl, suggesting that HOCl may modulate HypT protein activity directly. Survival rate was decreased in the absence of *hypT* under NaOCl treatment condition, confirming the defense function of *hypT* against HOCl. Based on the previous research that Met123, Met206 and Met230 play important role in sensing HOCl and mechanism of activation of HypT proposed by the co-worker, I confirmed that the specific oxidation of methionine residue 206 is important for activation of HypT. These results suggest that HypT mitigates the overall oxidative stress by reducing the ROS produced spontaneously in the cell to cope with external HOCl stress.

Keywords: *Salmonella*, HypT, HOCl, iron acquisition, methionine oxidation

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CONTENTS

Ⅰ. INTRODUCTION

Non-typhoidal *Salmonella* are one of the biggest causes of food poisoning and are making many hospitalizations and deaths worldwide [1]. Annually, 1 million cases of non-typhoidal *Salmonella* infection occurs in the United States with 400 deaths, and 80 million cases globally [2, 3]. Normally, infection by non-typhoidal *Salmonella* is a self-limiting diarrheal disease, so it heals naturally, but in the case of immunocompromised patients, it can develop into a systemic disease [4, 5]. To date, no vaccine has been developed that can elicit a robust immune response against intracellular invasion of *Salmonella* [2]. Also, treatment with antibiotics against *Salmonella* Typhimurium has been limited due to development of resistance and prolonged bacterial shedding [6]. Therefore, alternative treatment strategies are in need.

Human neutrophil is the first defense system to work against invading microorganisms, which phagocytize and localize the microorganisms in the phagosome [7]. Neutrophil kills bacteria by fusing azurophilic granules containing bactericidal substances in this phagosome and by generating reactive oxygen species (ROS) through respiratory burst [8, 9]. The myeloperoxidase (MPO) contained in this azurophilic granule generates

extremely potent oxidant hypochlorous acid (HOCl) using hydrogen peroxidase (H_2O_2), a product of respiratory burst [8]. HOCl has a strong oxidative effect on DNA, proteins, lipids, carbohydrates, etc., and is widely used as a disinfectant in hospitals, food industry and households due to its strong oxidizing effect [9, 10].

HOCl has a strong oxidizing power specifically for the methionine (Met) and cysteine (Cys) residues of the protein, and firstly oxidizes Met and Cys residues on contact with proteins [11, 12]. Studies have also shown that the specific oxidation of Met and Cys sometimes leads to the activation of proteins, such as sensors. For example, redox-regulated chaperone Hsp33, one of the defense mechanisms against HOCl, is unfolded by the HOCl in the redox switch domain of C-terminal and shows strong activity [13].

Oxygen (O_2) is an essential element of aerobic organism and plays an important role in ATP production. However, this indispensable oxygen metabolism produces reactive oxygen species (ROS) such as superoxide anion ($O^{2\cdot-}$) and hydrogen peroxide (H_2O_2). ROS has a strong oxidizing power and indiscriminately damages proteins, DNA, and lipids [14]. Among the produced ROS, hydrogen peroxide reacts with intracellular iron ion and generates extremely reactive hydroxyl radical ($OH\cdot$) through Fenton reaction [15].

$$Fe^{2+} + H_2O_2 \leftrightarrow Fe^{3+} + OH + OH\cdot$$

In addition to damaging several molecules with their oxidizing abilities, ROS causes genomic alteration by attacking chromosomal DNA or free nucleotides. To prevent damage and mutation caused by ROS, cells have defense mechanisms [16]. Although there exist defense mechanisms against these spontaneous oxidative stresses, when additional external oxidative stress is given, such spontaneous oxidative stress is also a great burden.

Recently, HOCl-specific transcription factor HypT has been studied in *Escherichia coli* (*E. coli*). HypT regulates several genes and provides a defense mechanism against HOCl [10]. However, it is still unclear how HypT recognizes HOCl and controls regulons. In this study, I found that HypT homologue present in *Salmonella enterica* serovar Typhimurium functions similar to *E. coli* HypT. And also, based on the protein structure, activation mechanism of protein HypT was identified at the molecular level.

Ⅱ. MATERIALS AND METHODS

2.1. Construction of bacterial deletion mutants.

Mutants of *Salmonella enterica* serovar Typhimurium SL1344 (STm-SL1344) were constructed using Lambda-Red recombination as described [17-19] or using transduction as described [20]. The whole genome sequence of *S*. Typhimurium was refered from Genbank.

2.1.1. Bacterial preparation for Lambda-Red recombination.

Strains used in this study are listed on the Table 1. All strains carrying plasmid pKD46-Amp^R (pKD46) used for Lambda-Red recombination were overnight cultured at 30℃ in 3 mL of Luria-Bertani (LB) medium with shaking (220 rpm) supplemented with following antibiotics as needed: ampicillin (50 μg/mL), kanamycin (50 μg/mL) and chloramphenicol (12.5 μg/mL). This overnight culture was inoculated into 50 mL of fresh LB medium (supplemented with appropriate antibiotics and 0.1 M of L-arabinose) at a ratio of 1/100 and incubated at 30℃ with shaking (220 rpm) for 2 hours reaching mid-log phase (main culture).

2.1.2. Construction of a *hypT*-deleted strain (Δ*hypT*) using Lambda-Red recombination.

First, the kanamycin resistance cassette from plasmid pKD13 was amplified using the following primers: *hypT*-P1-F-kan and *hypT*-P4-R-kan. The sequences of the primers are listed on the Table 2. Main culture of STm-SL1344 carrying plasmid pKD46 (STm-SL1344+pKD46) was prepared as described above. The cells were harvested by centrifugation (10,000 g, 7 min, 4℃) and re-suspended with 50 mL of cold sterilized water (washing). And then cells were washed 2 times more with 1 mL of sterilized water (13,000 g, 1 min, 4℃). Finally, the pellet was re-suspended with 100 μL of cold sterilized water. The PCR product was then introduced into electro-competent STm-SL1344+pKD46 by electroporation using MicroPulser™ Electroporator according to the manufacturer's instructions (Cat. #165-2100, Bio-Rad). Then, cells were recovered in 1 mL of super optimal catabolite (SOC) media at 30℃ with shaking (200 rpm) for 2 hours. Recovered cells were plated on to kanamycin (50 μg/mL) supplemented LB agar plate to select mutants, Δ*hypT*::kan. Homologous recombination was confirmed by PCR with following primers: *hypT*-out-F and *hypT*-in-R1 or *hypT*-out-R. And then, mutant was grown at 37℃ on kanamycin supplemented LB plate for curing.

Finally, FLP-producing plasmid pCP20 was introduced into Δ*hypT*::kan to remove kanamycin resistance cassette as described [21]. Gene disruption of *hypT* was confirmed by PCR with following primers: *hypT*-out-F and *hypT*-in-R1 or *hypT*-out-R.

2.1.3. Construction of a *hypT*/*rclR*-deleted strain (Δ*hypT*Δ*rclR*::cm) using Lambda-Red recombination.

The double mutant was constructed as described above based on the *hypT*-deleted strain. The chloramphenicol resistance cassette from plasmid pKD3 was amplified using the following primers: *rclR*-Red-R2 and *rclR*-Red-F. The PCR product was introduced into *hypT*-deleted strain carrying plasmid pKD46, and was selected using chloramphenicol resistance, *rclR*::cm. The chloramphenicol resistance cassette was then left unremoved. Finally, the deletion of *rclR* gene was confirmed by PCR with following primers: *rclR*-out-F and pKD3-c1-R.

2.1.4. Construction of a *rclR*-deleted strain (Δ*rclR*::cm) using transduction.

Bacteriophage P22 transduction was used to transfer marked deletion: Δ*rclR*::cm, from Δ*hypT*Δ*rclR*::cm as described [20]. Disruption of gene *rclR* was confirmed by PCR with following primers: *rclR*-out-F and pKD3-c1-R.

2.2. Plasmid construction for complementation study.

To construct a HypT expressing plasmid under the *lac* promoter, *hypT* gene of STm-SL1344 was PCR amplified with following primers: BamHI-*hypT*-F and HindIII-*hypT*-R, to contain restriction enzyme site of BamHI and HindIII. This amplified *hypT* gene was inserted between the BamHI and HindIII of pUHE21-2*lacI*^{q} [22]: pUHE21-2*lacI*^{q}::*hypT*. And then, the insert was confirmed by DNA sequencing using following primers: BamHI-*hypT*-F and HindIII-*hypT*-R.

