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2020년 8월

박사학위 논문

Nitric oxide sensing mechanisms of transcriptional regulators NsrR and RpoS in Salmonella enterica serovar Typhimurium

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지도교수 방 일 수

이 논문을 이학 박사학위신청 논문으로 제출함

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GENERAL ABSTRACT

Nitric oxide sensing mechanisms of transcriptional regulators NsrR and RpoS in *Salmonella enterica* serovar Typhimurium

살모넬라 전사 조절자 NsrR 과 RpoS 의 산화질소 인식 기전

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Ecological cycles of nitrogen in environment include production of the antimicrobial radical nitric oxide (NO). NO can further mediate the production of reactive nitrogen species (RNS) by chemical and biological reactions mostly with oxygen molecules in ecosystems including life systems, which inactivate various bacterial macromolecules including nucleic acids, lipids, proteins, and small molecular weight thiol compounds, ultimately causing abnormal metabolism and consequent bacteriostasis. In animal hosts, inducible NO synthases in professional phagocytes, in response to microbial infections, produce cytotoxic levels of NO, thus infectious bacteria must adapt and resist to NO and RNS for their successful pathogenesis.

Against nitrosative stresses, many bacteria have evolutionally conserved *hmp* gene encoding the flavohemoglobin Hmp that metabolize NO inside bacteria to less

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toxic molecules. And Hmp expression has been proved to be required for the virulence of several pathogens. A negative transcriptional regulator NsrR has also been conserved in many of Hmp-expressing bacteria. In circumstances of increasing cellular free iron, the tight regulation of *hmp* transcription by NsrR is highly important for bacterial fitness because Hmp overexpression, in the absence of NO, can potentiate the Fenton chemistry-mediated oxidative damages in bacteria. However, molecular details on the NsrR-dependent regulation on the *hmp* transcription in enterobacteria are remained less understood. In addition, it has been reported that Hmp expression contributes differentially to bacterial virulence depending on infection phases (acute vs persistent), which suggests that RNS produced by NO-mediated reactions with molecules in animal cells can be hardly detoxified by the sole function of the flavohemoglobin. It has been open question if bacteria operate additional gene regulation system for RNS detoxification. This study presents the stationary-phase sigma factor RpoS as a potential regulator for gene transcription in bacterial response to RNS.

Using Salmonella *enterica* serovar Typhimurium (*S.* Typhimurium) as a model strain, this study has aimed to understand molecular mechanisms of NsrR and RpoS by which they sense NO and regulate transcription of target genes.

The first chapter provides the molecular characteristics of NsrR in regulating the *hmp* transcription. Val-43 residue of NsrR from *S.* Typhimurium was identified as a critical amino acid residue of NsrR for interacting with target *hmp* promoter, which determined NO consumption rate, growth rate, as well as *hmp* transcription, in *S.* Typhimurium exposed to NO. Moreover, heterogeneous amino acid+ residues in the α3 motifs of DNA recognition helix of NsrRs from bacteria in different phyla suggested that the diversity of NsrR structure might be related with bacterial ecological niches under various pressures of NO.

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In the second chapter, roles of the stationary-phase sigma factor RpoS and the interaction between RpoS and its adaptor protein RssB in *Salmonella* RNS response were investigated. The RNS-activated increase of RpoS levels and the RpoS-dependent RNS responsive gene transcription were required for *Salmonella* survival in acidified nitrite-containing cultures that produce a potential consortium of RNS, thereby promoting *Salmonella* virulence in mice in an NO-dependent way. Increase of RpoS levels in *S.* Typhimurium exposed to RNS was mainly due to the RNS-mediated inactivation of the adaptor protein RssB whose function is required for transferring RpoS to ClpP and ClpX protease complex under normal growth conditions.

In summary, molecular characterizations of two regulator proteins regulating transcriptions of genes necessary for the NO detoxification and RNS resistance in *S.* Typhimurium have provided in this dissertation by means of analyses including *S.* Typhimurium transcriptome, NO/RNS biochemistry, protein informatics, and mouse infection models. The findings in this study have shed light on how bacteria senses NO and transmits this signal to target genes important for bacterial fitness under NO stress.

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Chapter I

Identification of Val-43 of NsrR as critical amino acid residue for nitric oxide response of *Salmonella* Typhimurium

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ABSTRACT

The flavohemoglobin Hmp being conserved in many bacteria primarily metabolize the antimicrobial radical nitric oxide (NO) and so promotes bacterial fitness under NO stress conditions. The *hmp* transcription is mainly regulated by the Fe-S cluster containing NsrR repressor in response to NO, in which the molecular mechanism of NsrR sensing NO is not fully understood. Here this study presents Val-43 of NsrR as a critical amino acid residue for *Salmonella* Typhimurium NsrR in regulating *hmp* transcription. Val-43 to Ala mutant NsrR isolated through random mutagenesis showed high binding affinity to target DNA regardless of NO exposure. And when expressed in *S.* Typhimurium in NO-containing cultures, this mutant NsrR caused severe reduction in *hmp* transcription, bacterial growth impairment, and slow NO consumption rate. On the contrary, expression of Val-43 to Glu mutant NsrR caused similar effect as that of *nsrR* null mutation that constitutively activated *hmp* transcription and NO metabolism. Homology analysis with amino acid sequences of NsrRs from various bacteria, and structure modelling analysis with the resolved structures of *Streptomyces coelicolor* NsrR (ScNsrR) and another NO-sensing Fe-S containing regulator IscR from *E. coli* (EcIscR) revealed that Val-43 of *Salmonella* NsrR is correspondent to Ala-44 of ScNsrR and Glu-43 of EcIscR, both of which are located in the DNA recognition helix (RH) α3 motif, respectively. Results suggest that amino acid polymorphism in the RH α3 motif may confer differential roles on the species-specific structure and function of NsrRs in regulating transcriptions of target genes including *hmp*.

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INTRODUCTION

Bacteria can be exposed to antimicrobial radicals, nitric oxide (NO) and NO-mediated reactive nitrogen species (RNS), which are produced in ecological cycles of nitrogen. Animal phagocytic cells also produce cytotoxic levels of NO that can diffuse inside bacteria and damage various bacterial macromolecules, causing abnormal metabolism and consequent bacteriostasis that can limit bacterial proliferation (Fang and Vazquez-Torres, 2019). Therefore, bacterial strategies to avoid the NO-mediated nitrosative stress are important virulence determinants for intracellular pathogens such as *Salmonella enterica* to persist their pathogenic life cycles in NO-producing animal hosts (Bang et al., 2006; Ruby et al., 2012). To cope with nitrosative stress, many bacteria have evolutionally conserved *hmp* gene encoding an ancestral globin, flavohemoglobin Hmp that metabolize NO to less toxic molecules. Thus, mutant bacteria lacking this enzyme are susceptible to nitrosative stress and much less virulent in animals (Forrester and Foster, 2012).

In *S. enterica* and many other species in β and γ proteobacteria, Hmp expression is regulated in the transcription of its gene *hmp* by the [Fe-S] cluster-containing protein NsrR (Forrester and Foster, 2012; Rodionov et al., 2005). As a transcriptional repressor, NsrR binds to specific DNA region containing consensus sequences around promoters of a set of target genes including *hmp*, but upon exposure to NO, inactivation of [Fe-S] cluster in NsrR causes releasing NsrR from target DNAs, thereby activating transcription of target genes (for review, (Sevilla et al., 2019)). Repression of *hmp* transcription by NsrR under conditions without nitrosative stress seems to be crucial for bacterial fitness, because Hmp overexpression, in the absence of NO, can rather damage bacterial redox homeostasis (Fig. 1) (Bang et al., 2006; Gilberthorpe et al.,

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2007; McLean et al., 2010). These features have rationalized the role of NsrR as a master regulator for bacterial gene expression specific for NO detoxification.

The Rrf2 family protein NsrR contains the [Fe-S] cluster coordinated by three cysteine residues in the C-terminal region (Tucker et al., 2010). Despite of controversial observations on the forms of [Fe-S] cluster ([2Fe-2S] or [4Fe-4S]) in NsrR proteins purified from *Neisseria gonorrhea*, *Bacillus subtilis*, and *Streptomyces coelicolor*, mutational studies on three conserved cysteine residues have revealed that [Fe-S] cluster in NsrR is essential for the repression of target gene transcription (Crack et al., 2015; Isabella et al., 2009; Kommineni et al., 2010) (Tucker et al., 2010). Accodingly, nitrosylation of [Fe-S] cluster lessens the binding affinity of the NsrR to target DNAs, thereby increasing expression of NO-metabolizing proteins (Crack et al., 2015; Isabella et al., 2009; Yukl et al., 2008). Moreover, the first NsrR crystal structure that identified from a gram (+) *Streptomyces coelicolor* (ScNsrR) revealed the [4Fe-4S] cluster stabilizing the dimeric ScNsrR in which the NO-displaceable ligand affected DNA binding ability of NsrR (Volbeda et al., 2017).

However, due to relatively different consensus DNA sequences and variable target genes of NsrRs across bacterial species, and lacking the defined molecular structures of NsrR in Gram (-) species, molecular mechanisms of how species in enterobacteria sense NO through NsrR have remained unexplored. As an attempt to resolve the molecular characteristics of NsrR in *S. enterica* Serovar Typhimurium (*S.* Typhimurium), this study has originally aimed to determine molecular structures of WT NsrR and mutant NsrR that can bind target genes regardless of NO exposure, the comparison of which can answer molecular pathway for NO sensing of NsrR. Although crystallization of both NsrR proteins has been unsuccessful in this study, identification

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of critical amino acid residue of NsrR for controlling its binding ability to target DNA reveals new characteristic of NO sensing mechanism of *S*. Typhimurium NsrR.

Initially, this study has used a mutant *nsrR* clone that a lab member previously isolated through random mutagenesis, as the most effective in repressing *hmp* transcription under nitrosative conditions (Lee, 2012). Further characterization of this mutant NsrR in this study has started to reveal important molecular details of NsrR function in response to NO.

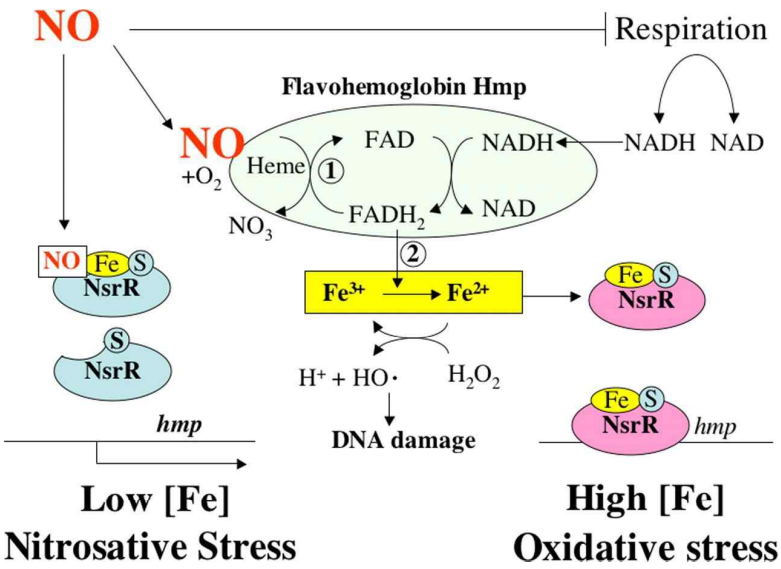

Figure 1. Model of redox homeostasis maintenance by the NsrR-regulated Hmp expression (Bang et al., 2006). Upon NO exposure, nitrosylation of NsrR derepresses *hmp* expression. Hmp detoxifies NO radical to less toxic nitrogen oxides (pathway ①), recovering bacterial respiration. In the absence of NO, electrons transferring from NADH to heme can be utilized by FAD to alternatively reduce ferric iron (Fe^{3+}) (pathway ②). Ferrous iron (Fe^{2+}) can potentiate the Fenton chemistry to produce oxidative damages. Hence, high levels of intracellular free iron promotes repression of *hmp* expression by the NsrR repressor.

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MATERIALS AND METHODS

Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium 14028S was used for WT parental strain and its isogenic gene mutants were used for this study and listed in Table 1. *E. coli* BL21(DE3) was used for expression and purification of NsrR proteins. All strains were cultured at 37℃, unless otherwise noted. Construction of gene mutations in *S.* typhimurium was performed by the PCR-mediated one-step gene mutation method using λ Red recombinase (Datsenko and Wanner, 2000). Further recombination processes of gene mutation including transduction to the fresh WT strain and removal of antibiotic cassettes in mutants were followed as described previously (Park et al., 2015). DNA primers used in this study are listed in Table 1. All mutants and plasmids constructed in this study were verified by DNA sequencing. For routine bacterial cultures, LB complex (Difco) or minimal E glucose (0.2%) (EG) media (Vogel and Bonner, 1956) were used. Antibiotics were added at the following concentrations to LB media when necessary: kanamycin (50 ug/ml), ampicillin (100 ug/ml), or chloramphenicol (20 ug/ml). For NO congeners, spermine NONOate (sperNO) was purchased from Cayman chemical, and S-nitrosoglutathione (GSNO) was synthesized by using the reaction of glutathione and acidified sodium nitrite, as reported (Hart, 1985a). All chemicals were purchased from Sigma-Aldrich, unless stated otherwise.

Site-directed mutation of *nsrR*

Site-directed mutation of *nsrR* on the plasmid was performed by two-step PCR using DNA primers containing bases changed for the designated mutation. For constructing any base mutation in *nsrR* gene, two PCR products of first PCR reactions

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with primers of pBAD-Fw and “primer for mutation”-Rev and “primer for mutation”-Fw and pBAD-Rev, respectively, were mixed, heat-denatured for 10 min at 95℃, and annealed slowly at RT. Using this mixture as the template, second PCR was performed with primers pBAD-Fw and pBAD-Rev. The purified PCR products corresponding the full *nsrR* gene size were cloned into pBAD30 using enzymes as described for random mutagenesis methods. To perform the site-directed mutation of *nsrR* in *S.* Typhimurium chromosome, I used the combinational recombination method using the Red recombination system and I-*Sce*I endonuclease (Blank et al., 2011). The desired mutation site was firstly recombined with Cm^R cassette and I-SceI recognition site using the Red recombinase with DNA product amplified from pWRG100 with primers listed in Table 1, then the cassette was replaced to mutated bases by I-SceI endonuclease encoded from pWRG99 and the 80mer-annealed dsDNA containing bases to be changed.

Measurement of *hmp* transcription

To measure *hmp* transcription, two complementary methods were used. When used with *hmp-lacZ* transcriptional fusion, β-galactosidase activity was measured essentially following the method described in (Maloy, 1990b). For measuring *hmp* mRNA levels, quantitative real time RT-PCR was performed as reported previously (Cho et al., 2015). Briefly, for measurung β-galactosidase activity, *S.* Typhimurium strains grown overnight in LB broth were diluted at 1:200 (v/v) in EG media either containing GSNO (500 uM) or not, and grown to early log phase (OD_{600}=0.5). For RT-PCR, log-phase cultures grown as aforementioned but without GSNO were divided, and the half of culture was treated with GSNO (500uM), then cultured more for 1h. Bacterial transcription was stopped by mixing a one-fifth volume of ice-cold

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phenol/ethanol (5% phenol in 95% ethanol) solution to the culture before harvesting the cells. Total RNA was extracted using RNAiso Plus reagent (Takara Bio), then subjected to RT-PCR analysis using a QuantiTect SYBR Green RT-PCR kit (Qiagen) with primer pairs *hmp*-Fw and *hmp*-Rev.

Measurement of nitric oxide susceptibility

Salmonella strains grown overnight in LB broth were diluted in PBS to OD_{600} = 1 before inoculating equal amount (OD_{600} = 0.02) into the EG media supplemented with or without GSNO (500μM or 1mM). Growth kinetics were determined by measuring OD_{600} every 30 minutes during 24 hours at 37°C with agitation in the BioScreen C Microbiology Microplate reader (Labsystems).

