2016년 8월

박사학위 논문

Nitric oxide-mediated repression of amino acids metabolism in *Salmonella* Typhimurium

조선대학교 대학원

치의생명공학과

박 윤 미

Nitric oxide-mediated repression of amino acids metabolism in *Salmonella* Typhimurium

산화질소에 의한 살모넬라 아미노산 대사의 억제

2016년 8월 25일

조선대학교 대학원

치의생명공학과

박 윤 미

Nitric oxide-mediated repression of amino acids metabolism in *Salmonella* Typhimurium

산화질소에 의한 살모넬라 아미노산 대사의 억제

지도교수 방 일 수

이 논문을 이학 박사학위 신청 논문으로 제출함

2016년 4월

조선대학교 대학원

치의생명공학과

박 윤 미

박윤미의 박사학위논문을 인준함

위원장 조선대학교 교 수 국 중 기 (인)

위 원 전남대학교 교 수 정 영 희 (인)

위 원 조선대학교 교 수 김 태 형 (인)

위 원 조선대학교 교 수 이 준 식 (인)

위 원 조선대학교 교 수 방 일 수 (인)

2016년 6월

조선대학교 대학원

CONTENS

List of Tables ····· x i

List of Figures ····· x ii

Chapter Ⅰ.

General abstract (in English) ····· 2

General introduction ····· 5

General introduction reference ····· 12

Chapter Ⅱ.

Nitrosative stress causes amino acid auxotrophy in *hmp* mutant *Salmonella* Typhimurium

Abstract (in Korean) ····· 18

A. Introduction ····· 19

B. Materials and Methods ····· 21

1. Bacterial strains and culture media ····· 21

2. Measurement of *Salmonella* susceptibility to nitrosative stress ····· 21

C. Results ····· 23

1. Amino acid supplementation restores growth of *hmp* mutant under nitrosative stress ····· 23

2. Cys and BCAA are required for NO resistance ····· 28

D. Discussion ····· 31

E. References ····· 34

Abstract (in English) ····· 38

Chapter Ⅲ.

Branched-chain amino acid supplementation promotes aerobic growth of *Salmonella* Typhimurium under nitrosative stress conditions

Abstract (in Korean) ····· 40

A. Introduction ····· 42

B. Materials and Methods ····· 47

1. Bacterial strains and culture media ····· 47
2. Measurement of *Salmonella* susceptibility to nitrosative stress ····· 50
3. Measurement of the *Salmonella* NO consumption rate ····· 51
4. Measurement of intramacrophage survival of *Salmonella* strains ····· 51
5. Measurement of nitrite concentration ····· 52
C. Results ····· 54
1. BCAA supplementation recovers aerobic growth of *hmp* mutant S. Typhimurium in NO-producing cultures ····· 54
2. BCAA supplementation does not change the NO-metabolizing activity of *S.* Typhimurium ····· 61
3. *ilvD* mutant *S.* Typhimurium in RAW 264.7 macrophages is auxotrophic for BCAA ····· 63
4. BCAA supplementation cause little effect on the replication of *hmp* mutant *S.* Typhimurium in NO-producing RAW 264.7 macrophages ····· 67

D. Discussion ····· 70

E. References ····· 76

Abstract (in English) ····· 83

Chapter Ⅳ.

Nitric oxide represses the transcription of the glutamine synthetase gene *glnA*, which causes glutamine auxotrophy in *Salmonella* Typhimurium

Abstract (in Korean) ····· 86

A. Introduction ····· 88

B. Materials and Methods ····· 90

1. Bacteria strains and growth conditions and chemicals····· 90

2. Construction of the *S.* Typhimurium ····· 94

3. Disk diffusion assay ····· 94

4. Measurement of NO consumption rate ····· 95

5. Measurement of Growth Kinetics ····· 95

6. quantification of glutamine associated genes transcription by real-time reverse transcription (RT-PCR) 96

7. beta-galactosidase assay (β-gal assay)..... 97

C. Results 98

1. Supplementation with Gln or Glu recovers the growth of *hmp* mutant *S.* Typhimurium in NO-producing cultures 98

2. Conversion of Glu to Gln is required for *Salmonella* growth in NO-producing cultures 104

3. Gln supplementation does not change the NO consumption rate of *S.* Typhimurium 111

4. *glnA* transcription is repressed under nitrosative conditions in an NRI/NRII-dependent manner 113

D. Discussion 117

E. References 123

Abstract (in English) 128

Chapter Ⅳ.