2.3. Single amino acid alteration of *hypT*.

To make a single mutation of methionine residue 206 (Met206) in the *hypT* gene, QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was applied. To change methionine 206 into leucine (M206L), pUHE21-2*lacI*^{q}::*hypT* was PCR amplified respectively using two sets of primers: BamHI-*hypT*-F / pt-M206L-*hypT*-R and HindIII-*hypT*-R / pt-

M206L-*hypT*-F as described in Figure 1 . And then, this two PCR product was amplified using each other as a template and also as a primer. After connecting two PCR fragments, it was inserted into pUHE21-2*lacI*^{q} vector: pUHE21-2*lacI*^{q}::*hypT*^{M206L}. The insert was DNA sequencing confirmed using following primers: BamHI-*hypT*-F and HindIII-*hypT*-R. The same method as above was used to substitute Met206 into glutamine (M206Q): pUHE21-2*lacI*^{q}::*hypT*^{M206Q}. Following primer sets are used for M206Q construction: BamHI-*hypT*-F / pt-M206Q-*hypT*-R and HindIII-*hypT*-R / pt-M206Q-*hypT*-F.

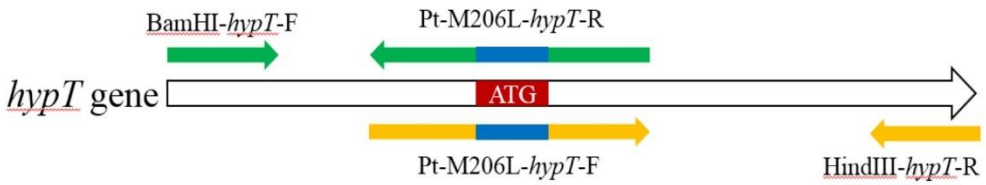


Figure 1. Construction of Met206 altered *hypT* gene.

2.4. RNA extraction and qRT-PCR.

Total RNA was isolated from STm-SL1344 strains. All strains were cultured overnight at 37℃ with shaking (220 rpm) in M9 medium (pH 5.8) supplemented with antibiotics as needed and sub-cultured in 2 mL of M9 medium with 1% overnight culture, antibiotics and 500 μM of IPTG as needed for 3.5 hours, reaching mid-log phase. If NaOCl treatment is needed, various concentrations of NaOCl was treated for 10 min. 2 volumes of RNAprotect bacterial reagent (Qiagen, Cat. #76506) was treated to 500 μM of samples prior to RNA extraction. Total RNA was isolated using RNeasy mini kit (Qiagen, Cat. #74524) according to the manufacturer's instruction. Ambion Turbo DNA-*free*™ (Ambion, Cat. #AM1907) was treated to remove residual DNA. The isolated DNA was then synthesized into cDNA using EcoDry™ Premix and random hexamers (Clontech, Cat. #639546). The synthesized cDNAs were mixed with 2×iQ SYBR Green Supermix (Bio-Rad, Cat. #170-8882) and RT-PCR was performed using CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The expression levels of mRNAs were normalized to the *gyrB* (DNA gyrase subunit B). Primer sets used for qRT-PCR are listed on the Table 3. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad software, San Diego, CA, USA).

2.5. Iron content measurement using ICP-AES.

STm-SL1344 strains were cultured overnight in LB medium supplemented with ampicillin (50 μg/mL) at 37℃ with shaking (220 rpm) and sub-cultured with 1% overnight culture, ampicillin and 500 μM of IPTG for 3 hours. 350 μM of NaOCl was treated for 1 hour and CFU was measured to determine the number of cells. 1 mL of cultures were harvested by centrifugation at 16,000 g, 4℃ for 2 min and the pellet was washed twice with triple-distilled water. Pellet was re-suspended in 100 μL of triple-distilled water and 2 volumes of 70% HNO_3 was treated to lyse the cells, lightly vortexed and heated at 75℃ for 5 min. The lysates were centrifuged at 16,000 g for 5 min and the 100 μL of supernatant was mixed with 2 mL of 1% HNO_3. And the iron content in the supernatants was analyzed by ICP-AES (Inductively coupled plasma atomic emission spectroscopy) using Varian 820-MS (Varian). The iron content was normalized to the number of cells [23]. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad software).

2.6. Electrophoretic mobility shift assay (EMSA) using a fluorescence-based method.

The 200 base pairs upstream of target operons (*sit*, *feo*, *fhu*) predicted to be the promoter region was PCR amplified. Primers used for PCR amplifications are listed on the Table 4. The EMSA was performed according to the manufacturer's instructions (ThermoFisher Scientific, Cat. #E33075). Briefly, PCR product and HypT protein complexes were separated by gel electrophoresis (7% poly-acrylamide gel, 1.0 mm). After electrophoresis, the poly-acrylamide gel was stained with SYBR Greed EMSA to stain DNA and imaged with Chemidoc (Bio-Rad). And then, the poly-acrylamide gel was re-stained with SYPRO Ruby EMSA to stain the protein and imaged with the Chemidoc again. Additionally, to prevent the non-specific binding between protein and DNA fragments, poly-dIdC was added.

2.7. DNaseI Foot printing.

The same region of the DNA was PCR amplified as used in EMSA, and the forward primer was tagged with a 6FAM dye in the 5 '. Primers used for PCR amplifications are listed on the Table 5. The DNaseI Foot printing was performed according to the protocol. Briefly, 200 ng of 6FAM tagged PCR

product was incubated with 800 ng of wild type HypT or HypT^{M206Q} for 15 min. 0.04 unit of DNaseI was treated for 30 sec. DNA fragments were analyzed using ABI3730xl DNA Analyzer (PE Applied Biosystems, Lincoln centre Drive Foster City, CA, USA). Analyzed DNA fragments were visualized using Peak Scanner software version 1.0 (Applied Biosystems, Lincoln centre Drive Foster City, CA, USA).

2.8. Viability assay.

Δ*rclR*::cm strains were cultured overnight in M9 medium (pH 5.8) supplemented with antibiotics (chloramphenicol 12.5 μg/mL, ampicillin 50 μg/mL) at 37℃ with shaking (220 rpm) and sub-cultured in 2 mL of M9 with 1% overnight culture, antibiotics and 500 μM of IPTG for 4 hours reaching mid-log phase. And then, 435 μM of NaOCl was treated for 15 min at 37℃ with shaking (220 rpm) as described by Drazic et al. with modifications [10]. After the NaOCl treatment, samples were serially diluted in PBS and dotted on the carbenicillin (100 μg/mL) supplemented LB plate. Plates were photographed with GelDoc EZ imager (Bio-Rad).

Table 1. Bacterial strains and plasmids

Strains	Description	Reference
***Salmonella enterica* serovar Typhimurium**		
SL1344	Wild type, Sm^R	[24]
	Δ*hypT*	This study
	Δ*hypT* Δ*rclR*::*cm*	This study
	Δ*rclR*::*cm*	This study
Escherichia coli		
DH5α	*gyrA96 recA1 relA1 endA1 thi-1 hsdR17 glnV44 deoR* Δ(*lacZYA-argF*)*U169* [Φ80d Δ(*lacZ*)*M15*]	[25]
Plasmids		
pKD46	Ap^R P_{BAD}-*gam-beta-exo oriR101 repA101*^{ts}	[17]
pKD13	Ap^R FRT Km^R FRT PS1 PS4 *oriR6Kγ*	[17]
pKD3	bla FRT cm FRT *oriR6K*	[17]
pCP20	Ap^R Cm^R *cI857* λP_R*flp oripSC101*^{ts}	[17]
pUHE21-2*lacI*^{q}	rep_{pMB1} Ap^R *lacI*^{q}	[26]
pUHE21-2*lacI*^{q}-*hypT*		This study
pUHE21-2*lacI*^{q}-*hypT*^{M206Q}		This study
pUHE21-2*lacI*^{q}-*hypT*^{M206L}		This study

a. Sm^R, streptomycin resistant; Ap^R, ampicillin resistant; Km^R, kanamycin resistant; Cm^R, chloramphenicol resistant.