Expression and purification of NsrR and Val-43 to Ala mutant NsrR

To express and purify NsrR and Val-43 to Ala (V43A) mutant NsrR proteins, I constructed pET28a plasmid-based clones containing WT *nsrR* and *nsrR*V43A genes PCR-amplified from chromosomal DNA and from pBAD30-*nsrR*V43A, respectively. These N-terminal His-tagged proteins were routinely expressed by culturing log-phase (OD_{600} ~ 0.5) cells of *E. coli* BL21 harboring clones in LB broth supplemented with IPTG (0.5 mM) for 16 hrs at 18℃. The harvested cells were resuspended in 50 ml lysis buffer containing 20 mM Tris-HCl, pH7.5, 500 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and DNase I. After sonication (2 10 sec pulses at 25% amplitude with a rest for 2 min on ice) and centrifugation (2000 x *g*), the filtrated supernatant (0.45 um pore) was passed through a column containing Ni-NTA agarose resins (Quiagen), using an Econo pump (0.5 ml / min). After protein binding, Ni-NTA resins were washed with 2 ml washing buffer (20 mM Tris-HCl

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pH7.5, 20 mM imidazole, 500 mM NaCl, 1 mM DTT) for 1 min, and His-tagged proteins were eluted with elution buffer (20 mM Tris-HCl pH 7.5, 500 mM imidazole. 500 mM NaCl). The purified His-tagged proteins were dialyzed using a dialysis membrane (MWCO 10KD; Spectrum Laboratories, Inc.) in a buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT) at 4℃ for overnight. The His-tag was removed by digestion with human α-thrombin (7 unit, 10,000x dilution in 2 mM $CaCl_2$) at RT for 6 hours. After re-subjection of digestion mixture onto the Ni-affinity column, the digested proteins in flow-through fractions were concentrated, and finally isolated by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) in the buffer (20 mM Tris, pH 7.5, 50 mM NaCl). To synthesize His-tagged NsrR proteins *in vitro*, TNT T7 Quick coupled transcription/translation system (Promega) was used following protocols provided. 1 ug of pET28a-*nsrR* or pET28a-*nsrR*V43A clone was added to the TNT Quick master mix and incubated at 30 ° C for 1 hour.

Electrophoretic mobility shift assay (EMSA)

EMSA reaction was carried out under ambient aerobic conditions in a protein-DNA binding buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 1mM DTT), and products were resolved on PAGE using 5% native gels in 0.5% TBE (Tris-borate-EDTA) buffer (Welgene). The DNA oligonucleotides for NsrR binding (Table 1) were labeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase (Promega), then annealed with the reverse-complementary oligonucleotides, producing dsDNA *hmp* probe containing the 19 bp NsrR binding consensus conserved in γ-proteobacteria (Rodionov et al., 2005). For all EMSA experiments, 0.1 ng of *hmp* DNA probe was used and reacted with NsrR proteins for

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15 minutes. For super shift assay using His-tagged proteins, an anti-His antibody (Santacruz) was added to the reactions.

Measurement of NO consumption rate

The NO consuming ability of *Salmonella* strains was analyzed essentially as described in the previous report (Park et al., 2015). Briefly, mid log-phase cells (OD_{600}=0.7) with or without treatment with GSNO (1mM) for 1 h were harvested and washed with phosphate-buffered saline (PBS) and resuspended in 10 mL pre-warmed PBS. These samples were then transferred into a multiport measurement chamber (NOCHM-4; WPI Inc., USA) equipped with an ISO-NOP NO sensor (WPI Inc.) connected to a free radical analyzer (TBR4100; WPI Inc.) at 37°C. The NO consumption rate was determined by measuring the remaining NO in the buffer after adding a fast-releasing NO donor ProliNONOate (Cayman Chemical). The data signal was obtained by using the Labchart program (WPI Inc.).

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Table 1. Bacterial strains, plasmids, and DNA oligomers used in this study

Strains	Genotype	Source or Reference
IB1	*S.* Typhimurium 14028s, Wild-type	ATCC
IB3	*hmp::*	This study
IB697	*Δnsr*R::FRT/pBAD30-*nsrR*::AP	This study
IB1076	*hmp-lacZ*::KM	This study
IB2106	*Δnsr*R::FRT *hmp-lacZ*::KM/pBAD30-*nsrR*::AP	This study
IB2108	*Δnsr*R::FRT *hmp-lacZ*::KM/pBAD30-*nsrR (Random mutant B4 : M17L* and *V43A)*::AP	This study
IB2088	*Δnsr*R::FRT *hmp-lacZ* ::KM/pBAD30-*nsrR M17L*::AP	This study
IB2089	*Δnsr*R::FRT *hmp-lacZ* ::KM/pBAD30-*nsrR V43A*::AP	This study
IB2109	*Δnsr*R::FRT/pBAD30::AP	This study
IB2110	*Δnsr*R::FRT/pBAD30-*nsrR*::AP	This study
IB2111	*Δnsr*R::FRT/pBAD30-*nsrR*(Random Mutant B4)::AP	This study
IB2080	*Δnsr*R::FRT/pBAD30-*nsrR M17L*::AP	This study
IB2081	*Δnsr*R::FRT/pBAD30-*nsrR V43A*::AP	This study
IB1433	WT/pBAD30-*nsrR*::AP	This study
IB2079	WT/pBAD30-*nsrR V43A*::AP	This study
IB1507	BL21/pET28a(+)-*nsrR* (C-terminal His-Tag)::KM	This study
IB1609	BL21/pET28a(+)-*nsrR V43A* (C-terminal His-Tag)::KM	This study
IB1646	*nsrR* V43A	This study
IB2305	*nsrR* V43M	This study
IB2306	*nsrR* V43E	This study
IB2307	*nsrR* V43I	This study

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Plasmid		
pBAD30	Gene expression vector	(Miller and Gennis, 1986)
pET28a	Gene expression vector	Novagen
pKD46	Plasmid encoding arabinose-inducible lamda red recombinase, temperature sensitive	(Datsenko and Wanner, 2000)
pWRG100	Plasmid carrying CM^r cassette and an I-*Sce*I recognition site	(Blank et al., 2011)
pWRG99	AHT-inducible I-*Sce*I	(Blank et al., 2011)

DNA Oligonucleotides	
	Sequence (5′ → 3′)
His-*nsrR* Fw	CAGTTAACGAGTTTCACCGA
His-*nsrR* Rev*	GCGCCGCC**CTCGAG** CTGGCCGCGCGGCACCAG CTCCAC CAGTAATAATTTATAAAGC
pBAD Fw	TCGCAACTCTCTACTGTTTC
pBAD Rev	GCCTGATACAGATTAAATCAG
nsrR M17L Fw	CTAATCTAT**TTG**GCGTCGTTA
nsrR M17L Rev	TAACGACGC**CAA**ATAGATTAG
nsrR V43A Fw	TAATCATATG**GCC**A AAATTATCA
nsrR V43A Rev	TGATAATTTT**GGC**CATATGATTA

Real-time PCR	
rpoD Fw	GTGAAATGGGCACTGTTGAACTG
rpoD Rev	TTCCAGCAGATAGGTAATGGCTTC
hmp Fw	CGCTACAGTAAAGGCCACCA
hmp Rev	CCCGACGATGTTGTACTGCT

DNA probe for Gel mobility shift assays	
NsrR-*hmp* Fw	AAGATGCATTTGATATACATCAAT

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NsrR-*hmp* Rev	ATTGATGTATATCAAATGCATCTT

Chromosomal *nsrR* mutant construction

NsrR I-SceI-cat Fw	GTGACAGAAGTCTACGGCGTGTCCCGTAATCATATG GCCACGCCTTACGCCCCGCCCTGC
NsrR I-SceI-cat Rev	GCGGTGACAAAGCCCGCCCGGCTAAGCTGATTGAT AATTTCTAGACTATATTACCCTGTT
V43A Fw	GTGACAGAAGTCTACGGCGTGTCCCGTAATCATATG GCCAAAATTATCAATCAGCTTAGCCGGGCGGGCT TTGTCACCGC
V43A Rev	GCGGTGACAAAGCCCGCCCGGCTAAGCTGATTGAT AATTTTGGCCATATGATTACGGGACACGCCGTAGAC TTCT GTCAC
V43M Fw	GTGACAGAAGTCTACGGCGTGTCCCGTAATCATATG ATGAAAATTATCAATCAGCTTAGC CGGGCGGGCTTTGTCACCGC
V43M Rev	GCGGTGACAAAGCCCGCCCGGCTAAGCTGATTGAT AATTTTCATCATATGATTACGGGACACGCCGTAGAC TTCTGTC AC
V43E Fw	GTGACAGAAGTCTACGGCGTGTCCCGTAATCATATG GAGAAAATTATCAATCAGCTTAGCCGGGCGGGCTTT GTCACCGC
V43E Rev	GCGGTGACAAAGCCCGCCCGGCTAAGCTGATTGAT AATTTTCTCCATATGATTACGGGACACGCCGTAGAC TTCTGTCAC
V43I Fw	GTGACAGAAGTCTACGGCGTGTCCCGTAATCATATG ATCAAAATTATCAA TCAGCTTAGC CGGGCGGGCT TTGTCACCGC
V43I Rev	GCGGTGACAAAGCCCGCCCGGCTAAGCTGATTGAT AATTTTGATCATATGATTACGGGACACGCCGTAGAC TTCTGTCAC

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RESULTS

Identification of mutant NsrR repressing *hmp* transcription under nitrosative conditions

To isolate mutant NsrR that can constitutively bind to *hmp* promoter and so cause repression of *hmp* transcription regardless of NO exposure, I chose a mutant *nsrR* clone that was isolated previously in the lab by random mutagenesis on the *nsrR* structural gene in a plasmid (Lee, 2012). *S.*Typhimurium strain harboring both this mutant clone (B4) and the *hmp-lacZ* operon fusion had displayed the lowest LacZ activity on the plate containing an NO congener, GSNO (Lee, 2012). This mutant clone was further analyzed in effects to *hmp* transcription. Fig. 2A, B shows the results of the *nsrR* mutant clone B4 representing the most effective in repressing *hmp* transcription under nitrosative conditions. By the mutant NsrR B4, β-galactosidase activity of *hmp-lacZ* and *hmp* mRNA levels were largely reduced compared with *S.* Typhimirium expressing WT NsrR. Both results showed that mutant NsrR B4 caused almost no induction of *hmp* transcription by NO treratment. The growth of *S.* Typhimurium harboring NsrR B4 clone was also severely impaired in GSNO-containing culture, demonstrating that repression of *hmp* transcription by this mutant NsrR is physiologically relevant (Fig. 2C). DNA sequence analysis revealed that this mutant clone B4 contained two mutations changing Met-17 to Lys and Val-43 to Ala (V43A). Further respective site-directed mutation for each single mutation revealed V43A mutation was mostly correspondent to the repression effect of mutant NsrR clone B4 on *hmp* transcription.

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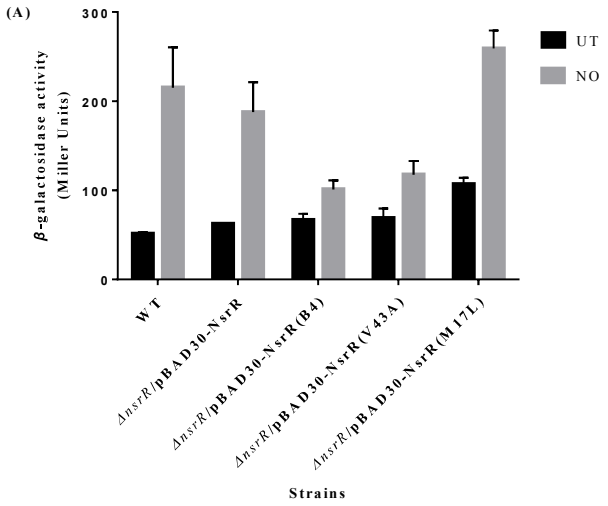

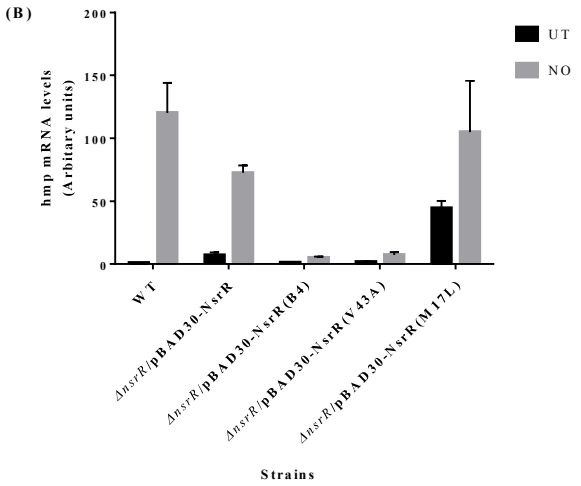

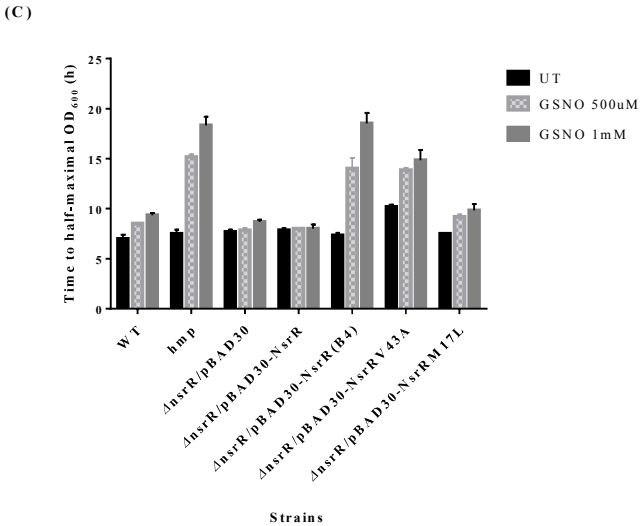

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Fig. 2. Identification of mutant *nsrR* clones regulating the NO mediated induction of *hmp* transcription. (A) β-galactosidase activities of *S.* Typhimurium strains containing the *hmp-lacZ* transcriptional fusion and also harboring mutant or WT *nsrR* gene on the plasmid. Strains were cultured with GSNO (500 uM) (NO) or without (UT) to log-phase (OD_{600} = 0.5). (B) Same set of strains used for (A) but without *hmp-lacZ* fusion were cultured to OD_{600} = 0.5, then half of cultures were collected and the other half were treated with GSNO (500 uM) and cultured for additional 1h, before purifying total RNA. (C) The bacterial growth is shown as the time to half-maximal OD_{600} based on the growth curve of each strain measured for 24h.

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V43A mutant NsrR binds to the *hmp* promoter with higher affinity than WT NsrR

To compare the binding capacity of purified V43A mutant NsrR and WT NsrR proteins to *hmp* promoter, electrophoretic mobility shift assay (EMSA) was employed (Fig. 2). V43A mutant NsrR was bound to the *hmp* DNA probe containing NsrR consensus sequence with higher efficiency than WT NsrR. And this observation was also confirmed by EMSA with *in vitro* translated NsrR proteins that showed DNA probe shifted by mutant NsrR under the same condition that WT NsrR could not lead the distinguishable shift (Fig. 3 A,B). In the absence of NO, as increased the concentration of both NsrR proteins, DNA binding affinity appeared proportionally increased, but detecting the shifted bands by WT NsrR-DNA complexes needed much longer developing time than those by V43A NsrR-DNA complexes (note intensities of unbound DNA probes between two gels in Fig. 3B). Of note, super-shifted bands caused by an anti-His antibody confirmed the specificity of NsrR-DNA complexes. When proteins were exposed to NO prior to DNA binding, the intensities of shifted bands by WT NsrR significantly reduced, while those by V43A NsrR almost unchanged. These results show that V43A mutation significantly increases the binding affinity of NsrR to target DNA, even in the presence of NO. To confirm different binding capabilities of WT NsrR and V43A mutant NsrR to target DNA in the cell, I transformed clone plasmids containing both the plasmid-born arabinose-inducible promoter and the natural promoter of *hmp* into WT *Salmonella*, and expressed at sequentially increased concentrations of arabinose. Surprisingly, *Salmonella* containing V43A NsrR clone repressed *hmp* transcription under NO stress without arabinose addition, suggesting that V43A mutant NsrR outcompetes WT NsrR in binding to *hmp* promoter to inhibit transcription. Whereas *Salmonella* overexpressing WT NsrR

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slightly reduced the NO-induced *hmp* transcription even in the highest concentration of arabinose (Fig. 3C). Taken together, Results demonstrate that V43A substitution mutant NsrR can bind to *hmp* promoter with high affinity, which causes inhibition of *hmp* transcription in *Salmonella* irrespective of NO exposure.