General conclusion····· 131

General abstract (in Korean) ····· 134

List of Tables

Chapter Ⅲ.

Table 1. Bacterial strains, plasmids, and DNA oligomers used in this study ····· 49

Chapter Ⅳ.

Table 1. Bacterial strains, plasmids used in this study ··· 91

Table 2. Primer sequences and used in this study····· 92

Table 3. Glutamine associated gene expression of other bacteria in RNS····· 120

List of Figures

Chapter Ⅱ.

Figure 1. Comparison of *S.* Typhimurium growth in minimal and complex media under nitrosative stress condition ····· 26

Figure 2. Casamino acid or amino acid pool restores growth of *hmp* mutant against nitrosative stress····· 27

Figure 3. Cys and BCAA promotes RNS resistance of *hmp* mutant *Salmonella* ····· 30

Chapter Ⅲ.

Figure 1. Effect of BCAA supplementation on the aerobic growth of *S.* Typhimurium ····· 58

Figure 2. Effect of BCAA supplementation on the semi-aerobic and anaerobic growth of *S.* Typhimurium ····· 60

Figure 3. Comparison of the NO consumption rate of *S.* Typhimurium in the presence or absence of BCAA

supplementation ····· 62

Figure 4. Effect of BCAA supplementation on the intra-macrophage survival of *S.* Typhimurium ····· 66

Figure 5. Effect of BCAA supplementation on the intramacrophage survival of *hmp* mutant *S.* Typhimurium····· 69

Chapter Ⅳ.

Figure 1. The effect of casamino acid (CAA) supplementation on *S.* Typhimurium resistance against NO ····· 101

Figure 2. The effect of amino acid supplementation on the growth of *S.* Typhimurium under nitrosative stress ····· 103

Figure 3. Conversion of Glu to Gln is required for the growth of *S.* Typhimurium under nitrosative stress ····· 109

Figure 4. L-Gln but not D-Gln is required for the growth of *S.* Typhimurium under nitrosative stress ····· 110

Figure 5. Comparison of the NO consumption rate of *S.*

Typhimurium in the presence or absence of Gln supplementation ····· 112

Figure 6. The effect of NO on the expression of *glnA*, *gltB*, and *gltD* ····· 116

Chapter Ⅴ.

Figure 1. The NO-mediated modification of metabolism in *S.* Typhimurium ····· 133

Chapter Ⅰ.

General abstract

Nitric Oxide (NO) is generated from phagocytic cells of host animals as an antimicrobial factor for invading pathogens. Thus, the proliferation of bacteria is controlled by NO and NO-derived reactive nitrogen species (RNS). Although many bacteria express NO-detoxifying enzymes, highly reactive NO still can inactivate a variety of bacterial macromolecules, causing abnormal bacterial metabolism and subsequent growth arrest. However, molecular details of bacterial metabolic alterations caused by NO are remained poorly understood. In this study, to determine the NO-caused changes of amino acids metabolism in an enteric pathogen *Salmonella enterica* serovar Typhimurium, I have employed a *hmp* mutant *S.* Typhimurium lacking the principal NO-metabolizing enzyme flavohemoglobin Hmp that experience severe nitrosative stress when exposed to NO. I have found that NO can cause amino acids auxotrophy in *S.* Typhimurium and biosynthetic reactions for branched-chain amino acids (BCAA; Leucine, Isoleucine, and Valine) and glutamine are disturbed by NO, but exogenous supplementation with corresponding amino acids can rescue auxotrophy under nitrosative stress conditions.