Table 2. Primers used for the construction of bacterial strains and plasmids

Primers	Sequences (5' to 3')
hypT-P1-F-kan	CCT CTT TGA CCG GGG AGC AAC GCC TTA CCG CCT GAG GTG TTG TAG GCT GGA GCT GCT TC
hypT-P4-R-kan	CGT AGA ATG CCC CTT CGG GCG GCT GAC AGG ATA ACG GTA ACT GTC AAA CAT GAG AAT TAA
hypT-out-F	TGA TTG ATT CCA CAG CGT CG
hypT-in-R1	CGT CTC TTC GAT GAG TGA AT
hypT-out-R	CAT TGA GCA GGT GTA TGC GC
rclR-Red-F	CCG CCT GCG GCG TCA AAC TGC AGG CGG GTT CAG CGT ACG TGT GTA GGC TGG AGC TGC TTC
rclR-Red-R2	TTA TCT CTA GTT TTG AAA ATA AAT CTG TTC TGG AGT CTA TAT GGG AAT TAG CCA TGG TCC
rclR-out-F	GCC GGT CTG CTA CCT GTT TTA
pKD3-c1-R	TTT TCA CCA TGG GCA AAT AT
BamHI-*hypT*-F	AAA GGA TCC ATG GAT GTA ACT GGA GCA GG
HindIII-*hypT*-R	AAA AAG CTT TTA CAG CGC GGC CTG AAG CC
pt-M206Q-*hypT*-F	AAC TCC TAT CAG GGC CGA TTG AT
pt-M206Q-*hypT*-R	ATC AAT CGG CCC TGA TAG GAG TT

pt-M206L-*hypT*-F	AAC TCC TAT CTT GGC CGA TTG AT
pt-M206L-*hypT*-R	ATC AAT CGG CCA AGA TAG GAG TT

Table 3. Primers used for qRT-PCR

Primers	Sequences (5' to 3')	Classification
***gyrB*-qRT-F**	ATA TCG GCG ACA CGG ATG AC	Control
***gyrB*-qRT-R**	CCT TCT TCC GGG TGA ATC CC	
***hypT*-qRT-F**	CTG GAG CAG GTT TGC ACA AT	*hypT* transcription
***hypT*-qRT-R**	GGC GAG ACC TGT CGG TTA AA	
***metN*-qRT-F1**	AGC GTT GTT AGC TCC TGA CC	Met, cys biosynthesis related genes
***metN*-qRT-R1**	AAA GTG TTC CAG CAG GGG AC	
***metB*-qRT-F1**	TAT GAC GCG TAA ACA GGC CA	
***metB*-qRT-R1**	CTA ATG CGC GCT GAA CAA CA	
***cysH*-qRT-F1**	GGA TAT CCG GGC GAA TCT GG	
***cysH*-qRT-R1**	AAG TCG ATC GCG TAA TGG CT	
***sitC*-qRT-F1**	TCA AAG GCT GGT CGC TCA TT	Iron acquisition related genes
***sitC*-qRT-R1**	CAC CAT AAA AAG GCC GAC GC	
***sitD*-qRT-F1**	AAC GCC CTT ATG GTT TCG GT	
***sitD*-qRT-R1**	GAC GAT GCC CAT CAC CGT AT	
***feoA*-qRT-F1**	ATT CAC TCC TGA CAC TGC GT	
***feoA*-qRT-R1**	AAT GAA GAG CCG GGC AAC A	
***feoB*-qRT-F1**	AAG GAC GAA ATG GAC GAC CC	
***feoB*-qRT-R1**	AAT GGC GAG CAG GAA CAT	

	CA
***fhuA*-qRT-F1**	CGT GTT GAG TTG ATG CGT GG
***fhuA*-qRT-R1**	GCC CCG TCA GAC GAT ATG AG
***fhuC*-qRT-F1**	CAC GCT TTT ACA CCC CCT CT
***fhuC*-qRT-R1**	CCT GTG GCA ATT GTT GAG GC

Table 4. Primers used for EMSA

Primers	Sequences (5' to 3')
Up200-*sit*-F	CGA TAC GTT TAC CAG GCA TC
Up200-*sit*-R	AGT ATC CCT CGC AAC AAT GT
Up200-*feo*-F	GCC ATA TCA ACA TCG GCG CA
Up200-*feo*-R	ACG ACC TAC TTG TTT CTT AC
Up200-*fhu*-F	TCG CCA TCC GGC AAA TCC TC
Up200-*fhu*-R	TGG TAT ATC TCT GAT GAA AG

Table 5. Primers used for DNase Foot printing

Primers	Sequences (5' to 3')	
FAM-Up200-*sit*-F	CGA TAC GTT TAC CAG GCA TC	5' 6FAM tagged
Up200-*sit*-R	AGT ATC CCT CGC AAC AAT GT	
FAM-Up200-*feo*-F	GCC ATA TCA ACA TCG GCG CA	5' 6FAM tagged
Up200-*feo*-R	ACG ACC TAC TTG TTT CTT AC	
FAM-Up200-*fhu*-F	TCG CCA TCC GGC AAA TCC TC	5' 6FAM tagged
Up200-*fhu*-R	TGG TAT ATC TCT GAT GAA AG	

Ⅲ. RESULT

3.1. Identification of HypT in *S*. Typhimurium.

An uncharacterized LysR-type transcription regulator (LTTR) gene was found as a candidate for *hypT* in *Salmonella enterica* serovar Typhimurium SL1344. This gene showed high sequence identity (82%) with the *hypT* of *Escherichia coli* str. K-12 substr. MG1655. And also, methionine residues (Met123, Met206 and Met230) that are important in sensing HOCl are conserved [27]. Therefore, uncharacterized LTTR gene was determined to be *hypT* in STm-SL1344 and proceeded the study.

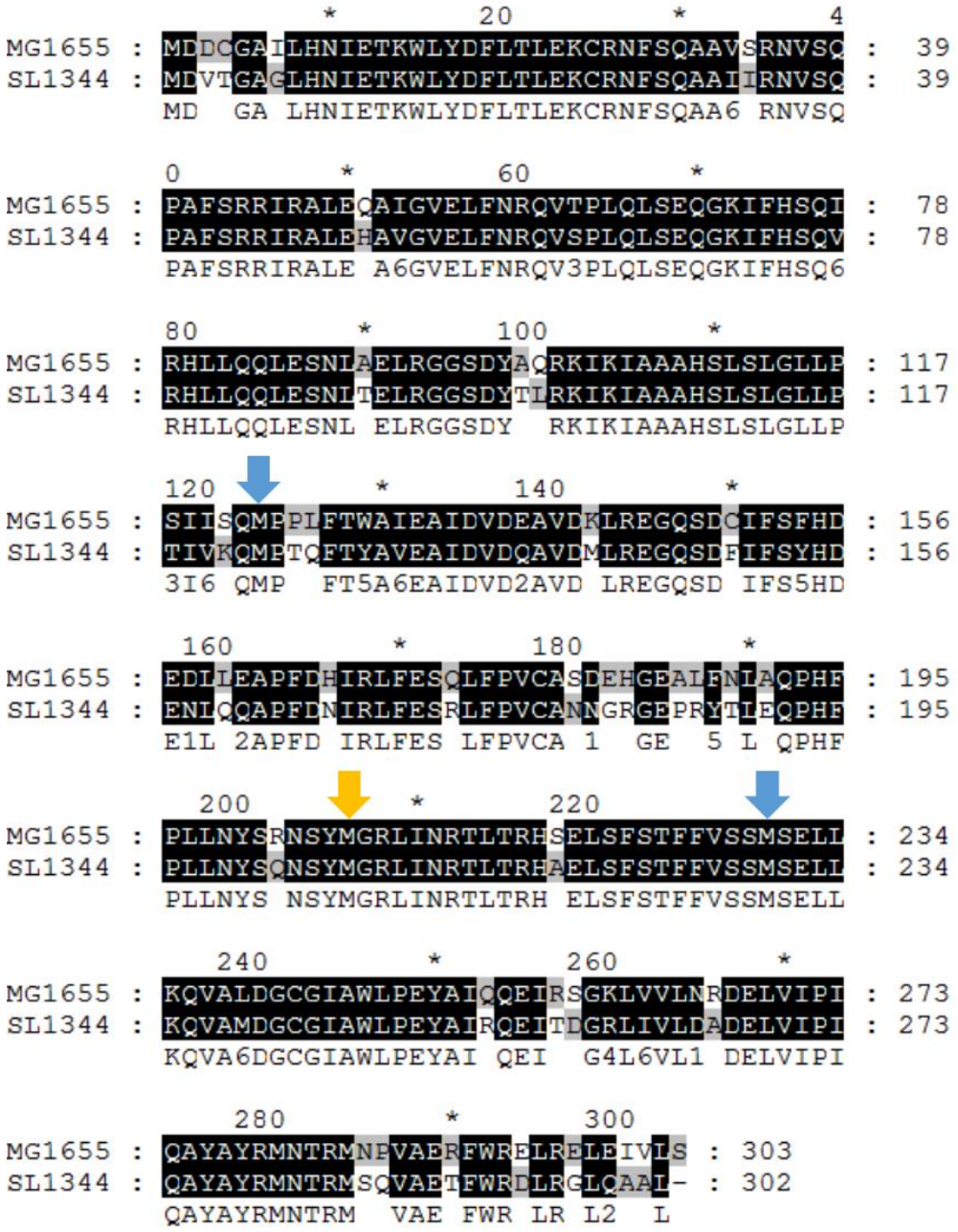


Figure 2. Alignment of HypT from *S.* Typhimurium and *E. coli*.