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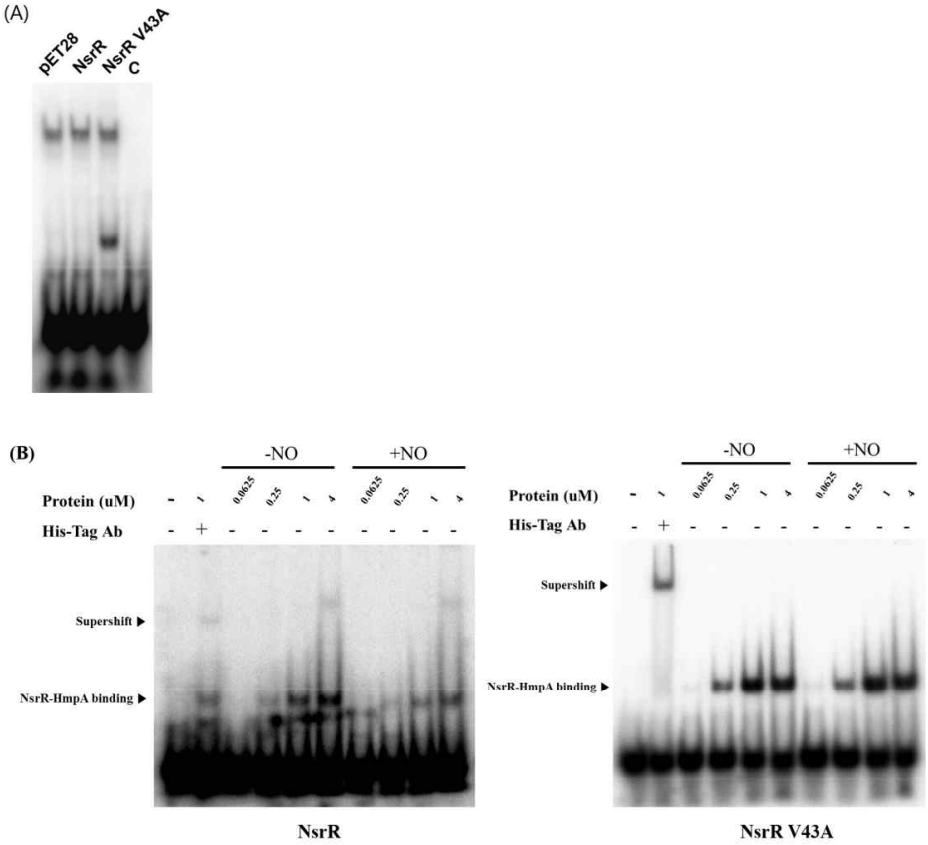

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Fig. 3. Higher binding affinity of V43A mutant NsrR to *hmp* promoter (A, B) EMSA of WT NsrR and V43A mutant NsrR proteins with *hmp* promoter DNA. (A) Mock lysate (pET28) and *in vtro*-translated NsrR proteins were incubated with ^{32}P-labeled *hmp* promoter DNA for 15 min before PAGE. C; free probe DNA. (B) Purified WT NsrR (left) and V43A mutant NsrR (right) proteins were subjected to EMSA as described for (A). Anti-His antibodies were added for supershift assay. GSNO (1 mM) was used for NO congener. (C) Comparative effect of WT and V43A mutant NsrR expression on *hmp* transcription. WT *S.* Typhimurium harboring either pBAD-*nsrR* or pBAD-*nsrR*V43A were cultured with various concentrations of L-arabinose to log-phase (OD_{600}=0.5) were treated or not with GSNO for 1h before purifying total RNA.

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The *nsrR*V43A and *nsrR*V43E mutations in *Salmonella* genome inversely modulate *hmp* transcription and subsequent NO metabolism

To investigate the importance of Val-43 residue of NsrR under more physiological conditions, I performed the site directed mutations on *nsrR* gene in *Salmonella* chromosome to express each V43A, V43M, V43E, and V43I mutant NsrR in single strain, respectively. V43A chromosomal mutation in *nsrR* (*nsrR*V43A) also caused severe reduction in *hmp* transcription and *Salmonella* growth rate in NO-producing cultures, compared with those of WT and other *nsrR* mutants, as observed in strains expressing V43A mutant NsrR from plasmid (Fig. 4A, B). On the contrary, in *nsrR*V43E mutant *Salmonella hmp* transcription was significantly increased in cultures without NO, suggesting the reduced binding affinity of this mutant NsrR to *hmp* promoter in *Salmonella*. V43M and V43I mutations exhibited normal NO-dependent regulation of *hmp* transcription and growth rate comparable to those of WT *Salmonella*. Next, NO consumption rates of *Salmonella* expressing WT and mutant NsrRs were measured using the fast NO-releasing congener ProliNO before and after NO stress was given with GSNO. NO metabolic activity of *nsrR*V43A mutant was not significantly increased by the GSNO pretreatment, while WT and other mutants *Salmonella* showed faster NO consumption by GSNO pretreatment (Fig. 4C). Without GSNO pretreatment, only *nsrR*V43E mutant consumed NO significantly, reflecting the increased *hmp* transcription in this mutant (Fig. 4C, A). WT, *nsrR*V43M, and *nsrR*V43I *Salmonella* strains showed similar increase of NO consumption rate by GSNO pretreatment. These results demonstrate the importance of Val-43 residue in NsrR, especially when substituted to Ala and Glu, on regulating *hmp* expression and subsequent NO metabolism in *Salmonella*.

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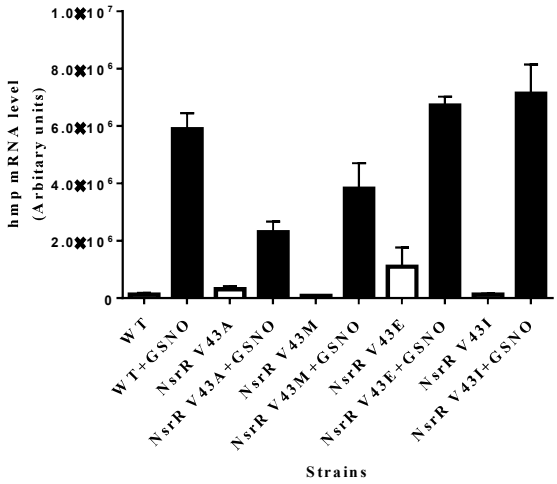
(A)



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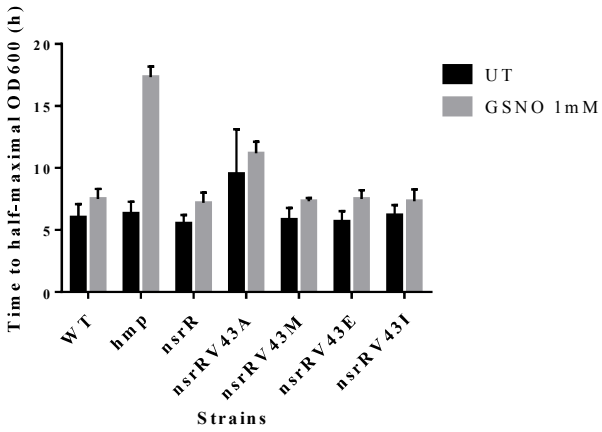

(C)

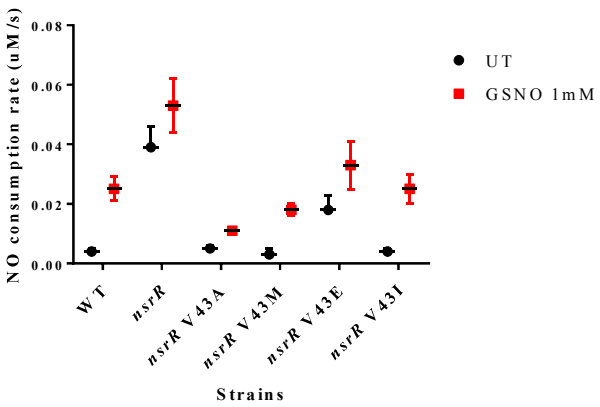

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Fig. 4. Effect of genomic mutation for substituting Val-43 of NsrR to other amino acids on *S.* typhimurium response to NO. Effects of Val-43 substitution mutations in *S.* Typhimurium chromosome were examined on hmp transcription (A), bacterial growth (B), and NO consumption rate (C). Measurements for *hmp* transcription and growth rate were performed as described in the legends for Fig. 1B and Fig1C, respectively. For measuring the NO consumption rate, *Salmonella* strains cultured to mid log-phase (OD600=0.7) were collected before and after treatment with GSNO for 1 hr, and same amount of cells were resuspended in pre-warmed PBS buffer. The concentration of NO that remained in each culture were recorded for 5 min. The results are expressed as the NO consumption rate calculated by the elapsed time to the change of NO concentration (2uM to 1uM).

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Potential structural diversity in the DNA recognition helix α3 of NsrRs among bacterial phyla

Discriminative effects of Val-43 substitutions to Ala and Glu in *S.* Typhimurium NsrR (StNsrR) on *hmp* transcription have proposed the necessity of comparative examination for NsrR diversities among bacteria. Also interestingly, recent study presented the first structure of NsrR from *Streptomyces* (ScNsrR) reveals that Ala-44 residue of ScNsrR is correspondent to Val-43 of *E. coli* NsrR (EcNsrR) or StNsrR, and Glu-43 of another Rrf2 family response regulator IscR of *E. coli* (EcIscR) that can be superposed to ScNsrR to understand relation of the [Fe-S] cluster and the DNA binding affinity and specificity (Volbeda et al., 2017).

Amino acids comparison of NsrR and IscR among species in 3 different phyla showed that three Cys residues in C-terminal regions are strongly conserved across species, displaying their importance as ligands for Fe-S cluster of these proteins (Fig. 4). But in the α3 motifs of DNA recognition helix (RH), strong homology of Val-43 as well as other amino acids conserved in NsrRs from enterobacteria are disappeared in NsrRs from *Staphylococcus*, *Bacillus*, and *Streptomyces*, and in IscRs from *E. coli* and *Pseudomona*s Glu-43 in IscRs that are correspondent to Val-43 of NsrRs in enterics was conserved in all species tested (Fig. 5).

Based on the defined structure of holo-ScNsrR dimer containing [4Fe-4S] cluster, StNsrR structure was predicted using SWISS-MODEL program, which presented Val-43 in RH α3 motifs oriented outward as observed for Ala-44 in ScNsrR (Fig. 6). Substituting Val-43 to Ala, Met, ILE, and Glu in StNsrR model also predicted structural locations of each amino acid side chains with differences in size and polarity, respectively. These analyses suggest that side chains of Val, Met, and Ile having similar properties may be related with similar phenotypes of corresponding *nsrR* mutants with

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those of WT *S.* Typhimurium, but significantly different properties of Ala and Glu side chains would be the cause for discriminative phenotypes observed in this study. Results also suggest that diverse amino acids composing RH domains of NsrR across bacterial phyla would make potential different mechanism in bacterial adaptation to NO.

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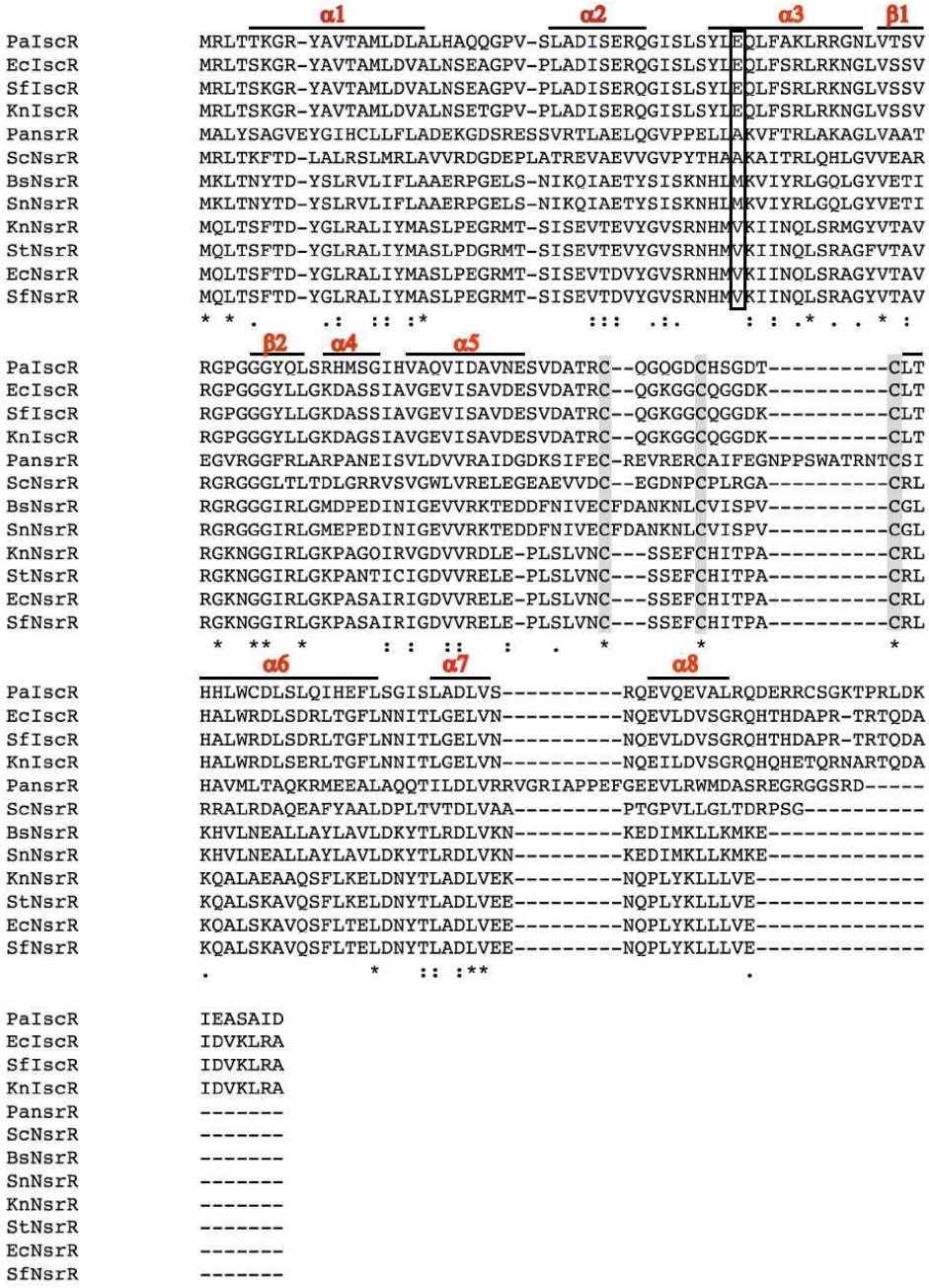

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Fig. 5. Comparison of amino acid sequences of NsrRs and IscRs from various bacterial species. Sequence alignment was obtained by using MUSCLE algorithm, and secondary structure elements were indicated in red based on *Streptomyces* NsrR structure. Conserved Cys residues are indicated in the shaded boxes, and Val-43 residues of NsrRs from enterobacteria were compared with corresponding amino acid residues of NsrRs from other species and of IscRs (empty box). Alignment analysis used amino acids of NsrRs are from Gram (–) species, *S.* Typhimurium, *Shigella flexneri, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *E. coli*, and from Gram (+) strains, *S. coelicolor, Bacillus subtilis,* and *Streptococcus pneumonia*. IscRs are from *P. aeruginosa, E. coli, S. flexneri,* and *K. pneumonia.* Symbols under amino acid alignment denotes as follows. *, totally conserved residues; :, highly conserved residues; ., residues conservatively substituted.