In NO-producing cultures, the growth of Hmp-deficient *Salmonella* is severely impaired but readily recovered by supplementing exogenous amino acid pool into the culture media, demonstrating the NO-induced amino acids auxotrophy in *S.* Typhimurium. A drop-out analysis excluding each

single amino acid from amino acid pool exhibits that BCAA are required for full recovery of bacterial growth under nitrosative conditions. Dihydroxyacid dehydratase (IlvD) and isopropylmalate isomerase complex (LeuCD) are essential enzymes for BCAA biosynthesis and both enzymes contain NO-targetable Fe-S clusters, so that their inactivation has been implicated as a cause of NO-mediated BCAA auxotrophy. Supplementation with BCAA recovers the growth of *hmp* mutant and mutants further lacking *ilvD* and *leuCD* in NO-producing culture in an oxygen-dependent manner. BCAA supplementation also recovers the replication of *ilvD* and *leuCD* mutants at wild-type levels inside RAW 264.7 macrophages that produces constant amount of NO regardless of varied supplemental BCAA concentrations.

In testing the effects of single amino acid supplementation on the NO-caused amino acid auxotrophy, I have found glutamine (Gln) as a major amino acid rescuing auxotrophy. Supplementation with Gln or glutamate (Glu), which is enzymatically interconvertible, can fully recover the growth of *hmp* mutant *S.* Typhimurium in NO-producing cultures. But, only Gln supplementation recovers the growth of mutants lacking genes for the biosynthesis and interconversion of Gln and Glu, demonstrating the dependence of Gln for Salmonella resistance against NO. In response to NO exposure, the transcription of *glnA* encoding glutamine synthetase is repressed in a way depending on the NRI/NRII two component response

regulators, suggesting that NO can disturb bacterial signal transduction system sensing nitrogen availability.

Supplementation with BCAA or Gln causes no effect on NO metabolic activity of *Salmonella*, suggesting the BCAA- or Gln-promoted NO resistance independence of NO metabolism itself.

Results in this study suggest that NO may damage reactions for maintaining levels of BCAA and Gln in Salmonella, resulting in auxotrophy for these amino acids, but which can be overcome by bacterial taking up them from surroundings.

General introduction

Salmonella are gram-negative bacteria of the enterobacteriaceae family. They are rod-shaped (bacilli), non-spore-forming, peritrichous flagellated motile enteric pathogens and include over 2,500 serovars. The cell diameters of *Salmonella* are 0.7 and 1.5 μm, while the lengths range from 2 to 5 μm. The genetic structure of *salmonella* is similar to the well-known genetic structure of *Escherichia coli* [1]. The genome of *Salmonella* is known to contain more than 4,000 genes and a sequence of 4,809,037 bp [1]. As chemotrophs, these bacteria obtain energy from the oxidation and reactive reduction of organic nutrient sources. *Salmonella* are facultative anaerobes that can survive with or without oxygen.

Salmonella are common gastrointestinal pathogens that infect animal and human hosts through contamination of food or water or by directly colonizing hosts [2, 3]. Infection can lead to typhoid fever and severe systemic illness [1]. Typhoid fever caused by *Salmonella enterica* serovar Typhi afflicts more than 20 million individuals each year, and 200,000 of the human deaths caused by *salmonella* infection each year are in developing countries [3-5]. However, there are no effective vaccines against typhoid fever in children [4]. In contrast, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) causes simple gastroenteritis, food poisoning (salmonellosis), and a process associated with intestinal inflammation and diarrhea in humans, but these *salmonella* can cause

deadly typhoid fever and systemic illness in mice [3]. Typhoid fever states in humans and mice are similar; thus, mouse models infected with *S.* Typhimurium are used for research into human typhoid infections [3].

After orally infected, *Salmonella* arrives at the gastrointestinal tract, which provides a primary cellular barrier [3]. *Salmonella* invade the host by using the major pathway of transcytosis through M cells as a major invasion route [2, 3] or through uptake by non-phagocytic enterocytes that induce the bacterial effector protein *Salmonella* pathogenicity Island 1 (SPI-1) in the virulence-associated type 3 secretion system (T3SS) [3]. In addition, *salmonella* may be uptaken by $CX3CR1^{+}$ macrophages/dendritic cells [3]. After subsequent phagocytosis by macrophages, *Salmonella* form the *salmonella*-containing vacuole (SCV), a membrane-bound compartment [6]. Therein, *Salmonella* induce second T3SS encoded by *Salmonella* pathogenicity Island 2 (SPI-2) for avoidance of antimicrobial activities and modification of cellular trafficking, thereby allowing for intracellular survival and replication within the SCV [3, 7]. These organisms disseminate to the mesenteric lymph nodes, liver, and spleen and stimulate the formation of microabscesses and persistent infection [2, 3]. Phagocytes provide limited nutrients, and antimicrobial radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) inhibit replication and also kill ingested organisms [2].