HypT of *E. coli* K-12 MG1655 was used as a reference and HypT of STm-

SL1344 was aligned. The gene showed 82% identity and 90% positives. The three conserved methionines that sense HOCl are indicated by arrows. Among them, Met206 is indicated by yellow arrow which is predicted to be the key residue of protein activation.

3.2. The expression of the iron acquisition related genes is repressed by HypT in *Salmonella*.

qRT-PCR was performed to find target genes regulated by HypT in *Salmonella*. Previous studies have shown that HypT in *E. coli* regulates several genes to counteract HOCl oxidative stress. When the protein is exposed to HOCl, cysteine and methionine are firstly oxidized. Therefore, biosynthesis of cysteine and methionine is increased in order to replenish them [10]. In addition, in order to alleviate oxidative stress, HypT lowers the expression of genes involved in iron acquisition to reduce the production of hydroxyl radicals that are continuously produced by intracellular free iron ions [28, 29]. Since HypT is thought to work similarly to *E. coli* in *Salmonella*, genes associated with cysteine, methionine biosynthesis and genes involved in iron acquisition were selected as candidates.

Transcription levels of putative target genes (*feoA*, *feoB*, *fhuA*, *fhuC*, *sitC*,

sitD, *metN*, *metB* and *cysH*) were compared in STm-SL1344 strains under HOCl-treated condition and non-treated condition. As a result, deletion of *hypT* (Δ*hypT*+pUHE21-2*lacI*^{q}) increased transcription levels of iron acquisition related genes by about 2-fold compared to the wild type strain (SL1344+pUHE21-2*lacI*^{q}). And complementation with the *hypT*-expressing plasmid (Δ*hypT*+pUHE21-2*lacI*^{q}::*hypT*) restored the transcription level of iron acquisition related genes to the that of wild type strain (Figure 3). However, the absence of *hypT* did not show any significant changes in the transcription level of cysteine, methionine biosynthetic genes. This result suggests that *Salmonella* HypT plays a role in lowering the expression level of iron acquisition genes and does not involved in methionine or cysteine biosynthesis, unlike *E. coli*.

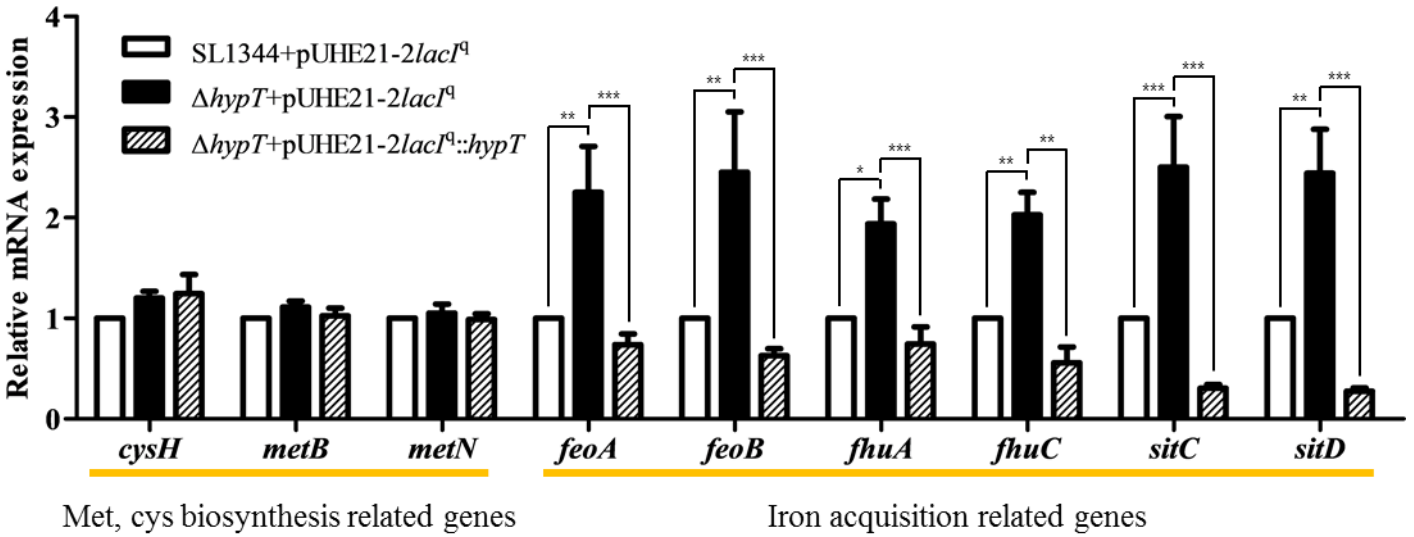


Figure 3. Transcriptional regulation by HypT in *Salmonella*.

Transcription levels of putative target genes under HOCl stress. From the left, each bar represents wild type (SL1344) and *hypT*-deleted (Δ*hypT*) strains carrying control plasmid (pUHE21-2*lacI*^{q}) or *hypT*-deleted strain with *hypT*-expressing complementation plasmid (Δ*hypT*+pUHE21-2*lacI*^{q}::*hypT*). The expression levels of mRNAs are normalized to the *gyrB* and the data represent four independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

3.3. Intracellular iron content regulated by HypT.

The iron content in the cells was measured to see if the repression of the iron acquisition related genes by HypT (Figure 3) is consistent with the phenotype. ICP-AES (Inductively coupled plasma atomic emission spectroscopy) was used to measure intracellular iron content. Briefly introducing, an atom is energized to the excited state, and then the radiation is measured when it returns to the ground state. The intensity of the radiation is proportional to the concentration of the element in the sample [30]. As a result, about 1.5-fold higher iron content was detected in *hypT*-deleted strain (Δ*hypT*+pUHE21-2*lacI*^{q}) compared to the wild type strain (SL1344+ pUHE21-2*lacI*^{q}) and the complementation of the *hypT* gene (Δ*hypT*+pUHE21-2*lacI*^{q}::*hypT*) restored intracellular iron content to beyond the wild type level. Thus, it was confirmed that the difference in gene expression was consistent with the phenotype.

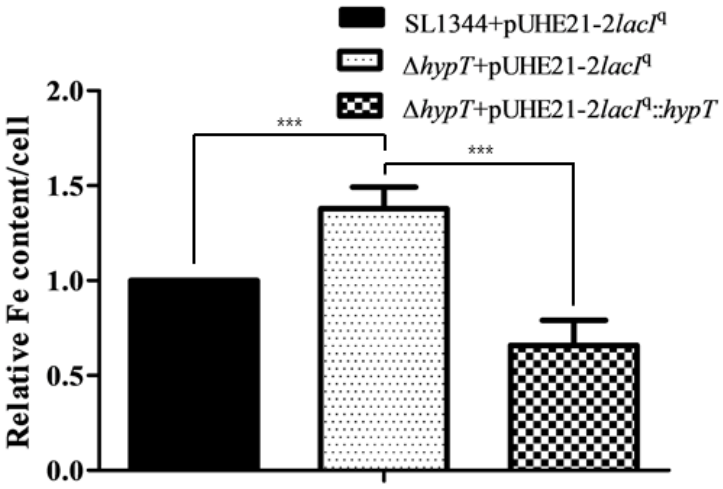


Figure 4. Intracellular iron content of the *S*. Typhimurium strains.

Iron content of the 3 strains used in qRT-PCR (Figure 3) was measured using ICP-AES after exposure to NaOCl. Iron content was divided by the number of the cells to determine relative iron content per cell. The data represent three independent experiments. ***P < 0.001.

3.4. HOCl stress lowers the expression of iron acquisition related genes.

To confirm whether the *Salmonella* actually works to lower the iron uptake under HOCl stress (to reduce the spontaneously-produced hydroxyl radical through Fenton reaction [15]), the expression levels of iron acquisition related genes were measured under NaOCl treated or non-treated conditions by qRT-PCR. When the NaOCl was treated to the wild type strain (STm-

SL1344), the expression levels of the iron acquisition related genes were decreased by about 2 times. These results indicate that *Salmonella* actually protect itself from HOCl oxidative stress by lowering the iron uptake.