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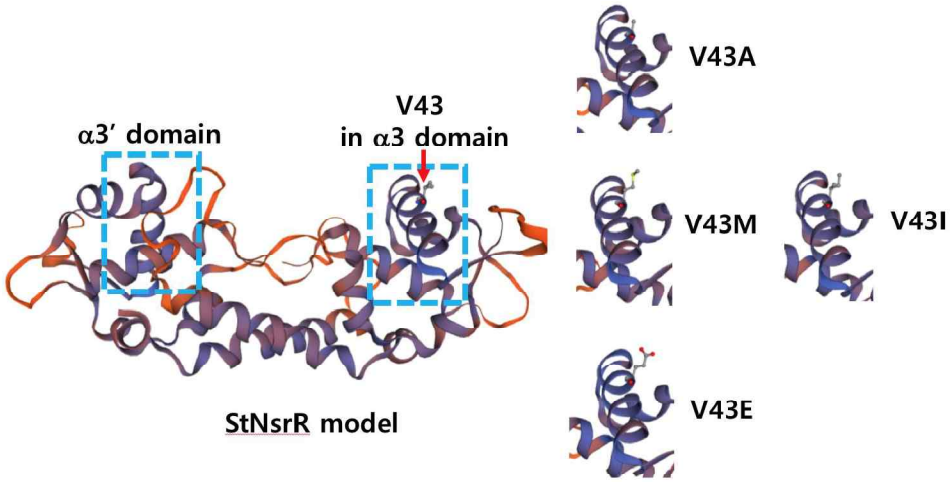

Fig. 6. Model of WT and Val-43 mutant NsrR proteins in *S.* Typhimurium. Model structure was obtained using SWISS-MODEL based on *S. coelicoler* NsrR dimer (PDB ID 5N07). The DNA recognition helix α3 domains of two monomers were indicated with dotted boxes, and α3 domains containing Val-43 and its substituted amino acid residues are shown separately in part, respectively, in that Val-43 (arrow) and its amino acid variants are indicated with atomistic details on each location of α 3 domains.

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DISCUSSION

The first structure of NsrR from *S. coelicolor* has revealed dimeric holo-NsrR containing [4Fe-4S] cluster degradable on NO exposure (Volbeda et al., 2017). ScNsrR [4Fe-4S] cluster coordinated by three Cys residues in one monomer and one Asp from the other monomer seems to stabilize ScNsrR in interacting with the target DNA backbone. To predict the interaction between ScNsrR and the target DNA in *hmp* promoter, they superposed ScNsrR structure with the structure of apo-EcIscR bound to target DNA, and proposed that, difference in residues Thr-41, Ala-44, and Lys-45 in the α3 RH of ScNsrR from structurally corresponding residues, Ser-40, Glu-43, and Gln-44 in EcIscR, would make distinct DNA sequences that two regulators could distinguish different sets of target genes (Rajagopalan et al., 2013; Volbeda et al., 2017).

In this study, the Val-43 residue of StNsrR has also been predicted to locate in the the α3 RH of NsrR based on structure modelling from crystal structure of ScNsrR (Fig. 6). Interestingly, Val-43 that conserved strongly in enterobacteria is correspondent to Ala-44 in ScNsrR, and to Glu-43 in EcIscR (Fig. 5), suggesting that these amino acid differences would affect functions of respective proteins during interactions with their target DNAs. Also in this paper that performed substitution mutagenesis on StNsrR, Ala-43 mutant NsrR was bound to *hmp* promoter DNA with higher affinity than Val-43 NsrR, and significantly repressed *hmp* transcription even in the presence of NO (Fig. 3, 4). Moreover, Glu-43 NsrR derepressed *hmp* transcription regardless of NO exposure, as in the case of interactions between type 1 site DNA and apo-EcIscR containing Glu43 residue in the α3 RH (Fig. 4). Structure of apo-EcIscR bound to a target DNA in *hya* promoter shows that Glu-43 is critical for apo-IscR to selectively bind to C7C8 and C7'8' bases in the major groove of type 2 site, but not to T7'T8' bases in type 1 site (Rajagopalan et al., 2013). It can be assumed that T5G6 and T5'G6' bases in the

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NsrR target sequence of *hmp* promoter maybe cause a repulsive interaction with polar carboxyl side chain of Glu-43, as apo-IscR Glu-43 does to T7'T8' bases in type 1 sequences. Taken together, these slight amino acid differences in α3 RH of ScNsrR, StNsrR, and EcIscR seems to elicit the specific binding affinity to their respective target DNA in response to NO. The identification of *Salmonella* NsrR structure in future will warrant the importance of amino acid residues in α3 RH of NsrR for bacterial NO metabolism in response to NO.

At the beginning, this study aimed to identify molecular structure of NsrR, with or without binding to target DNA, by X-ray crystallography analysis. In initial trial using methods for the purification of full sized StNsrR, it was unsuccessful to get NsrR crystals. In a secondary attempt to get enough NsrRs for crystallization, the smaller StNsrR containing 1-130 amino acids (StNsrR130) was constructed, and crystals of the NsrR130 bound to *hmp* promoter DNA were obtained (Fig. 7), Although it was unsuccessful to get proper X-ray diffraction, StNsrR130 showed a dimeric form possible, suggesting that StNsrR may form a potential dimer structure similar with ScNsrR. Hopefully, protocols used to concentrate and crystallize NsrR proteins in this study would be helpful for related scientists eager to resolve the structure of NsrR from enterobacteria.

In conclusion, this study identified Val-43 in StNsrR as the crucial amino acid residue for the regulation of *hmp* transcription in *Salmonella* responding to and detoxifying NO. Strong homologies in amino acid sequences of NsrRs in enterobacteria suggest that observations in this study would be a small but important coreprint to understand bacterial NO response in enteric pathogens that must cope with NO stress in animal hosts.

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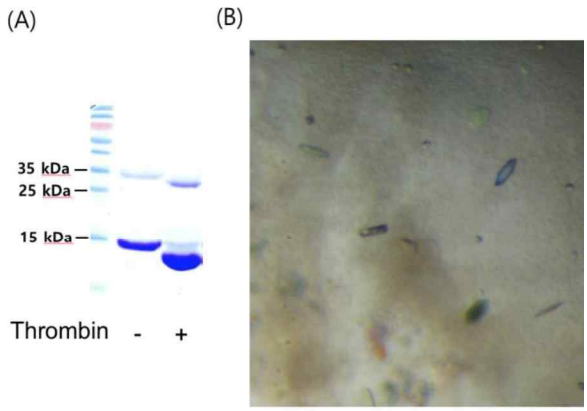

Fig. 7. Crystal of NsrR130 complexed with *hmp* promoter DNA. (A) Purification of *S.* Typhimurium NsrR130. His-NsrR130 was purified by Q-Sepharose and size-exclusion column chromatography and treated with thrombin, then analyzed by PAGE. (B) Crystal images of *Salmonella* NsrR130 complexed with *hmp* promoter DNA. Crystals were grown in a drop consisting of 0.2 *M* sodium bromide, 10%(*w*/*v*) glycerol, 20%(*w*/*v*) polyethylene glycol 3350, 100 m*M* Tris–HCl pH 8.5 at 293 K.

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Chapter II.

The stationary-phase sigma factor RpoS promotes *Salmonella* survival and virulence under reactive nitrogen species stress conditions

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ABSTRACT

In bacterial response to environmental changes including interactions with animal hosts, bacteria have evolved to utilize alternative sigma factors that rapidly activate transcription of selected sets of genes. But, little is known about corresponding sigma factors to promote bacterial resistance against antimicrobials, nitric oxide (NO) and NO-mediated reactive nitrogen species (RNS). Herein, this study shows that *Salmonella* Typhimurium fitness under nitrosative stress conditions requires the stationary-phase sigma factor RpoS. RpoS was required for *Salmonella* survival in acidified nitrite-containing cultures, and for virulence in NO-producing mice. Nitrosative stress increased RpoS levels and RpoS-dependent RNS responsive gene transcription in *S.* Typhimuriumis. Increase of RpoS level was mainly due to NO-mediated inactivation of the RpoS proteolysis process through the adaptor protein RssB, independently of actions of anti-adaptor proteins, IraD, IraM, and IraP. RssB was nitrosylated upon NO exposure, and mutations of Cys-57 and Cys-171 residues of RssB inhibited RpoS proteolysis, demonstrating these Cys residues as potential RNS targets. Transcriptome analysis revealed that RpoS modulated 16% of acidified nitrite-inducible gene transcription in *S.* Typhimurium. *S.* Typhimurium mutants lacking highly RpoS-dependent acidified nitrite-inducible genes showed hyper-susceptibility to acidified nitrite comparable to that of *rpoS* mutant. Moreover, virulence of attenuated *rpoS* mutant was restored in iNOS$^{-/-}$ mice, exhibiting physiological importance of RpoS for *Salmonella* pathogenesis in NO-producing animals. Results have extended role of RpoS in *Salmonella* fitness under nitrosative stress, appreciating this sigma factor as the master regulator for bacterial stress response.

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INTRODUCTION

The innate immunity of animal host produces a variety of antimicrobial factors against bacterial infections. Among them, nitric oxide (NO) and NO-mediated reactive nitrogen species (RNS) play important roles in abrogating bacterial proliferation inside host animals (Fang, 2004). RNS can be generated by a variety of biological and chemical reactions. In animal endogenous RNS sources are largely derived from NO synthetase function producing NO from L-arginine and oxygen. Most remarkably, upon being activated by bacterial infections, inducible nitric oxide synthase (iNOS) of phagocytic cells produce cytotoxic levels of NO. Moreover, NO can be chemically transformed to other potent RNS such as nitrogen dioxide ($NO_2\bullet$), dinitrogen trioxide (N_2O_3), peroxynitrite ($ONOO^-$), dinitrosyl iron complexes, nitrosothiols and nitroxyl (HNO) (Fang, 2004). Exogenously, dietary nitrate (NO_3) can also be transformed to these RNS through the successive reactions including the reduction of NO_3 to nitrite (NO_2) by nitrate reductases of normal flora, and protonation of NO_2 in acidic environments, such as the dental caries lesion and the stomach acidity, produces nitrous acid (HNO_2). HNO_2 then spontaneously generate N_2O_3, NO and NO_2, which can be found in the acidified nitrite chemistry (Lundberg et al., 2004). Accordingly, synergistic antimicrobial activities of RNS can be found in acidic compartments in host cell organelles such as acidic phagosomes that produces NO and generates NO_2. (McCollister et al., 2007).

To cope with RNS stresses, many bacteria express the principal NO metabolizing enzyme flavohemoglobin Hmp that metabolizes most of NO entering into bacteria to less toxic NO_3 and N_2O under aerobic and anaerobic conditions, respectively (Forrester and Foster, 2012). And the importance of NO detoxification in bacterial pathogenesis was representatively demonstrated in a study with *Salmonella enterica*

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serovar Typhimurium (*S.* Typhimurium), in which *hmp* mutant showed high susceptibility to nitrosative stress and much reduced virulence in a *Salmonella*-resistant mouse strain (Bang et al., 2006). But in acute phase infection models with the *Salmonella*-susceptible mice, *hmp* mutant still shows virulence comparable to that of WT *S.* Typhimurium, while WT *S.* Typhimurium turns to be more virulent in the iNOS$^{-/-}$ mice than parental WT mice (Bang et al., 2006; Mastroeni et al., 2000). Differences in mouse virulence of *hmp* mutant *S.* Typhimurium suggest that NO reaction with other molecules in animal cells may produce RNS that are hardly metabolized by Hmp alone.

To screen for genes playing potential important roles in RNS resistance of *S.* Typhimum, my previous study in the thesis for Master of Science had employed a robust screening methods in which RNS-sensitive mutants can be selected on the solid culture media containing acidified nitrite that produces various RNS. And an *rpoS* mutant lacking the stationary sigma factor RpoS had found as being hypersensitive to acidified nitrite.

In many proteobacteria including *E. coli* and *Salmonella*, RpoS has been regarded as the master alternative sigma factor for general stress response. And RpoS levels are modulated at multiple levels including transcription, translation, and proteolysis. RpoS proteolysis is coordinated by interactions between the adaptor protein RssB and one of anti-adaptor proteins, IraD, IraM, and IraP, before RpoS is transferred to ClpX and ClpP protease complex (Fig. 1) (Park and Bang, 2013). RpoS accumulated in stationary phase cells or in response to environmental stresses regulates the transcription of genes involved in different physiological functions, and *rpoS* mutation causes reduced virulence in some pathogenic bacteria (Hengge, 2011). However, despite of extensive studies more than 30 years, the role of RpoS in RNS stress response remained unexplored.

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This study presents that nitrosative stress increases RpoS levels in *S.* Typhimurium by inactivating the adaptor protein RssB, and that RpoS directs transcription of RNS-responsible genes, thereby potentiating *Salmonella* virulence in NO-producing mice.

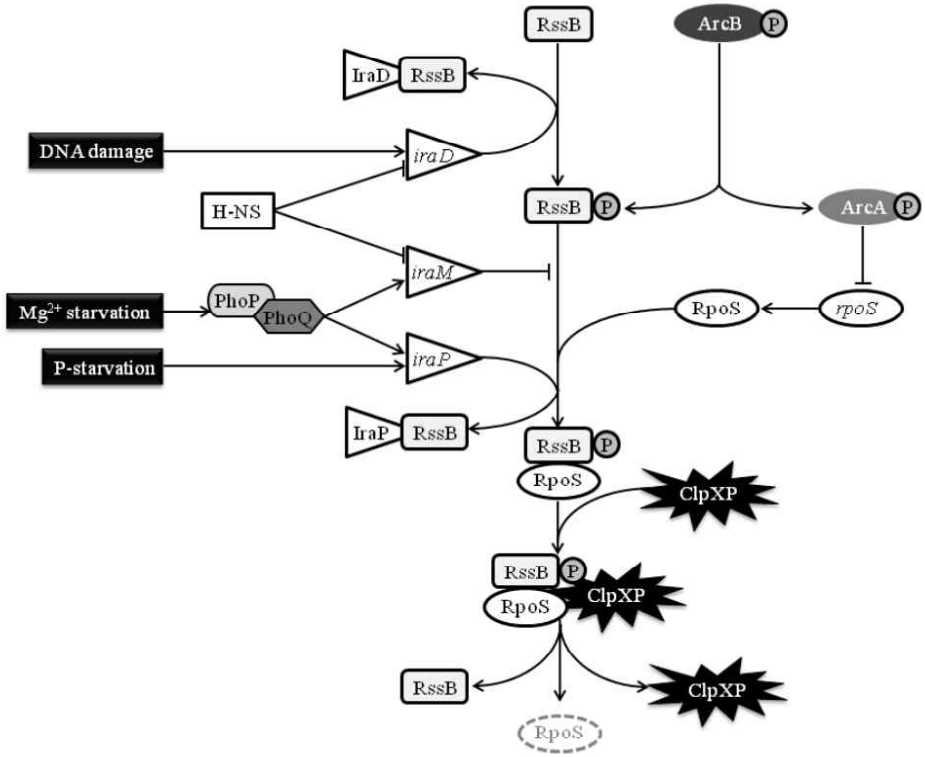

Fig. 1. Regulation of RpoS proteolysis by adaptor protein RssB and anti-adaptor proteins. In log-phase cells, the adaptor protein RssB delivers RpoS to ClpXP protease. But, under DNA-damaging conditions or conditions depleting phosphate or magnesium in the cell, one of anti-adaptor proteins, IraD, IraM, and IraP, interacts with RssB to inhibit RssB binding to RpoS, thereby protecting RpoS from proteolysis.

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MATERIALS AND METHODS

Strains, media and chemicals

Bacterial strains used in this study are listed in Table 1. All *S.* Typhimurium strains are derivative of 14028S. Techniques for standard molecular biology and *Salmonella* genetics were performed according to protocols as described by (Sambrook and Russel, 2001) and (Maloy, 1990a). Construction of mutant *Salmonella* strains was performed by the PCR-mediated one-step gene mutation method using λ Red recombinase as described previously (Bang et al., 2006; Datsenko and Wanner, 2000), and DNA primers used for mutant construction are listed in Table 1. Luria-Bertani (LB) complex medium and minimal E medium containing 0.2% glucose were used routinely for the bacterial culture. Most chemicals were purchased from Sigma-Aldrich, unless stated otherwise. Spermine-NONOate was purchased from Cayman Chemical (Ann Arbor, MI). Acidified nitrite was provided by adding sodium nitrite into 100mM MES buffered- media or buffers adjusted to pH 5.0 with HCl. Sodium nitrite solution was made fresh for each experiment. GSNO was synthesized as described by (Hart, 1985b) .