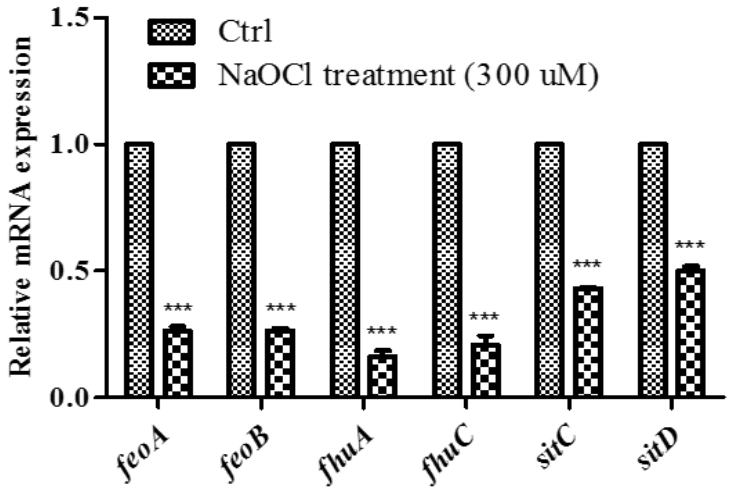
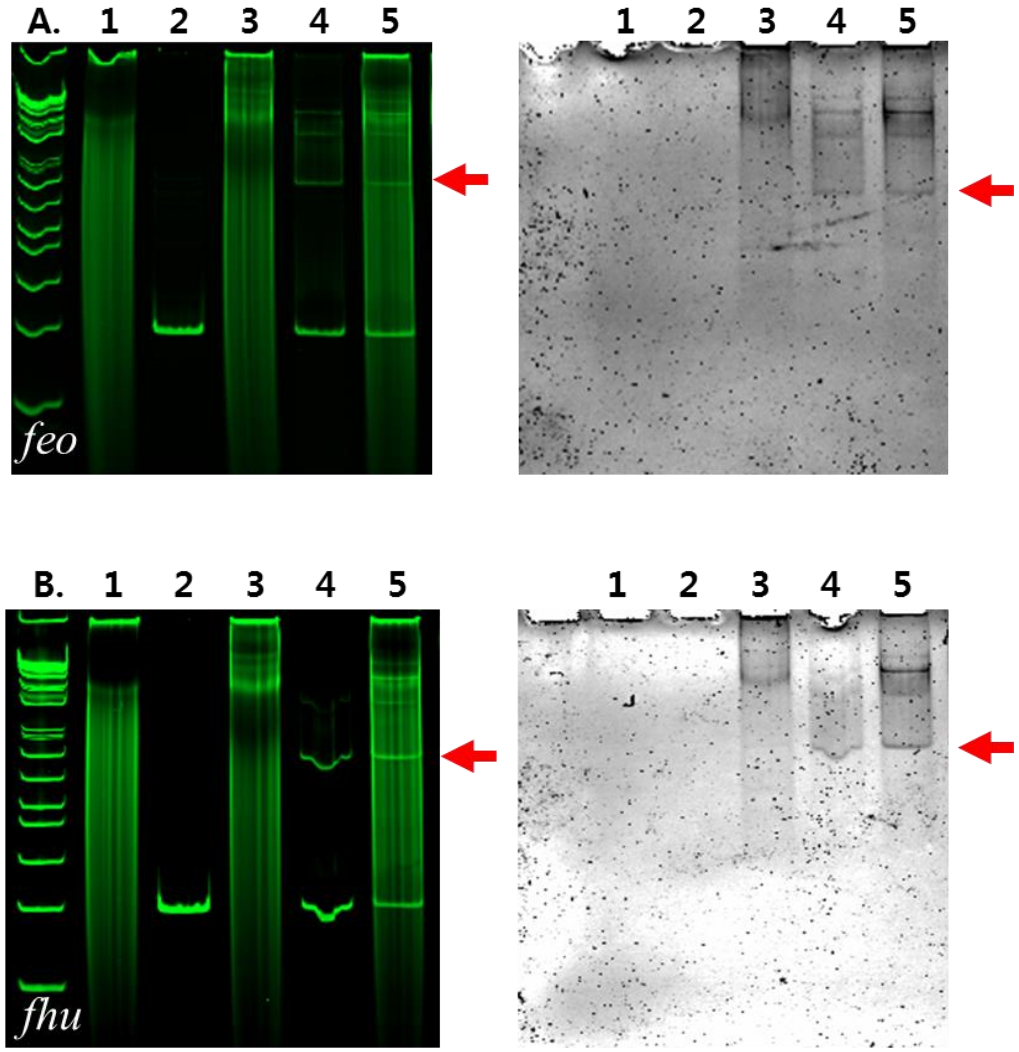


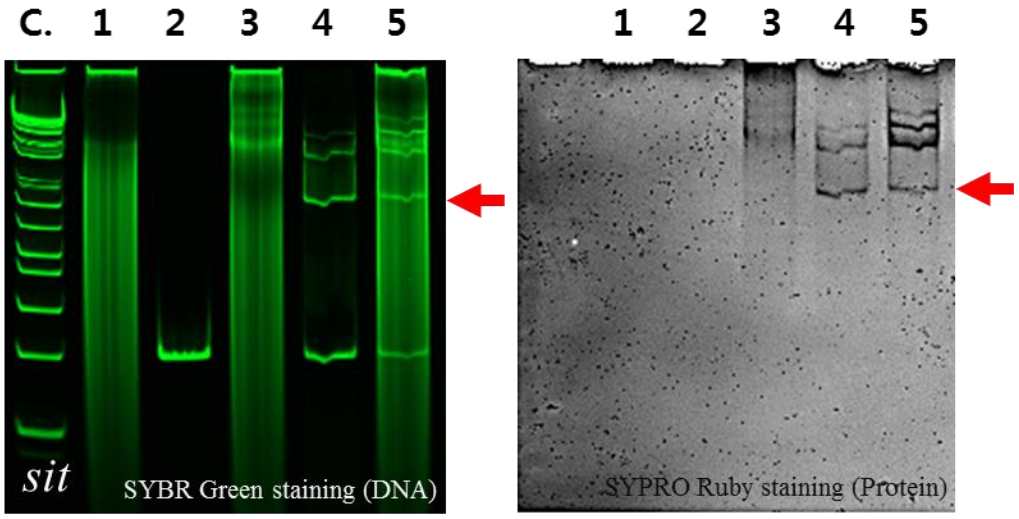
Figure 5. The expression levels of iron acquisition related genes in the absence (Ctrl) or presence of NaOCl.

Under the NaOCl treated condition, the transcription levels of iron acquisition related genes were decreased. The expression levels of mRNAs are normalized to the *gyrB* and the data represent four independent experiments. ***P < 0.001 in comparison to Ctrl.

3.5. HypT directly binds to control the regulons.

Previous results suggest that *Salmonella* HypT works similar to HypT of *E. coli*. However, in previous studies, HypT has only been studied to reduce the expression of iron acquisition related genes, but it has not been identified how to control regulons. To investigate how HypT regulates the iron acquisition operons, EMSA (Electrophoretic mobility shift assay) was conducted. The upstream 200 base pairs of each operon (*feo*, *fhu*, *sit*) predicted to contain a promoter region was PCR amplified and incubated with HypT. As a result of gel electrophoresis of HypT and PCR product, band shift occurred (indicated by a red arrow). These result indicate that HypT binds directly to the promoter region of the target gene. However, further studied are needed to identify the sequences needed for HypT binding.


A.
1
2
3
4
5
feo
1
2
3
4
5
B.
1
2
3
4
5
fhu
1
2
3
4
5



D.

Lane	1	2	3	4	5
Protein (ng)	0	0	500	250	500
DNA (ng)	0	100	0	100	100
Poly-dIdC (ng)	500	0	500	0	500

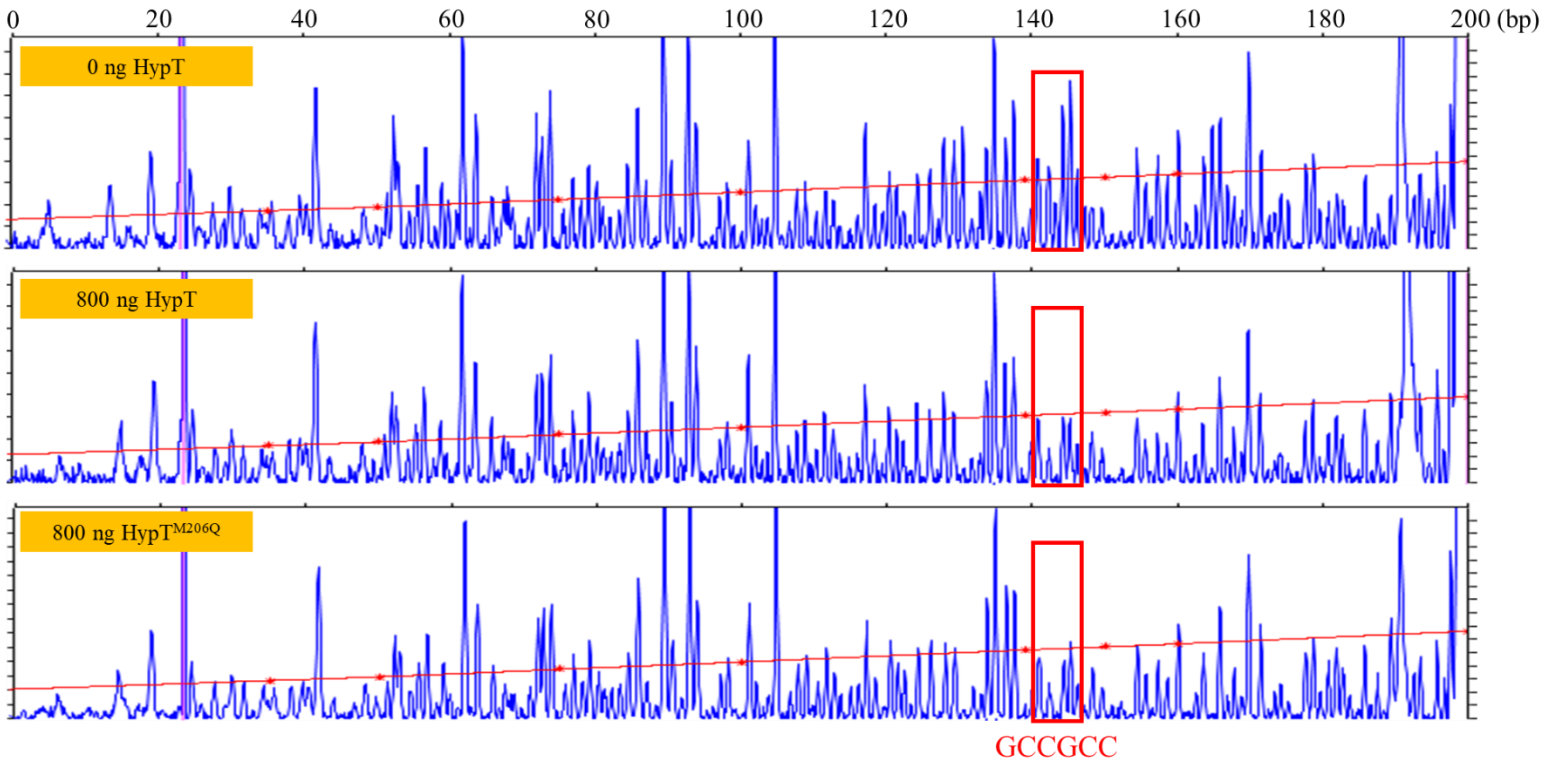
Figure 6. Electrophoretic mobility shift assay (EMSA) of HypT and predicted promoter region (*feo*, *fhu*, *sit* operon).

Lane 1; Poly-dIdC only, Lane 2; DNA only, Lane3; Protein mixed with poly-dIdC, Lane 4; Protein mixed with DNA, Lane 5; Protein mixed with DNA and poly-dIdC. Detailed information about the lanes is on (D). The left pictures represent the SYBR Green stained gels for DNA, and the right pictures represent the re-stained gels by SYPRO Ruby stain for protein. To reduce the non-specific binding between DNA and protein, poly-dIdC was added. The up-shifted bands which indicate binding between DNA and protein, is not affected by poly-dIdC addition (Lane 5).

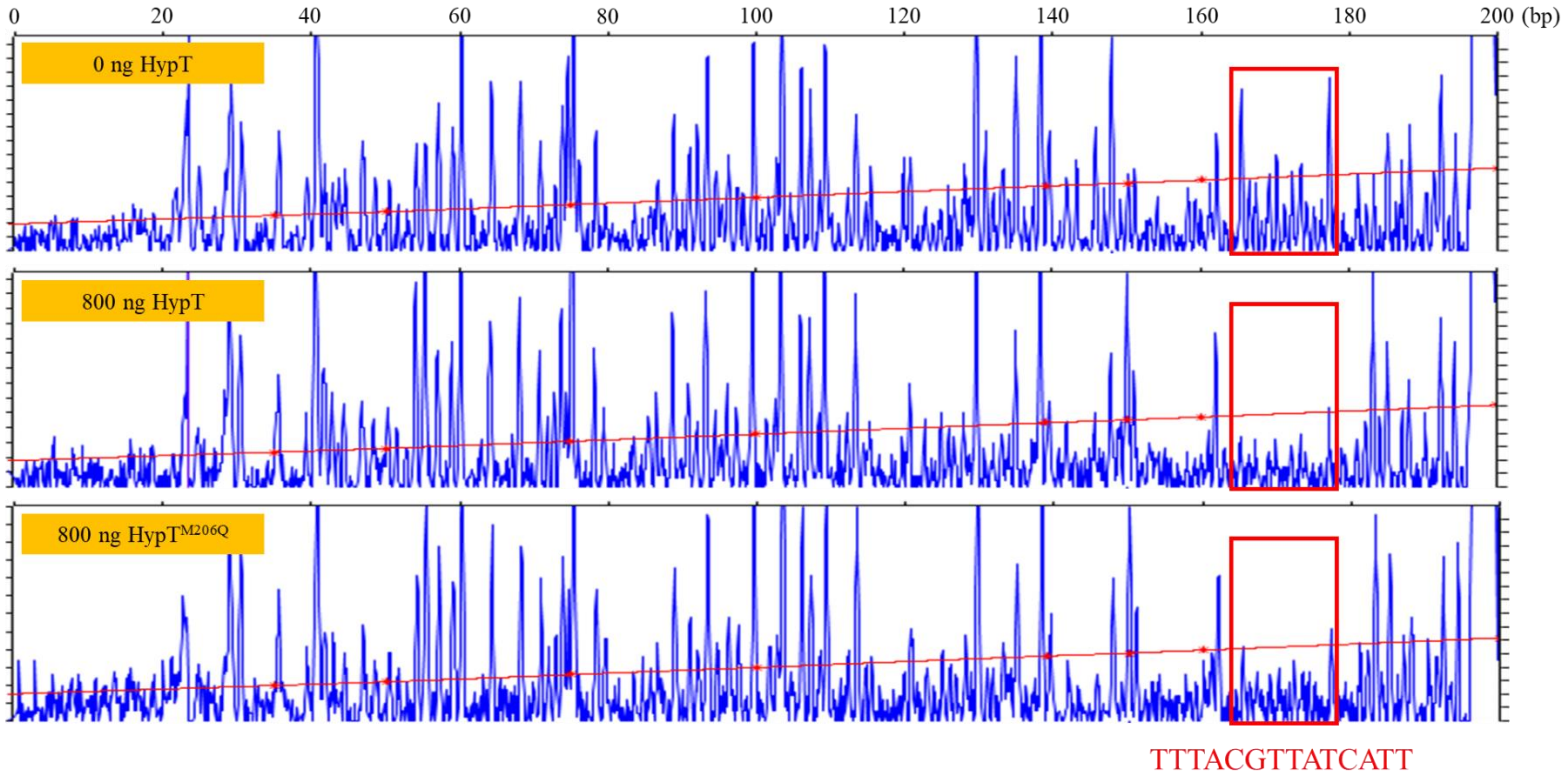
3.6. HypT binds to the upstream of the regulon.

To specify the HypT binding region, DNaseI Foot printing was performed. This method is based on the principle that when a protein and a DNA bind to each other, the part is protected by DNase digestion. The area marked in red box indicates the protection region and the sequence in the binding part is also marked in red. HypT and $HypT^{M206Q}$ have the same binding sites for each operon.

A.


0
20
40
60
80
100
120
140
160
180
200 (bp)
0 ng HypT
800 ng HypT
800 ng HypT^{M206Q}
GCCGCC

B.


0
20
40
60
80
100
120
140
160
180
200 (bp)
0 ng HypT
800 ng HypT
800 ng HypT^{M206Q}
TTTACGTTATCATT

C.