RNS susceptibility assays.

Salmonella susceptibility to RNS was determined by measuring either growth kinetics or bacterial survival rate after challenge with NO donors. Cells grown overnight in LB media were diluted in PBS to $OD_{600} = 0.01$, then equal amount of cells were inoculated into wells of microplate containing fresh LB, LB buffered with MES (pH5.0) or minimal E glucose media supplemented with or not various NO donors. Growth kinetics was measured by determining the optical density (OD_{600}) at 37°C during incubation in a Bioscreen C Microbiology Microplate reader (Growth Curves, USA). For measuring *Salmonella* survival rate after RNS challenge in the acidic condition,

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cells grown overnight in LB media were diluted in PBS to OD_{600} = 0.01, and challenged to PBS buffered with 50mM MES (pH5.0) containing either 3mM Spermine-NONOate or 3mM sodium nitrite. For anaerobic condition, the challenge buffer and culture media were flushed with O_2-free N_2 gas for 30 min. RNS challenging and bacteria culture were performed in an anaerobic chamber (Bactron I, Sheldon Manufacturing). Viable counts were measured by diluting cells in PBS and plating onto LB agar at timed intervals. Percent survival was calculated by dividing cfu at time points after challenge by cfu before challenge and multiplying by 100. SOD (superoxide dismutase, Sigma-Aldrich; 300 unit/ml) was added to the RNS challenging buffer to scavenge superoxide if needed.

Expression and purification of RssB

The *rssB* gene was PCR-amplified from WT *S.* Typhimurium chromosome with primers His-Tag RssB Fw and RssB Rev (SalI) by using iPFU polymerase (Promega). Purified PCR products were digested with NdeI and SalI and cloned into the pET28a vector (Novagen) digested with same enzymess. Overnight cultures of *E. coli* BL21 harboring pET28a-*rssB* were sub-cultured into 1.5L of LB medium (1:200) at 37℃. Log-phase cultures (OD_{600} ~ 0.5) were overexpressed by addition of 500uM IPTG and cultured for 16 hrs at 18℃. The harvested cells were resuspended in 50ml lysis buffer containing 20mM Tris-HCl pH7.5, 500mM NaCl, 5mM $MgCl_2$, 10mM imidazole, 1mM EDTA, 1mM DTT, 1mM PMSF and a little DNase I. After sonication (2 10 sec pulses at 25% amplitude with a rest for 2 min on ice) and centrifugation (2000 x *g*), the fitrated supernatant (0.45um syringe filter) was passed through a column containing Ni-NTA agarose resins (Quiagen), and His-RssB protein binding was performed using an Econo pump (0.5ml / min). After protein binding, Ni-NTA agarose resins were

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washed to 2ml/min by washing buffer (20mM Tris-HCl pH7.5, 20mM imidazole, 500mM NaCl, 1mM DTT) and His-RssB was eluted by elution buffer (20mM Tris-HCl pH 7.5, 500mM imidazole. 500mM NaCl). When necessary, The His-tag was removed by digestion with human α-thrombin (7 unit, 10,000x dilution in 2 mM $CaCl_2$) at RT for 6 hours. After re-subjection of digestion mixture onto the Ni-affinity column, the digested proteins in flow-through fractions were concentrated, and finally isolated by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) in the buffer (20 mM Tris, pH 7.5, 50 mM NaCl).

Biotin-switch assay

S-Nitrosylation of His-RssB was examined by detecting biotin that switches nitrosylated thiols in RssB following the methods described in (Husain et al., 2010). Briefly, purified RssB proteins in HDN buffer (250 mM Hepes pH 7.7, 1mM DTPA, 0.1 mM neocuproine) were treated with or not GSNO (100 uM). To block free thiols, S-Methyl methanethiosulfonate (25 mM) added to the samples, then mixture was incubated at 50°C for 30 min with intermittent vortexing every 5 min. MMTS was removed by precipitation with acetone, then samples were washed 4 time with 1 ml of 70% acetone before resuspending them in HDN buffer supplemented with 1% SDS. Then biotin-switch was accomplished adding ascorbate (10mM) and biotin-HPDP (0.8 mM) (Pierce) to the samples, and incubated for 1 h. After precipitation and washing with acetone, biotinylated His-RssB proteins were further selected by incubating with NeutrAvidin beads (Pierce) following protocol offered by manufacturer. Finally, biotinylated His-RssB was identified in immunoblot analysis by using an anti-His tag antibody (Santacruz).

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Immunoblot analysis

Preparation of cell lysate, electrophoresis and protein transfer to polyvinylidene difluoride membranes were performed as previously described by (Bang et al., 2000). The membrane was probed with 1:2,000 dilution of a murine monoclonal IgG antibody to RpoS (Neoclone) or to His-tag in TBST (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.05% [vol/vol] Tween 20) buffer overnight at 4°C. After several washing in TBST buffer, the blots were incubated with a 1:5,000 dilution of secondary anti-mouse IgG-peroxidase conjugate (Sigma Co.) in TBST buffer for 1 hour. Proteins were visualized by using the PowerOpti-ECL Western Blotting Reagent (Anigen) and the Fuji Las-3000 luminescent image analyzer (Fuji). A representative picture of at least three independent experiments was shown.

cDNA microarray analysis and quantitative reverse transcription PCR

RNS-inducible genes between WT and *rpoS* mutant *Salmonella* were compared by performing *Salmonella* microarray analysis as described in (Bang et al., 2005). Bacteria grown overnight in LB were diluted to 1:1,000 in LB media buffered with MES (100mM), adjusted to either pH 7.0 or pH5.0, and incubated to early log-phase (OD_{600} ~ 0.4). Then cells from both pH cultures were collected before and after treatment with sodium nitrite (3mM) for 1 hour. Total RNA was purified using Qiagen RNA purification kit after stopping bacterial transcription by adding a 1/5X volume of an ice-cold mixture solution of ethanol-phenol (95%/5%) to the bacterial culture. cDNA labeling and hybridization to *Salmonella* array chips were performed as described previously (Bader et al., 2003). Microarray analyses were repeated two times for each conditions, in which each RNA samples labeled in duplicate with Cy3 and Cy5 for

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hybridization with counterpart samples in each microarray analysis. Transcription profiles were provided in Table S1.

Mouse virulence

Eight to ten week-old C57BL/6 and congenic *iNOS*$^{-/-}$ mice were infected *p.o*. with 10^7 CFU of the indicated *Salmonella* strains. Mouse survival was monitored over time, and sick mice were sacrificed by euthanization, and counted as numbers of dead mice on next day.

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Table 1. Bacterial strains and plasmids used in this study.

Strains	Genotype	Source or Reference
IB1	*S.* Typhimurium 14028s, Wild-type	ATCC
IB3	*hmp::*	This study
IB1006	*rpoS :: Tn10dCm*	This study
IB1101	*ΔsodC :: Cm*	This study
IB1115	*ΔsodC ΔkatE ΔkatN* :: Cm	This study
IB1172	*ΔrssB*	This study
EF644	*Δcya-1400 ::Km /* pF379A /pMF383	(Matthew Moreno et al., 2000)
EF682	*Δcya-1400 ::Km /* pAF404/pMF383	(Matthew Moreno et al., 2000)
IB1678	*RssB C57G*	This study
IB1679	*RssB C123G*	This study
IB1697	*RssB C171G*	This study
IB1698	*RssB C202G*	This study
IB1703	*RssB C316G*	This study
IB1704	*RssB C321G*	This study
IB1660	*RssB 6 cys→6Gly chromosomal DNA mutant (no AB)*	This study
IB2297	*RssB C57S*	This study
IB2298	*RssB C123S*	This study
IB2299	*RssB C171S*	This study
IB2300	*RssB C202S*	This study
IB2301	*RssB C316S*	This study
IB2302	*RssB C321S*	This study
IB1503	*Solu BL21 /* pET28a (+)-*RssB clone :: Km*	This study
IB1346	*ΔycgB ΔyliH ΔyecG ΔSTM3688 ΔwraB ΔyhcO ΔygaU ΔybaY ΔuspA ΔSTM1324 ΔotsB ΔyqjI ΔyeaH ΔyhhA ΔyeaG*	This study

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IB1347	*ΔycgB ΔyliH ΔyecG ΔSTM3688 ΔwraB ΔyhcO ΔygaU ΔybaY ΔuspA ΔSTM1324 ΔotsB ΔyqjI ΔyeaH ΔyhhA ΔyeaG Δdps :: Cm*	This study

Plasmid

pKD3	Plasmid carrying the FRT-Cm^r-FRT- cassette	(Datsenko and Wanner, 2000)
pKD4	Plasmid carrying the FRT-Km^r-FRT- cassette	(Datsenko and Wanner, 2000)
pTP233	Plasmid encoding IPTG-inducible lamda red recombinase	(Datsenko and Wanner, 2000)
pCP20	Plasmid expressing the FLP recombinase	(Datsenko and Wanner, 2000)
pET28a	Gene expression vector	Novagen
pBAD18	Gene expression vesctor	(Miller and Gennis, 1986)
pBAD33	Gene expression vesctor	(Miller and Gennis, 1986)
pKD46	Plasmid encoding arabinose-inducible lamda red recombinase, temperature sensitive	(Datsenko and Wanner, 2000)
pWRG100	Plasmid carrying CM^r cassette and an I-*Sce*I recognition site	(Blank et al., 2011)
pWRG99	AHT-inducible I-*Sce*I	(Blank et al., 2011)
pMF379A	*rpoS^{UK1}-'cyaA(T18) in pT18, Ap^R*	(Matthew Moreno et al., 2000)
pMF383	*cyaA*(T25)'- *rssB* in pT25 Cm^R	(Matthew Moreno et al., 2000)
pAF404	*cyaA*(T18)'- *clpX*^{K12} in pT18, Ap^R	(Matthew Moreno et al., 2000)

DNA Oligonucleotides

	Sequence (5′ → 3′)
RssB-P1-Fw	CCCTGATCTCATGATATGTGATATCGCTATGCC GAGAATG CATATGAATATCCTCCTTAG

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RssB-P2-Rev	GCAATATCCAGCACCAGTCCAGGCTGATCGGC GGAGACGA GTGTAGGCTGGAGCTGCTTC
RssB Fw	CTGTCCGCA AATCTCTACGC
RssB Rev	CGAATTAAGGGCAGCCATTTAA
His-Tag RssB Fw	GAGAGCATATGACGCAGCCA
RssB Rev (SalI)	TAACACTCGTCGACAA AGGCC

Chromosomal *rssB* mutant construction

rssB C57 I-SceI-cat Fw	CAGTTTGAGACCGTTCATTCTCGGCATAGCGA TATCACCT CGCCTTACGCCCCGCCCTGC
rssB C57 I-SceI-cat Rev	CCCTGGAGTTGATGGGGCGTTTTACCCCTGAT CTCATGATCTAGACTATATTACCCTGTT
C57G Fw	CAGTTTGAGACCGTTCATTCTCGGCATAGCGA TATCACCTATCATGAGATCAGGGGTAAAACGC C CCATCAACTC CAGGG
C57G Rev	CCCTGGAGTTGATGGGGCGTTTTACCCCTGAT CTCATGATAGGTGATATCGCTATGCCGAGAAT GAACGGTCTCAAACTG
rssB C123 I-SceI-cat Fw	TTCAACCCGCGAGTTAAACATATTAGGGTATA AACCAGCGCGCCTTACGCCCCGCCCTGC
rssB C123 I-SceI-cat Rev	AGCCGGTAAAAGATCTCAACCGCTTGCGGGAA ACCGTATTCTAGACTATATTACCCTGTT
C123G Fw	TTCAACCCGCGAGTTAAACATATTAGGGTA TAAACCAGCGAATACGGTTTCCCGCAAGCG GTTGAGATCT TTTACCGGCT
C123G Rev	AGCCGGTAAAAGATCTCAACCGCTTGCGGGAA ACCGTATTCGCTGGTTTATACCCTAATATGTTT AACTCGCGGG TTG AA
rssB C171 I-SceI-cat Fw	ATCGGCGGAGACGAGCTGCCGGTAATTAATGC GACCATGACGCCTTACGCCCCGCCCTGC
rssB C171 I-SceI-cat Rev	GTTGCAGGAATTGCAGCCCCCGGTACAGCAGG TGATTTCCTAGACTATATTACCCTGTT
C171G Fw	GCGGAGACGAGCTGCCGGTAATTAATGCGACC ATGAGAAATCACCTGCTGTACCGGGGGCTGCA ATTCCTGC
C171G Rev	TGCAGGAATTGCAGCCCCCGGTACAGCAGGTG ATTTCTCATGGTCGCATTAATTACCGGCAGCTC GTCTCCGC
rssB C202 I-SceI-cat Fw	TAACACGCCGTTATCGCCCGCCCGGGTGACAT CCAGACCACGCCTTACGCCCCGCCCTGC

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rssB C202 I-SceI-cat Rev	TGGATATTGCGCCGCTATCTGATAACGAACTG GCATTTTACTAGACTATATTACCCTGTT
C202G Fw	TATCGCCCGCCCGGGTGACATCCAGACCATAA AATGCCAGTTCGTTATCAGATAGCGGCGCAAT ATCC
C202G Rev	GGATATTGCGCCGCTATCTGATAACGAACTGG CATTTTATGGTCTGGATGTCACCCGGGCGGGC GATA
rssB C316 I-SceI-cat Fw	CTCCTGCGCCCCATATTTGACACTGCCACGAG TCACCACGCGCCTTACGCCCCGCCCTGC
rssB C316 I-SceI-cat Rev	AGGAACATTGGGTAACGCCTACCTTAACCAAT TAAGCCAGCTAGACTATATTACCCTGTT
C316G Fw	GCAGACGCCCTCCTGCGCCCCATATTTGACAC TGCCACGAGTCACCACGCTGGCTTAATTGGTT AAGGT AGGCGTTAC
C316G Rev	GTAACGCCTACCTTAACCAATTAAGCCAGCGT GGTGACTCGTGGCAGTGTCAAATATGGGGCGC AGGAGGGCGTCTGC
rssB C321 I-SceI-cat Fw	CGCAGACGCCCTCCTGCGCCCCATATTTGACC CTGCCACGCGCCTTACGCCCCGCCCTGC
rssB C321 I-SceI-cat Rev	GGTAACCCCTACCTTAACCAATTAAGCCAGCG TTGCGACTCTAGACTATATTACCCTGTT
C321G Fw	GCAGACGCCCTCCTGCGCCCCATATTTGACCC TGCCACGAGTCGCAACGCTGGCTTAATTGGTT AAGGTAGGCGTTAC
C321G Rev	GTAACGCCTACCTTAACCAATTAAGCCAGCGT TGCGACTCGTGGCAGGGTCAAATATGGGGCGC AGGAGGGCGTCTGC
C57S Fw	CAGTTTGAGACCGTTCATTCTCGGCATAGCGA TATCCGATATCATGAGATCAGGGGTAAAACGC C CCATCAACTC CAGGG
C57S Rev	CCCTGGAGTTGATGGGGCGTTTTACCCCTGAT CTCATGATATCGGATATCGCTATGCCGAGAAT GAACGGTCTCAAACTG
C123S Fw	TTCAACCCGCGAGTTAAACATATTAGGGTA TAACGAAGCGAATACGGTTTCCCGCAAGCG GTTGAGATCT TTTACCGGCT
C123S Rev	AGCCGGTAAAAGATCTCAACCGCTTGCGGGAA ACCGTATTCGCTTCGTTATACCCTAATATGTTT AACTCGCGGG TTG AA

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C171S Fw	GCGGAGACGAGCTGCCGGTAATTAATGCGCG AATGAGAAATCACCTGCTGTACCGGGGGCTGC AATTCCTGC
C171S Rev	TGCAGGAATTGCAGCCCCCGGTACAGCAGGTG ATTTCTCATTCGCGCATTAATTACCGGCAGCTC GTCTCCGC
C202S Fw	TATCGCCCGCCCGGGTGACATCCAGCGAATAA AATGCCAGTTCGTTATCAGATAGCGGC GCAATATCC
C202S Rev	GGATATTGCGCCGCTATCTGATAACGAACTGG CATTTTATTCGCTGGATGTCACCCGGGCGGGC GATA
C316S Fw	GCAGACGCCCTCCTGCGCCCCATATTTGACAC TGCCACGAGTCCGAACGCTGGCTTAATTGGTT AAGGT AGGCGTTAC
C316S Rev	GTAACGCCTACCTTAACCAATTAAGCCAGCGT TCGGACTCGTGGCAGTGTCAAATATGGGGCGC AGGAGGGCGTCTGC
C321S Fw	GCAGACGCCCTCCTGCGCCCCATATTTGCGAC TGCCACGAGTCGCAACGCTGGCTTAATTGGTT AAGGTAGGCGTTAC
C321S Rev	GTAACGCCTACCTTAACCAATTAAGCCAGCGT TGCGACTCGTGGCAGTCGCAAATATGGGGCGC AGGAGGGCGTCTGC
ybaY P1 Fw	GCGATATCTCTGGCGGCCTGCGCTGATAAAAG CGCTGATAGTGTAGGCTGGAGCTGCTTC
ybaY P2 Rev	ACCGCTTTTTGCGACAGCACCTTAGACGGCGC ATCAGCCACATATGAATATCCTCCTTAG
ybaY Fw	ACTCGTGCACATCGTAAGTGG
ybaY Rev	ATGGGTTAAACGGCAACACA
dps P1 Fw	ACCGCACTGACCGATCATCTGGATACTATG GCCGAGCGCGCATATGAATATCCTCCTTAG
dps P2 Rev	GGCGGTAAAGATATCGGCAGTGTCTTCATC TTTGGCTTCAGTGTAGGCTGGAGCTGCTTC
dps Fw	GGAATTTATCGAGGTCGCGT
dps Rev	TCATGAGATGCTGGATGGCT
yliH P1 Fw	AAGCTGATAAACGCGCGTATCGATCTTGCC GCTTATCTGCGTGTAGGCTGGAGCTGCTTC

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yliH P2 Rev	AACAAACGGCGGCGGCTGCAAGCGCGCCTT CGGCACGGCGCATATGAATATCCTCCTTAG
yliH Fw	ATCTGCAATTGCGAAAAGCG
yliH Rev	GGAGCTCGCGATTCAGTTCA
wraB P1 Fw	CGGATTACGAAGCCATTATTTTTGGTACGC CAACCCGGTTCATATGAATATCCTCCTTAG
wraB P2 Rev	AACCGGGTTGGCGTACCAAAAATAATGGCA TCGTAATCCGGTGTAGGCTGGAGCTGCTTC
wraB Fw	AGAACTGAACACGCTGCCGA
wraB Rev	TGTCAGGCCAGATGCGTACC
yeaH P1 Fw	TCAAGGTCAGGCCAGCCAGGACGGCGAAGG CCAGGATGAGCATATGAATATCCTCCTTAG
yeaH P2 Rev	GGTACGCCGTTTGAGGTGAAACCGGCGCGG TGAGTTTTATGTGTAGGCTGGAGCTGCTTC
yeaH Fw	CGCGCCAGAGAGTTTTGTAG
yeaH Rev	CCAGAATGATCGCATTGAGC
STM1324 P1 Fw	ACTGCGTAATGAACTGCCAGGCGGAGAAGT TCATGCCGCACATATGAATATCCTCCTTAG
STM1324 P2 Rev	TCCAGCGGGCGGGGCGAGAGGTAGTCCATG ACTAAAAAGCGTGTAGGCTGGAGCTGCTTC
STM1324 Fw	GTTGCCCAAGAATAAACGCA
STM1324 Rev	TTTAAGCGAGCAAGTCGGTG
ycgB P1 Fw	TGAACGAGGGCTGGGCGACCTTCTGGCACT ACACCATCCTGTGTAGGCTGGAGCTGCTTC
ycgB P2 Rev	ACTGACAAATACGTTTGATATCCTGGAACA TGGCAAAGCCCATATGAATATCCTCCTTAG
ycgB Fw	TTATCCGCAGAAACAGACGC
ycgB Rev	GGCAATATCCGGAAACCAGT
yecG P1 Fw	TCACCAGCTACTGGCTAAAGCGGTCTCTAT CGCCCGCCCTGTGTAGGCTGGAGCTGCTTC
yecG P2 Rev	GCCAAAATATGCTGGCTTAACTCACCTGAT GCGATGAAAGCATATGAATATCCTCCTTAG
yecG Fw	CATATTCTTGTCGCCGTTGC

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yecG Rev	CCGCAAATGACCAGATCAAC
otsB P1 Fw	CCCACTTTTACGGAAATTCCGCCAGCGTGA TTCACCACGCGTGTAGGCTGGAGCTGCTTC
otsB P2 Rev	CGCAACACGTTACGCAGCACTGGCCGCAAC TGGCGTTGCACATATGAATATCCTCCTTAG
otsB Fw	CAGACATCGGGTACGCTTTC
otsB Rev	CAGGCTCCGGAACATGAAG
ygaU P1 Fw	TCTGGGACGCGGTAACCGCTAATCATGATA AGGACGATCACATATGAATATCCTCCTTAG
ygaU P2 Rev	ACCTGATCGTCAACGCTGCTAATCCCGGCG ATATTCCCCAGTGTAGGCTGGAGCTGCTTC
ygaU Fw	AAAACTGACTTTCAGCCGCC
ygaU Rev	CAAGCGAGGTGAATATGGGA
yqjII P1 Fw	ATGCAAAATCAACATGAAGGGTGTTGCAAA AACCGGGATCGTGTAGGCTGGAGCTGCTTC
yqjII P2 Rev	CCGGGTCAGGATATCAAGAATGACCAGCCG AAGTTCCCCGCATATGAATATCCTCCTTAG
yqjII Fw	CTGCGGATAAACCACAGCGA
yqjII Rev	AATCCAGTTCGGCGATTTCC
yhcO P1 Fw	CCAAAGCGATTTTTATCGCGAGTTTACGCA GACGTTTGGTCATATGAATATCCTCCTTAG
yhcO P2 Rev	AACGAACTCAATCTCTAGCGGTAGGGGTAA GATATCGCTCGTGTAGGCTGGAGCTGCTTC
yhcO Fw	CGCAGTTTATCAGGCAGGTG
yhcO Rev	GGGCTAAAAGCGTTTCTGGA
yhhA P1 Fw	ACTACTTATTACGGCTTTGTTACCCTTTGC TGTATTGGCCGTGTAGGCTGGAGCTGCTTC
yhhA P2 Rev	CTGTCCTGCTCTGTCCTGCTCTGGGAATGG TTTAGCATCCCATATGAATATCCTCCTTAG
yhhA Fw	TTTAATCCTG GCCCACAACG
yhhA Rev	GTCTGCGAAT TCTGGTGTAT AC

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uspA P1 Fw	TATTCTGACCTGTACACCGGTCTGATTGAC GTCAATCTGGGTGTAGGCTGGAGCTGCTTC
uspA P2 Rev	AGAAGACATCAGTTTGCTCCAGAAGTCCTG GTGATGACCGCATATGAATATCCTCCTTAG
uspA Fw	AACGCGAAAATCTCCCTCAT
uspA Rev	TGTCAACGTGAACGGTGTTG
STM3688 P1 Fw	AATAGGTGAGAACGTACCACTTCTTATTGA TAAAGCCGTCCATATGAATATCCTCCTTAG
STM3688 P2 Rev	GGTCGGTACAGCCGCCGGTAATATTCCAGA CGCTGCAAATGTGTAGGCTGGAGCTGCTTC
STM3688 Fw	CCGCCGTTAACCTCTTTCTT
STM3688 Rev	GGTCCCACATGGAGGATCTA

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RESULTS

The previous results for comprehension of this study: RpoS is required for *S.* Typhimurium resistance to the acidified nitrite (Park, 2010).

This section mostly presents previous results in my thesis for Master of Science, with reevaluation of results and reassortment of data, for readers to understand background and experimental conditions for this study

To screen gene mutations causing the retarded growth of *S.* Typhimurium on the acidified nitrite (AN), my previous study (Park, 2010) used the acidic LB agar (pH5.0) containing 3mM nitrite, and found that an *rpoS*::Tn10 mutant was much more susceptible than WT. In the liquid culture with same condition for AN, the growth of *rpoS* mutant was also significantly impaired, compared with that of WT (Fig. 2A). When log-phase cells were exposed to AN, *rpoS* mutation caused drastic reduction in *Salmonella* survival up to 10,000 fold in aerobic cultures and 100 fold in anaerobic cultures, respectively (Fig. 2B), indicating that RpoS plays important roles in anti-nitrosative stress response, regardless of oxygen availability. The result in anaerobic cultures was unexpected, because RpoS had been known to activate transcription of key genes encoding enzymes for metabolism of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide that can potentiate cytotoxic effects of NO (Brunelli et al., 1995; Woodmansee and Imlay, 2003), so that the effect of *rpoS* mutation to *Salmonella* AN resistance was expected to be mostly from the inadequate expression of these enzymes. However, mutant *S.* Typhimurium lacking all RpoS-dependent genes encoding catalases [*katE* and *katN*, (Robbe-Saule et al., 2001)] and superoxide dismutase [*sodC*II (Fang et al., 1999)] was resistant to AN as much as that of WT (Fig. 2C). Supplementation with superoxide dismutase to the AN challenge buffer significantly restored the survival of *rpoS* mutant under both aerobic and anaerobic

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conditions, but there was still about 100 fold decrease in the survival rate of *rpoS* mutants (Fig. 2D), suggesting that RpoS-dependent resistance to RNS could be resulted from RpoS-directed transcription of other sets of genes as well as conventional genes for ROS detoxification.

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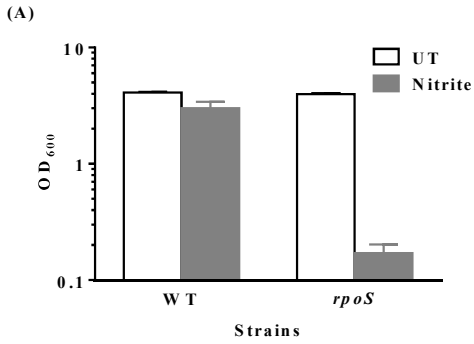

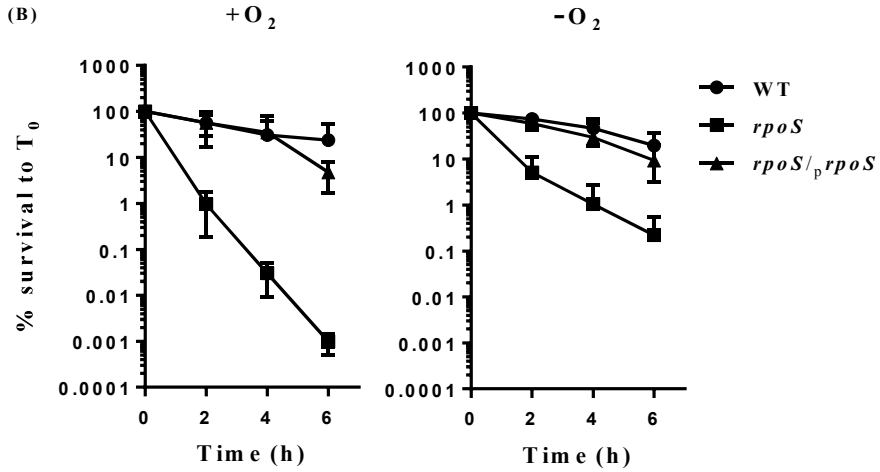

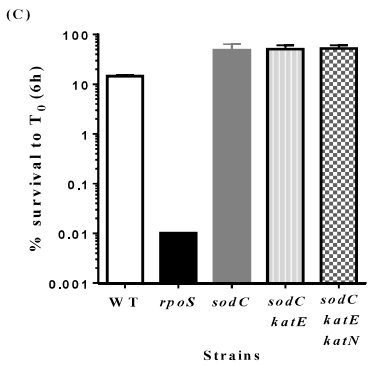

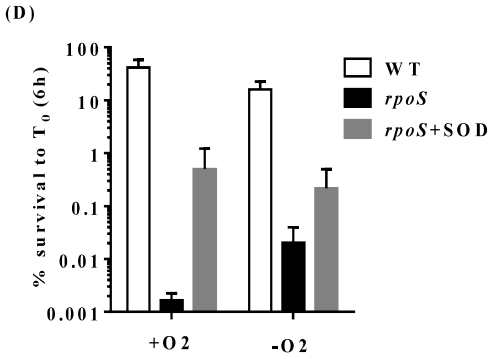

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Fig. 2. RpoS is required for *Salmonella* resistance to acidified nitrite. (A) OD_{600} values were measured for WT and *rpoS* mutant *Salmonella* cultured for 16 h in LB-Mes broth (pH 5.0) containing sodium nitrite (2mM) (nitrite) or not (UT). (B, C) *Salmonella* strains cultured overnight were challenged to nitrite (3mM) containing buffer (PBS-Mes, pH5.0) under aerobic or anarerobic conditions. (D) superoxide dismutase was added to challenging buffer for *rpoS* mutant. Results are shown as the mean +/- SD from three independent experiments

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RNS stress increses RpoS levels by inhibiting the RssB mediated proteolysis of RpoS

As the general stress response regulator, RpoS levels are increased in bacteria that responds to environmental changes causing stationary-phase-like state (Gottesman, 2019). To examine whether RpoS levels are changed by AN exposure, this study chose an AN condition containing a minimum concentration of nitrite (3mM) inhibiting *Salmonella* growth in LB media (pH 5.0) (Fig. 3A). RpoS levels in log-phase cells were very low as known to be from the rapid proteolysis of RpoS, but upon AN exposure, they were highly increased to comparable levels with those of stationary-phase cells or of log-phase cells of *rssB* mutant lacking the adaptor protein RssB (Fig. 3B). Adding the NO congener spermineNONOate (1mM) to log-phase culture of WT also increased RpoS levels comparable to those of AN-treated cells, demonstrating that RNS stress increases RpoS levels in *S.* typhimurium during log-phase.

Because RpoS levels in *rssB* mutant in log-phase cultures were not additionally increased by RNS stress, it can be hypothesized that the RssB-mediated proteolysis could play regulatory role on RpoS expression in *S.* Typhimurium under RNS stress. To examine this, this study observed the effect of RNS to the RpoS proteolysis in a condition that high levels of RpoS in stationary-phase cells can be degraded timely after treatment with translation-inhibiting antibiotic chloramphenicol (Fig. 3C). In fact, RpoS levels in *S.* Typhimurium were largely decreased for 1 hour after chloramphenicol treatment. However, RpoS levels did not change in cells treated with NO and chloramphenicol together, indicating that RNS stress can inhibit RpoS proteolysis. Of note, this proteolysis was not affected by H_2O_2 treatment. H_2O_2 is known to increase RpoS levels in log-phase *E. coli* by sRNAs (small RNAs)-activated translation and by inhibiting RpoS proteolysis through the action of an anti-adaptor

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IraD that binds to and inhibit RssB in interactions with RpoS (Gottesman, 2019). In fact, the RssB-mediated RpoS proteolysis can be modulated by interactions between RssB and one of anti-adaptor proteins IraD, IraM, or IraP, in which each anti-adaptor inactivates RssB in its binding to RpoS, thereby increasing RpoS levels in log-phase bacteria (Gottesman, 2019). In AN-treated *Salmonella*, however, deletion mutations in genes for these anti-adaptors did not affect RpoS levels (Fig. 3D), suggesting that RNS inhibition on RssB-directed RpoS proteolysis would be rather directly operated without anti-adaptor actions. Next, to examine the RNS effect to protein interactions in RssB-RpoS-ClpX complexes, this study employed a bacterial two-hybrid system utilizing the cAMP-dependent *lacZ* reporter and the reconstitution of adenylate cyclase activity depending on close interactions between two test proteins fused to the separate fragments of adenyl cyclase catalytic domain (Karimova et al., 1998). When cells were treated with NO, there was significant reduction in β-galactosidase activity of *E. coli cya* mutant harboring two plasmids expressing Cya'-RssB and RpoS-'Cya, but little changes of β-galactosidase activity observed in the strain expressing ClpX-'Cya and Cya'-RpoS that cultured in the presence and absence of NO (Fig. 3E). Taken together, RNS stress can specifically inhibit the RssB and RpoS interaction, increasing RpoS levels in *Salmonella*.