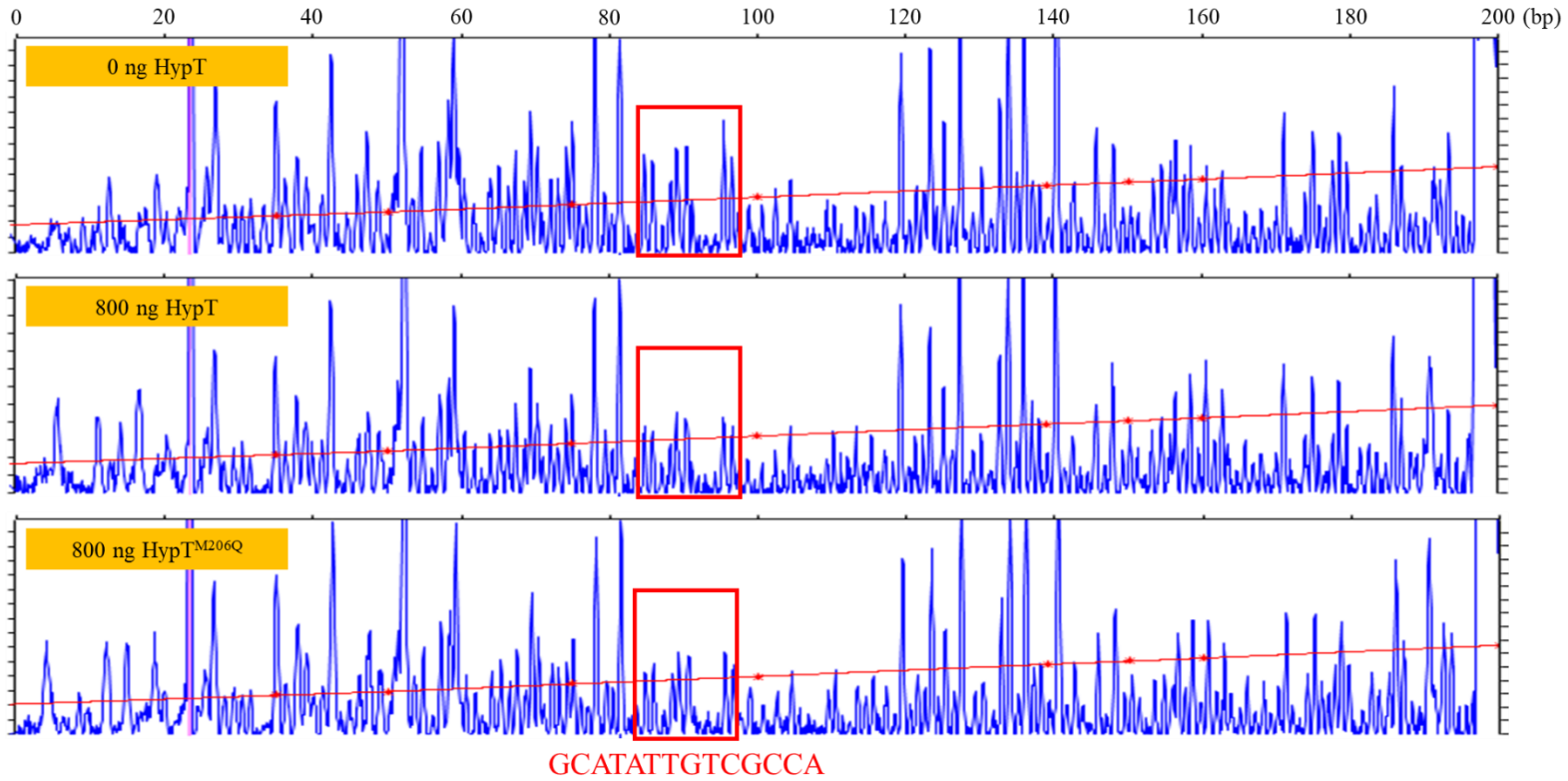

0
20
40
60
80
100
120
140
160
180
200 (bp)
0 ng HypT
800 ng HypT
800 ng HypT^M206Q
GCATATTGTCGCCA

Figure 7. HypT binding region of upstream 200 bps.

DNaseI Foot printing of predicted promoter regions (*feo*, *fhu*, *sit* operons). Figures A, B, and C show experimental results for the upstream 200 bps of *feo*, *fhu*, and *sit* operon, respectively.

3.7. Gene expression of *hypT* is not affected by NaOCl concentration.

To determine whether *hypT* gene expression is regulated by HOCl, expression level of *hypT* is measured by qRT-PCR under various concentrations of NaOCl treatment in STm-SL1344. Although the concentration of NaOCl increased, the mRNA transcription level of *hypT* did not show any significant difference. These results suggest that HypT undergoes direct modulation by HOCl to regulate its activity.

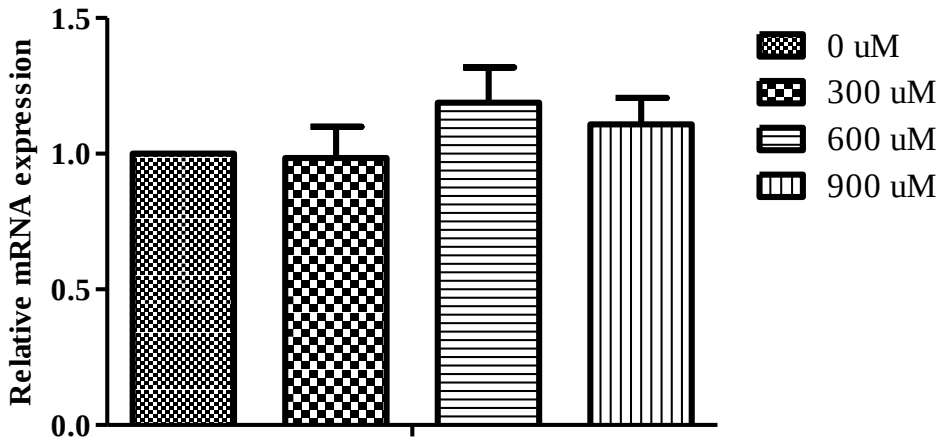


Figure 8. Transcription level of *hypT* as NaOCl concentration increased.

A qRT-PCR revealed that the concentration of NaOCl did not affect the expression level of *hypT* in *S*. Typhimurium SL1344. The expression levels of mRNA are normalized to the *gyrB* and the data represent four independent experiments.

3.8. HypT provides a defense against HOCl.

To investigate the defensive role of HypT against HOCl stress in *Salmonella*, viability assay was conducted using STm-SL1344 strains used in qRT-PCR (SL1344+pUHE21-2*lacI*^{q}, Δ*hypT*+pUHE21-2*lacI*^{q}, Δ*hypT*+pUHE21-2*lacI*^{q}::*hypT*). However, the *hypT*-deleted strain showed no difference in viability against HOCl stress compared to the wild-type strain (data not shown). This phenomenon was predicted to be due to the additional defensive mechanism of *Salmonella* against HOCl, which covers the effect of HypT. Therefore, I removed the *rclR*, one of the defense mechanisms against HOCl in *Salmonella*, to highlight the effect of HypT [31]. In the mutant construction, deletion of *rclR* based on the *hypT*-deleted strain (Δ*hypT*) was possible through Lambda-Red recombination; (Δ*hypT*Δ*rclR*::cm), but the *rclR* was not deleted from the wild type strain and was constructed using transduction. After construction of 3 strains (Δ*rclR*::cm+pUHE21-2*lacI*^{q}, Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}, Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}::*hypT*), viability assay was conducted (figure 9). Three strains showed no difference in the growth rate in the absence of NaOCl (Left picture, first three lines). The absence of *hypT* in the NaOCl treated groups showed 1 or 2 log reduced viability. And complementation with *hypT*-expressing plasmid restored its viability above the wild type (Right picture, first three lines). These results

indicate that *hypT* is indeed a defense mechanism against HOCl stress in *Salmonella*.

3.9. Met206 plays an important role in activation of HypT.

Previous studies have shown that Met123, Met206, and Met230 residues play an important role in the activation of HypT. Therefore, these three residues were replaced by glutamine, which has a form similar to methionine sulfoxide, which is an oxidized form of methionine. As a result, substitutions that mimic this oxidation have allowed HypT to regulate genes to more powerful levels. In addition, substitution with isoleucine, a form similar to un-oxidized methionine, resulted in the expression of genes similar to those without *hypT*. So they concluded that oxidation of Met123, Met206 and Met230 is important for activation of HypT [27].

Based on previous research, my co-worker proposed structural mechanism of HypT activation (Inseong Jo, unpublished data). Based on the structure of the putative ligand binding site, Met206 was predicted to play the most important role in the activation of HypT. Briefly, HOCl binds to the ligand binding site of HypT and then specifically oxidizes Met206, which causes the

structural change of HypT as the Met-SO (oxidized Met206) exits the hydrophobic pocket. Also, structural change was detected by substitution of Met206 of HypT to glutamine when compared to the wild type structure of HypT (Inseong Jo, unpublished data).