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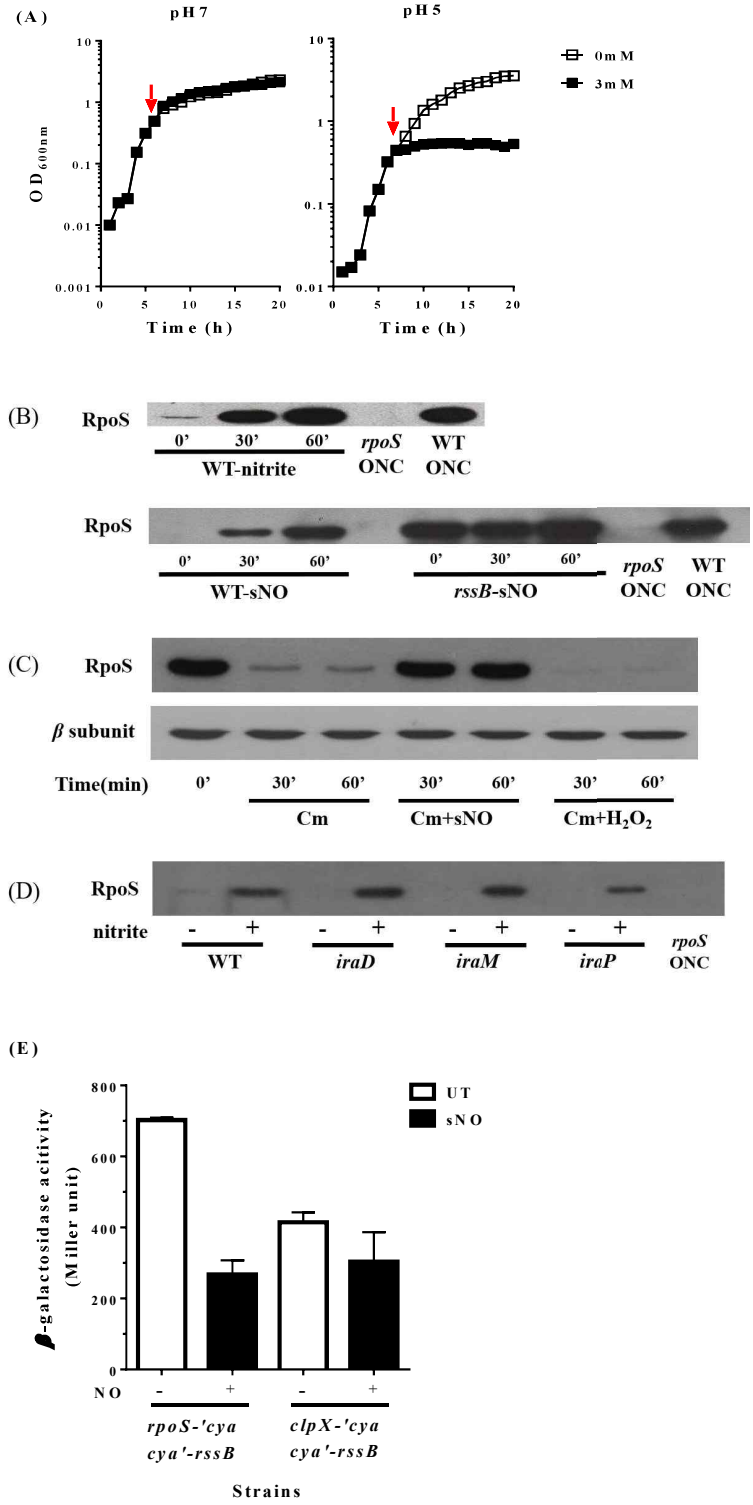

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Fig. 3. RpoS levels in *Salmonella* under RNS stress conditions. (A) Minimum inhibitory concentration of nitrite (3mM) for *Salmonell*a growth in log-phase. (B-D) RpoS levels were measured by immunoblot analysis. Data shown are representatives of those from three independent experiments. (B, D) *Salmonella* strains grown to log-phase (OD_{600}=0.3) in LB Mes (pH 5.0) broth were treated with nitrite (3mM) for 1 h. Equal amount of proteins (10 ug) from *Salmonella* taken at 30 min-intervals were comparatively analyzed with samples from overnight cultured (ONC) WT and *rpoS* mutant *Salmonella* used as positive and negative controls for RpoS expression, respectively. Levels of RNA polymerase β-subunit were used as an internal loading control for each sample. (C) RpoS degradation was monitored when stationary-phase *Salmonella* were treated with chloramphenicol (Cm) in the presence and absence of spermineNONOate (sNO; 1mM) or hydrogen peroxide (1mM) for 1 h. (E) RNS effects to *in vivo* interactions between RssB, RpoS, and ClpX were measured. *E. coli cya* mutant harboring clones for two hybrid pairs were cultured with IPTG and appropriate antibiotics. β-galactosidase activity was assayed in log-phase (OD_{600} = 0.5) cells cultured with or without (UT) spermineNONOate (sNO; 1mM). Results are shown as the mean +/- SD from three independent experiments.

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Nitrosylation of RssB modulates RpoS proteolysis

Because RssB contains 6 cysteine residues, it is hypothesized that thiols in some Cys residues in RssB can be vulnerable to RNS attack and play important roles for RssB activity in RpoS proteolysis. Firstly, using a biotin-switch analysis, RssB was found to by nitrosylated by exposing NO to purified His-tagged RssB (Fig. 4A). Then each Cys residues were substituted into Gly and Ser by mutating corresponding nucleotides in Salmonella chromosome, respectively. 6 sets of both Cys to Gly and Cys to Ser mutations showed similar phenotypes with respect to RpoS levels in *S.* Typhimurium, in the analyses performed for this study. Mutations on Cys-57 and Cys-171 residues absolutely recovered RpoS levels in chloramphenicol-treated cells, comparable to those in cells without any treatment, and did not change RpoS levels in cells threated with both NO and chloramphenicol (Fig. 4B). This result suggest that these Cys residues may be critical targets of RNS to inactivate RssB activity. These two Cys mutations also increased RpoS levels in log-phase cultures in the absence of RNS stress, highlighting their importance in RssB-mediated RpoS proteolysis (Fig. 4C). On the contrary, in both mutants expressing Cys-202 to Gly and Cys-202 to Ser mutant RssBs, there was no detectable RpoS under all culture conditions even in stationary-phase (data not shown), suggesting that Cys202 residue may be important for RssB to determine the pace of RpoS proteolysis. The SWISS-MODEL program-predicted structure of *Salmonella* RssB, based on the crystal structure of *Pseudomonas* RssB, suggest two potential intermolecular disulfides between Cys-57 in one monomer and Cys-171 in the other monomer that can be located proximate to each other if two RssB monomers were disposed in an antiparallel symmetry (Fig. 5). Although the crystal structure of *Escherichia* RssB was defined in a closed form bound with IraD for inhibiting RssB interaction with RpoS (Dorich et al., 2019), the open form and dimer possibility of

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RssB in *E. coli* and *Salmonella* have not been resolved yet. However, this program-based prediction of *Salmonella* RssB structure with the finding of the two Cys residues playing important roles on RpoS proteolysis suggest that RssB may exist in an open dimer form being active for delivering RpoS to ClpXP protease.

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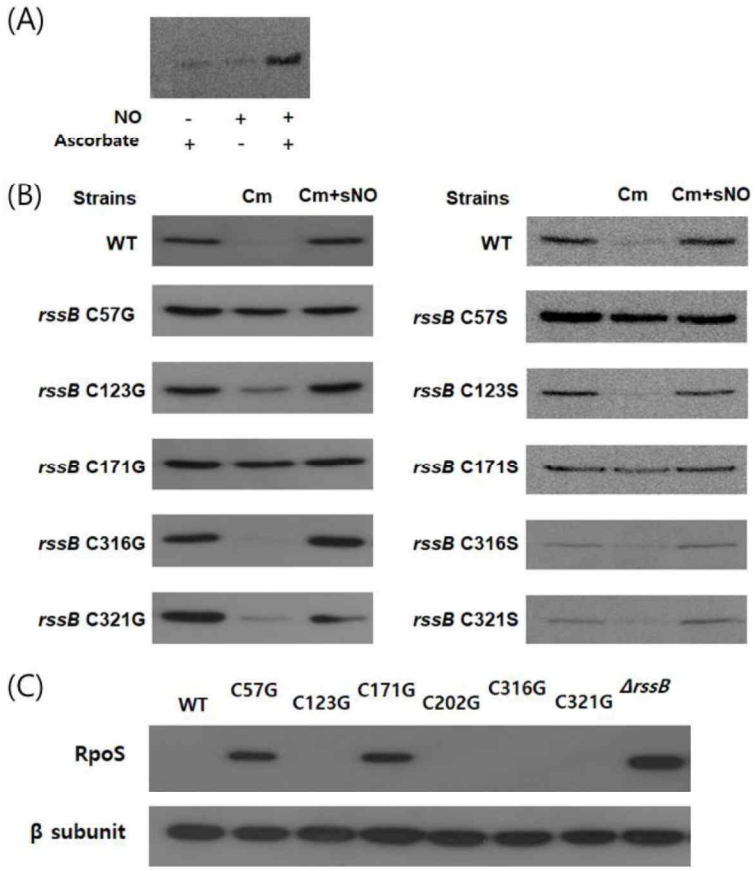

Fig. 4. Nitrosylation of RssB and important Cys residues of RssB for RpoS proteolysis. (A) Purified His-RssB was treated with GSNO (100uM) for 1 h, and biotin-switched in the presence and absence of ascorbate as described in the methods. Eluted His-RssB proteins from NeurAvidin-biotin complexes were examined by immunoblot analysis with an anti-His tag antibody. (B-C) Immunoblot analysis for measuring RpoS levels in *Salmonella*. (B) Overnight cultures of WT and *rssB* mutant *Salmonella* expressing various Cys mutant RssBs were treated with Cm with and without spermineNONOate (sNO) as described for Fig. 2C. (C) Log-phase cultures of WT and *rssB* mutants were analyzed for measuring their RpoS levels. Levels of RNA polymerase β-subunit were used as an internal loading control for each sample. Data shown are the representatives from at least three independent experiments.

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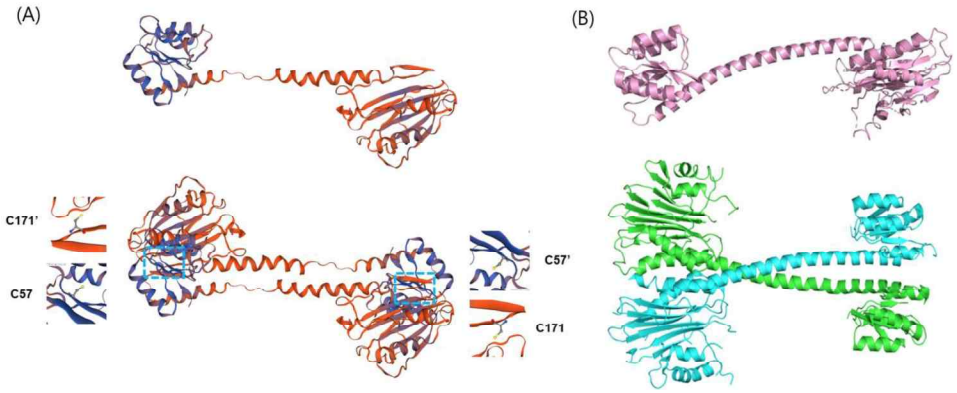

Fig. 5. Model of RssB protein in *Salmonella*. *Salmonella* RssB model structure (A) was obtained using SWISS-MODEL, based on the monomer and homo dimer structures of RssB-like protein from *Pseudomonas aeruginosa* (PDB ID 3EQ2) (B). In (A), the dotted box indicates possible locations for potential disulfide bonds between Cys-57 residue in one monomer and Cys-171 residue in the other monomer, when disposed in an antiparallel symmetry, in which each Cys residues are indicated in atomistic details.

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Transcription profile of genes differentially expressed by RpoS under RNS stress

To identify genes regulated by RpoS when *S.* Typhimurium were exposed to RNS stress, transcriptome was anlalyzed with cDNA microarray profiles of total RNA purified from WT and *rpoS* mutant *Salmonella* in log-phase cultures, before and after AN exposure under same condition for measuring RpoS levels aforementioned. The results show that 1204 genes were upregulated and 1365 genes were downregulated in WT by AN, respectively, more than 2 fold with p value < 0.05. In these AN-regulated genes, 366 genes were differentially transcribed more than 2 fold by *rpoS* mutation, indicating that ranscription of up to 15% of AN-regulated genes was related with RpoS expression. Genes regulated by AN were subjected to COGs (clusters of orthologous groups) analysis [http://www.ncbi.nlm.nih.gov/COG (Galperin et al., 2015)], and AN-regulated genes whose expression were affected by *rpoS* mutation were also classified in COGs category (Table 2). As expected from previous genetic studies on *Salmonella* stringent response under growth arrest conditions, genes for translation system (category I) and cell wall biogenesis (category M) were much more counted in genes downregulated by AN than genes upregulated by AN. Categories that had significantly more upregulated genes than downregulated genes by AN includes carbohydrate metabolism (G), transcription (K), and inorganic ion metabolism (P). In these categories G, K, and P, genes whose transcriptions were affected by RpoS were also comparatively more counted than genes in other categories, suggesting that *Salmonella* function in these categories would significantly depend on RpoS-dependent gene transcription. Because COG categories having more upregulated genes by AN also have significantly more RpoS-regulated genes than other categories, and because AN dramatically increases RpoS levels, it can be hypothesized that genes with high RpoS-dependence on their AN-inducible transcription will be responsible for the RpoS-dependent AN resistance.

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To test this, highly RpoS-dependent AN inducible genes (abbreviated hereafter as HRAI) were selected with criteria that included genes whose transcriptions were induced more than 10 fold by AN and also differently induced in WT more than 5 fold than in *rpoS* mutant. Each HRAI gene was mutated, and compared their mutation effect on the AN resistance with those of WT and *rpoS* mutant. However, each HRAI gene mutation caused little effect on AN challenge of *S.* typhimurium (Data not shown). Then multiple gene mutants were constructed, in which HRAI genes were accumulatively deficient following the order of RpoS dependence on their induction of transcription. On each occasions when multiple gene mutants constructed accumulatively and successfully, mutants were challenged to AN. Mutants that accumulated 14 gene mutations showed little difference in survival on AN challenge, comparable to WT. But after adding *dps* mutation as 15^{th}, mutants showed reduced survival rate similar to that of *rpoS* mutant (Fig. 6), demonstrating a concerted function of RpoS-dependent AN-inducible genes for S. Typhimurium resistance to AN. Of 15 HRAI genes that required for AN resistance, *ycgB, wraB, ybaY, ygaU, otsB, yeaH,* and *dps* genes were known to be regulated by RpoS, but the RpoS relation with the transcription of other HRAI genes was not known or proved yet. These results illustrates the importance of the RpoS-dependent transcription of AN-inducible genes, as well as the increase of RpoS levels by AN, in protecting *Salmonella* from RNS stress.