Based on these results, to demonstrate the activation of HypT by specific oxidation of Met206, a plasmid expressing HypT with substituted Met206 was constructed. Met206 was replaced with leucine (M206L) to mimic un-oxidized methionine, and Met206 was replaced with glutamine (M206Q) to mimic the oxidized form. Complementation strains (Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}::*hypT*, Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}::*hypT*^{M206Q}, Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}::*hypT*^{M206L}) showed no difference in the growth rate in the absence of NaOCl (Figure 8, left picture, last three lines). When NaOCl was treated, M206Q showed same level of viability as the wild type HypT complementation strain, indicating that HypT works normally. However, in the case of M206L, the viability was reduced by about 2 logs compared to the complementation strain or M206Q, indicating that HypT is not working properly (Figure 9, right picture, last three lines). These results suggest that the specific oxidation of Met206 plays an important role in the activation of HypT.

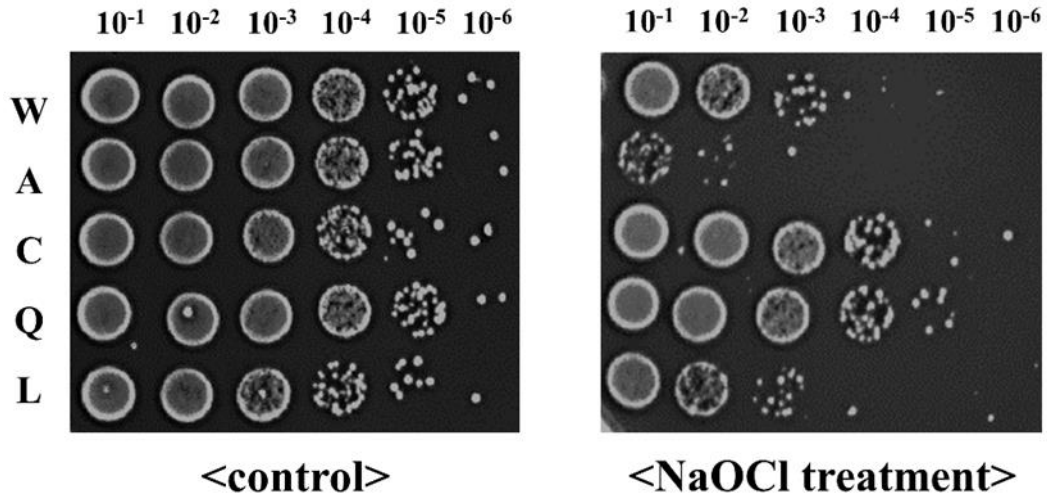


Figure 9. Viability assay under NaOCl treatment.

Left picture represent NaOCl non-treated condition, right picture represent NaOCl treated condition. (W; Δ*rclR*::cm+pUHE21-2*lacI*^{q}, A; Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}, C; Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}::*hypT*, Q; Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}::*hypT*^{M206Q}, L; Δ*hypT*Δ*rclR*::cm+pUHE21-2*lacI*^{q}::*hypT*^{M206L}). HypT plays a protective role against HOCl stress and oxidation of Met206 induces activation of HypT.

Ⅳ. DISCUSSION

This study identified the characteristics of HypT, a HOCl-specific transcription factor in *Salmonella*. HypT has been studied to alleviate oxidative stress by increasing the biosynthesis of methionine and cysteine and inhibiting iron uptake in *E. coli* [27]. The LTTR gene with high identity to *E. coli hypT* was found in *Salmonella enterica* serovar Typhimurium SL1344. As *Salmonella* HypT was predicted to work similar to *E. coli* HypT, several genes that are known to controlled by HypT in *E. coli* were selected as candidates and transcription levels were measured. As a result, *Salmonella* HypT repressed iron acquisition related genes similar to *E. coli* HypT but does not regulate methionine and cysteine biosynthetic genes (Figure 3). These differences in the gene expression related to iron acquisition were also consistent in the phenotype, so that HypT was functionally confirmed that it plays a role in lowering the levels of iron ions to reduce spontaneous oxidative stress in the cells (Figure 4). In addition, repression of iron acquisition also functioned in the absence of HOCl, but showed a stronger repressive effect in the presence of HOCl.

HypT has been studied to regulate several genes in *E. coli*, but it is not known how it regulate regulons. The EMSA and DNaseI Foot printing results

suggested that HypT regulates regulons by directly binding to the upstream of the regulon (Figure 6, 7).

In addition, the gene expression of *hypT* was not affected by HOCl (Figure 8). This suggests that HOCl regulates activity of HypT through direct modulation. To provide this more accurately, further experiments such as measuring the amount of protein expression by NaOCl concentrations are needed.

The co-worker proposed an activation mechanism based on the structure of HypT protein (Inseong Jo, unpublished data). When HOCl is attached to the ligand binding site of HypT, the nearest Met206 is selectively oxidized due to the specifically strong oxidative potential for methionine. The oxidized Met206 (methionine sulfoxide) escapes from the hydrophobic pocket due to the increased polarity, and triggers conformational change of HypT. This conformational change was confirmed by crystallization of HypT replacing methionine residue 206 to glutamine which mimics oxidized methionine (methionine sulfoxide).

The activation mechanism proposed above was confirmed, since the oxidation mimetic model (M206Q) exhibits a higher survival rate than the non-oxidative mimetic model (M206L) under HOCl stress (Figure 9).

In conclusion, a collaborative study revealed the mechanism of HypT-mediated defense, a HOCl-specific transcription factor in *Salmonella*. When HOCl is attached to the ligand binding site of HypT, the nearest methionine, Met206, is selectively oxidized due to the specifically strong oxidizing power to methionine. This oxidized methionine (methionine sulfoxide) escapes the hydrophobic pocket and triggers a conformational change in the protein HypT. And this conformational change resulted in enhanced repressive function to the iron acquisition operons. HypT directly binds to the upstream of the iron acquisition operon to repress their transcription level. Resulting from this, lowered amount of free iron ions in the cell reduces the formation of hydroxyl radicals produced by the Fenton reaction [15]. As a result, it is suggested that HypT alleviates the overall oxidative stress to cope with external HOCl stress by reducing the ROS spontaneously generated in the cell.

Understanding the bacterial HypT-mediated defense mechanism will help to develop therapeutic agents that can further enhance the bactericidal function of neutrophils, or to develop more powerful disinfectants by counteracting bacterial defense mechanism.

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국문 초록

살모넬라 타이피뮤리움 (*Salmonella enterica* serovar Typhimurium)은 식중독의 주요 원인 중 하나인 식품 유래 병원균이다. 많은 연구에도 불구하고, 효과적인 백신이 개발되어 있지 않으며, 항생제 치료 또한 어려움이 있기 때문에 대안적인 치료 방법이 강구된다. 차아염소산 (hypochlorous acid, HOCl)은 면역 체계에서 침입한 균을 죽이기 위해 생성된다. 대장균에서 HOCl에 대한 특이적 전사인자인 HypT가 연구된 바 있으며, HypT는 여러 유전자를 조절하여 HOCl에 의한 손상을 경감시킨다고 알려져 있으나, 아직 정확한 작용 기전은 밝혀져 있지 않다. 본 연구에서는 살모넬라 타이피뮤리움에서 HypT의 작용 기전을 밝혔다. 대장균에서 이루어진 이전 연구 결과를 바탕으로 후보 유전자를 선출하여 전사 수준을 비교한 결과, *hypT* 결여 균주에서 철분 획득 관련 유전자의 발현이 더 높게 나타났으며, HypT의 작용은 HOCl의 부재에도 일어나지만 HOCl이 존재할 시에 더욱 강력한 전사 조절 능력을 가지는 것으로 나타났다. 이러한 유전자 발현의 차이는 표현형에도 이어져 *hypT* 결여 시에 더 높은 수준의 세포 내 철분 함량이 검출되었다.

또한 HypT는 철분 획득 관련 오페론 상단 부에 직접적으로 결합하여 전사를 조절하는 것으로 나타났으며, 유전자 *hypT*의 발현은 HOCl에 영향을 받지 않는 것으로 보아 HOCl에 의해 직접적인 HypT 단백질 활성의 조절이 일어나는 것으로 예측되었다. NaOCl 처리 조건 하에서 *hypT*의 결여 시에 생존성이 감소하여, HypT의 HOCl에 대한 방어 기능을 확인하였다. 또한 메티오닌 123, 206, 230번 잔기가 HOCl을 감지하는 데에 중요한 역할을 한다는 기존의 연구 결과에 더해 공동 연구자가 제시한 HypT의 활성화 기작을 바탕으로 메티오닌 206번 잔기의 특이적인 산화가 HypT의 활성화에 중요함을 확인하였다. 이러한 연구 결과들은 HypT는 외부 HOCl 스트레스에 대응하기 위해, 세포 내에서 생성되는 ROS를 줄임으로써 전체적인 산화 스트레스를 경감시키고, 생존성을 확보함을 제시한다.

주요어 : 살모넬라, HypT, 차아염소산, 철 이온, 메티오닌 산화

학번 : 2016-21724