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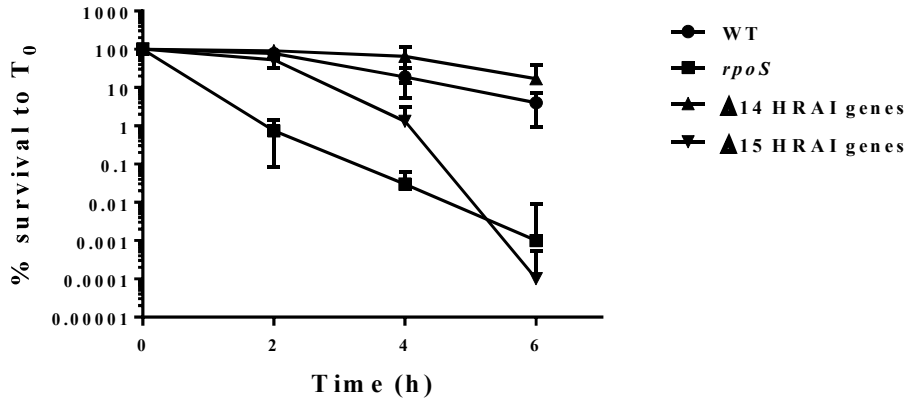
(A)



(B)

Genes	RpoS-dependence*	Function
ybaY	6.0253	glycoprotein/polysaccharide metabolism
dps	7.513	stress response DNA-binding protein; starvation induced resistance to H2O2
yliH	15.41	putative cytoplasmic protein
wraB	11.17	trp-repressor binding protein
yeaH	5.2096	putative cytoplasmic protein
STM1324	5.4587	putative cytoplasmic protein
ycgB	34.15	putative cytoplasmic protein
yecG	15.65	putative universal stress protein
otsB	5.9975	trehalose-6-phosphate phophatase, biosynthetic
ygaU	6.965	putative LysM domain
yqjI	5.0849	putative transcriptional regulator
yhcO	10.03	putative cytoplasmic protein
yhhA	12.2	putative outer membrane protein
uspA	5.3759	universal stress protein A
STM3688	14.01	putative cytoplasmic protein

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Fig. 6. Contribution of RpoS dependent genes on the *Salmonella* resistance to acidified nitrite. (A) WT and mutant *Salmonella* lacking *rpoS* or HRAI (highly RpoS-dependent acidified nitrite-inducible) genes (B) were challenged to acidified nitrite as described in legend for Fig. 1B. The *dps* mutation added to 14 HRAI gene mutants, and denoted as 15 HRAI genes. Data are shown as the mean +/- SD from three independent experiments. In (B), the RpoS-dependence (*) of HRAI genes was calculated as the ratio of the fold induction of gene transcription in WT / fold induction of gene transcription in *rpoS* mutant, by acidified nitrite.

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Table 2. AN-responsive genes in *S.* Typhimurium categorized with COG analysis

Category	Gene functions	Number of genes by AN		Number of genes differently expressed by RpoS
		up regulation	down regulation	
A	**RNA processing and modification**	1	0	0
B	**Chromatin structure and dynamics**	0	0	0
C	**Energy production and conversion**	48	70	28
D	**Cell cycle control, cell division, chromosome partitioning**	4	10	1
E	**Amino acid transport and metabolism**	85	79	20
F	**Nucleotide transport and metabolism**	16	41	0
G	**Carbohydrate transport and metabolism**	79	53	34
H	**Coenzyme transport and metabolism**	27	43	5
I	**Lipid transport and metabolism**	10	34	7

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J	**Translation, ribosomal structure and biogenesis**	35	157	2
K	**Transcription**	89	49	13
L	**Replication, recombination and repair**	29	46	0
M	**Cell wall/ membrane/ envelope biogenesis**	24	100	14
N	**Cell motility**	16	32	3
O	**Posttranslational modification, protein turnover, chaperones**	36	31	8
P	**Inorganic ion transport and metabolism**	71	32	13
Q	**Secondary metabolites biosynthesis, transport and catabolism**	16	16	4
R	**General function prediction only**	84	62	18
S	**Function unknown**	95	69	24
T	**Signal transduction mechanisms**	31	28	8
U	**Intracellular trafficking, secretion, and vesicular transport**	6	20	1

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V	**Defense mechanisms**	10	13	1
X	**Mobilome: prophages, transposons**	0	0	0
Y	**Nuclear structure**	0	0	0
Z	**Cytoskeleton**	0	0	0
Not in COG		197	234	38

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RpoS promotes *Salmonella* virulence in nitric oxide-producing mice

Experimental infection studies with several pathogens including *Salmonella* have shown the reduced virulence of *rpoS* mutant (Hengge, 2011). To test whether NO and RNS produced in mouse are involved in the RpoS-dependent virulence of *Salmonella*, C57/BL6 and its isogenic iNOS$^{-/-}$ mice were infected perorally with WT and *rpoS* mutant *S.* Typhimurium. As expected, infection with *rpoS* mutant did not change the survival of C57/BL6 mice while WT *Salmonella* killed most of mice, exhibiting the attenuated virulence of *rpoS* mutant (Fig. 7). In iNOS$^{-/-}$ mice, however, the virulence of *rpoS* mutant was largely recovered as it caused the reduced mice survival rate comparable to that infected with WT *Salmonella* (Fig. 7). Results demonstrate that RpoS is required for *Salmonella* virulence in NO-producing mice, suggesting the physiological role of the RpoS-directed transcription of AN-inducible genes on the *Salmonella* RNS resistance in animal hosts.

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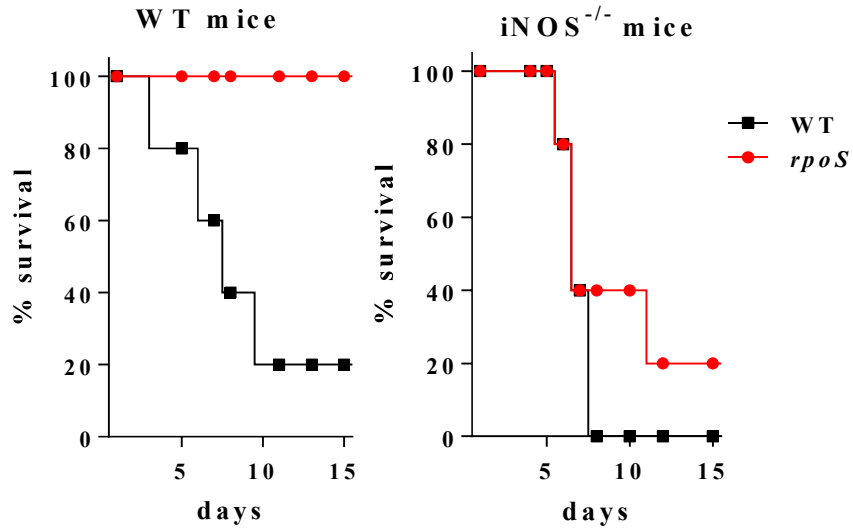

Fig. 7. *Salmonella* virulence requires RpoS in NO-producing mice. C57BL/6 and congenic *iNOS*$^{-/-}$ mice were infected perorally with 10^7 cfu of WT or *rpoS* mutant *S.* Typhimurium. Data shown are from independently duplicated experiments of 10 mice each.

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DISCUSSION

The importance of gaseous NO radical as an antimicrobial agent has been well accepted in many studies about innate immunity of animals expressing iNOS that produces cytotoxic levels of NO against bacterial infections. However, due to complicated chemistry and its high reactivity to biomolecules inside the cell, how diverse in NO-mediated RNS and how potent in their respective and potential antimicrobial activities are remained less understood. In this study, acidified nitrite (AN) have been chosen to screen *Salmonella* genes necessary for bacterial RNS resistance. AN-mediated RNS can be synthesized in various environments of animal hosts which includes stomach, saliva in some acidic environment of oral cavity, and bacteria containing vacuoles and phagosomes in phagocytic cells, where chemical and biological reactions with nitrate and nitrite can be continuously operated under acidic pH (Lundberg et al., 2004; Vazquez-Torres and Baumler, 2016). Nitrite acidification produces HNO_2 that potentiate antimicrobial activity by decomposing to nitrogen oxides including N_2O_3, N_2O_4, and NO, which are involved in successive nitrosylation reactions inactivating various biomolecules.

As the general stress sigma factor, RpoS has also been required for the acid tolerance response (ATR) of *Salmonella* in that a transient acid adaptation at mild acidic pH (pH4.4 ~ pH5.8) induces *de novo* synthesis of acid shock proteins including RpoS itself, resulting in increase of *Salmonella* survival rate at strong acid (~ pH 3) for several hours (Foster, 1999; Lee et al., 1995). And RpoS has been shown to contribute *Salmonella* virulence in mice when infected perorally, implicating the importance of RpoS-mediated acid tolerance response (Wilmes-Riesenberg et al., 1997). This study have shown the restoration of virulence of attenuated *rpoS* mutant in $iNOS^{-/-}$ mice

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infected perorally (Fig. 7), suggesting that the NO-mediated RNS generated under acidic pH in mice would potentiate the role of RpoS regulon in *Salmonella* pathogenesis.

As in the cases of ATR or AN resistance found in this study, increasing RpoS levels are helpful for many bacteria to induce RpoS-dependent gene transcription, thereby they resist to harsh environments (Fig. 2) (Gottesman, 2019). Among various levels and factors for regulating RpoS, this study have found that RNS can nitrosylate RssB in a direct manner, inhibiting RssB from RpoS binding without any aid of anti-adaptor proteins IraD, IraM, or IraP, which saves RpoS from proteolysis by ClpX and ClpP protease complex (Fig. 3). And importance of Cys-57 and Cys-171 residues of RssB in the RssB-mediated RpoS proteolysis has been shown for the first time in this study performed under physiological conditions with base-substituted mutation in *rssB* gene on the *Salmonella* chromosome, which suggest a potential S-nitrosylation on thiol residues of these cysteines (Fig. 4). Although it has been unsuccessful for concentrating *Salmonella* RssB enough for X-ray crystallography analysis because of protein precipitation by methods used in this study, the SWISS-MODEL algorism have predicted a potential RssB structure that may be formed as dimer by a possible disulfide bond between two cysteine residues (Cys-57 and Cys-171') if two monomers are located in a antiparallel symmetry (Fig. 5). With resolved RssB structure in a closed form bound with IraD (Dorich et al., 2019), future structural studies on the open form of RssB will shed light on the importance of these Cys residues of RssB in interactions with RpoS and/or NO.

For adapting to various environmental changes, *Salmonella* synthesize and utilize 6 alternative sigma factors, as well as the housekeeping sigma RpoD, recruiting RNA polymerase complex to initiate transcriptions of specific sets of genes encoding proteins for bacterial fitness. Transcriptome analysis performed in this study for

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understanding the *Salmonella* response to RNS have revealed that almost a half of genes in *Salmonella* genome are regulated by AN, in which RpoS is related with about 15 % of them, exhibiting the important role of RpoS as the alternative sigma factor for *Salmonella* response to RNS (Table 2). Despite of unsuccessful attempts to identify any single gene mutation causing *Salmonella* susceptibility to AN, mutants lacking 15 highly RpoS-dependent AN inducible genes showed a drastic reduction in AN resistance comparable to that of *rpoS* mutant (Fig. 6). In 15 genes for this mutant, *dps* gene encoding the DNA binding protein of stationary phase cells is required for protecting *Salmonella* DNA from reactive oxygen species (ROS) including hydroxyl radicals (Calhoun and Kwon, 2011; Grant et al., 1998). Interestingly, *dps* single mutant has not shown significant reduction in AN resistance, suggesting that ROS may be hardly included in RNS generated by AN in *Salmonella*, in accordance with finding of stable AN resistance of mutant *S.* Typhimurium lacking catalases and superoxide mutase (Fig. 2).

In conclusion, this study has identified RpoS sigma factor as the principal regulator of gene transcriptions for *Salmonella* survival under RNS stress conditions, in which RNS undermine the RssB-mediated RpoS proteolysis, resulting in the increase of RpoS expression that can trigger transcription of RpoS-dependent genes for *Salmonella* fitness against RNS. Overall, this study has expanded the role of RpoS to bacterial RNS stress response, highlighting RpoS as the genuine general stress sigma factor. Being as important as basic bacteriology with respect to stress response, the finding of RpoS-dependent *Salmonella* resistance to acidified nitrite will also be practically helpful to develop strategies for controlling the *Salmonella*-involved food poisoning from livestock products that are widely treated with acidified nitrite for

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preservation. Inhibiting RpoS activity in *Salmonella* will be useful means in preventing *Salmonella* infection by a variety of routes.

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국문초록

살모넬라 전사 조절자 NsrR 과 RpoS 의 산화질소 인식 기전

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환경에서 질소의 생태주기는 항균성 라디칼 산화 질소 (NO)의 생성을 포함한다. NO는 생명 시스템을 포함한 생태계에서 주로 산소분자와 화학 및 생물학적 반응에 의해 반응성 질소 종 (RNS)의 생성을 추가로 매개 할 수 있다. 이는 핵산, 지질, 단백질 및 저 분자량 티올 화합물을 비롯한 다양한 박테리아 거대 분자를 불활성화시켜 궁극적으로 비정상적인 대사 및 정균작용을 유발한다. 동물숙주에서 식세포의 inducible NO synthases (iNOS)는 미생물 감염에 반응하여 세포 독성 수준의 NO 를 생성한다. 따라서 감염성 박테리아는 성공적인 발병을 위해 NO 와 활성질소종에 적응하고 저항해야 한다. 질산화적 스트레스에 대항하여 많은 박테리아는 NO 를 약한 독성 분자로 대사하는 플라보헤모글로빈 Hmp 를 암호화하는 hmp 유전자를 보존 한다. 그리고 여러 병원체의 독성을 위해 Hmp 발현이 요구되는 것으로 입증되었다. 음성 전사 조절 인자 NsrR 은 많은 Hmp- 발현 박테리아에서 보존되어왔다. Free iron 이 증가하는 상황에서 NsrR 에 의한 hmp 의 전사 조절은 박테리아에게 매우 중요하다. NO 가 없을 때 hmp 과발현은 박테리아에서 펜톤-매개 산화 손상을 강화시킬 수 있기 때문이다. 그러나, 장내 세균에서 hmp 전사에 대한 NsrR-의존적 조절은 분자적으로 잘 알려지지 않았다. 또한, Hmp 발현은 감염 단계 (급성 vs 지속성)에 따라 박테리아 독성에 차등 적으로 기여하는 것으로 보고

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되었으며, 이는 동물 세포에서 NO- 매개 반응에 의해 생성 된 활성질소종이 플라보헤모글로빈의 단독 기능에 의해 거의 해독 될 수 없음을 시사한다. 박테리아가 RNS 해독을위한 추가 유전자 조절 시스템을 작동시키는 지에 대한 의문이 제기되어왔다. 이 논문에서는 stationary-phase sigma factor RpoS 를 활성질소종에 대한 박테리아 반응에서 유전자 전사에 대한 잠재적 인 조절자로 예상하였다. Salmonella enterica serovar Typhimurium (S. Typhimurium)을 모델 균주로 사용하여, 본 연구는 NO 를 감지하고 표적 유전자의 전사를 조절하는 NsrR 및 RpoS 의 분자 메커니즘을 이해하는 것을 목표로 하였다.

첫 번째 장은 hmp 전사 조절에있어서 NsrR 의 분자 특성을 제공하였다. S. Typhimurium 에서 NsrR 의 Val-43 잔기는 NO 에 노출 된 S. Typhimurium 에서 NO 소비 속도, 성장속도 및 hmp 의 전사를 조절하는 hmp 프로모터와 상호작용하기 위한 NsrR 의 중요한 아미노산 잔기로 확인되었다. 또한 다른 문(phyla)에서 박테리아로부터 NsrR 의 DNA 인식나선의 α3 motifs 의 heterogeneous 아미노산 잔기는 다양한 NO 조건에서 박테리아 ecological niches 와 관련될 수 있음을 제안하였다.

두 번째 장에서, stationary-phase sigma factor RpoS 의 역할 및 살모넬라 RNS 반응에서 RpoS 와 그 어댑터 단백질 RssB 사이의 상호 작용을 연구하였다. 활성질소종에 의한 RpoS 의 증가 및 RpoS-의존적 활성질소종 반응 유전자의 전사는 활성질소종을 생성하는 산성화 된 아질산염 함유 배지에서 살모넬라 생존에 필요하여, NO- 의존적 방식으로 마우스에서 살모넬라 독성을 촉진시킨다. RNS 에 노출 된 S. Typhimurium 에서 RpoS 의 증가는 주로 정상적인 성장 조건 하에서 RpoS 를 ClpP 및 ClpX 프로테아제 복합체로 전달하는데 필요한 adaptor protein RssB 의 활성질소종에 의한 비활성화에 의한 것이다.

요약하면, S. Typhimurium 에서 NO 해독 및 RNS 저항에 필요한 유전자의 전사를 조절하는 2 개의 조절 단백질의 분자 특성 분석을 S. Typhimurium transcriptome, NO / RNS 생화학, 단백질 정보학 및 마우스 감염을 포함한 분석을 통해 연구를 진행하였다. 연구 결과 박테리아가 NO 를 감지하는 방법과 NO 스트레스 하에서 박테리아의 적응에 중요한 유전자를 대상으로 이 신호를 전달하는지 밝혔다.