Bacterial pathogens such as *Salmonella enterica* serovar Typhimurium can be restricted by NO produced in host cells as a component of innate immune response [8]. ROS include superoxide anion (O_2^-), which in turn can be converted to hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) [8]. The O_2^- enters the periplasmic space of *Salmonella* through porins [9]. RNS include the lipophilic nitric oxide (NO) that can be converted to nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and nitrosothols (RSNO) [8, 10]. Moreover, as a strong nitrogen and oxidizing antimicrobial radical, peroxynitrite ($ONOO^-$) generated from NO and O_2^- further damages macromolecules including lipid, protein, and nucleic acids [11]. Inside macrophages, ROS cause an explosive oxidative chemistry that correlates with an early bacterial killing reaction [2]. however, ROS can be removed by bacterial enzymes such as superoxide dismutases (SODs), catalases, and peroxidases [8, 11, 13]. NO can pass through the diffuse bacteria membrane to interact with NO targets, including various multiple bacterial macromolecules such as lipids, nucleic acids, DNA, DNA-associated proteins, metal prosthetic groups of key molecular targets of the electron transport chain, iron-sulfur (Fe-S) clusters, and transcription factors causing abnormal metabolism [9, 14, 15]. *Salmonella* have evolved enzymes that metabolize RNS, including NO: flavohemoglobin Hmp, flavorubredoxin NorV, and periplasm cytochrome c nitrite reductase NrfA [8, 10]. Flavohemoglobin Hmp detoxifies almost all NO by catalyzing

the reaction of both oxygen and NO to produce nitrate (NO_3^-)under aerobic environments, and of the reduction of NO to N_2O under anaerobic environments [16, 17], NorV can reduce NO to nitrous oxide (N_2O) [16]; and NrfA reduces NO_2^- to ammonia (NH_3) under anaerobic environments [18]. In addition, $ONOO^-$ are reduced to NO_2^- by alkyl hydroperoxidase (AhpC) [9].

Although the roles of enzymes for detoxification of ROS and NO/RNS in bacterial survival and replication have been known well, there are considerable controversies in their role in virulence models using animal hosts. Especially, NO-related persistent infection of *Salmonella* is expected to be quite different from the ROS-mediated early period of infection, in terms of interactions between bacteria and host cells. During comparatively long period of infection, host-produced NO can damages bacterial metabolism to cause deficiency in nutrient required for replication, but bacteria may compensate it by taking up nutrient from host cells. However, the molecular mechanisms for this interaction remains largely unexplored. In this study, we have found that, without detoxification by flavohemoglobin, NO and RNS can cause amino acid auxotrophy in *S.* Typhimurium that can be recovered by supplying exogenous amino acids. Moreover, I have found that the biosynthesis pathways of branched amino acids and glutamine are NO targets.

In animals including humans, branched-chain amino acids (BCAA: isoleucine [Ile], leucine [Leu], and valine [Val]) are essential amino

acids because BCAAs cannot synthesize themselves. However, most bacteria can synthesize BCAAs that have aliphatic side-chains as branches, using oxaloacetate and pyruvate [24]. The BCAA biosynthesis pathway involves two essential iron-sulfur clusters containing the enzymes dihydroxyacid dehydratase (IlvD) and isopropylmalate isomerase (LeuCD) [22]. The IlvD enzyme is needed for the biosynthesis of each BCAA, but the leuCD enzyme is needed for the synthesis of only Leu. The iron-sulfur clusters exist in 3 types of form: 2Fe-2S, 3Fe-4S, and 4Fe-4S [25]. The IlvD is involved in 4Fe-4S clusters of enzymes that may be targets of NO cytotoxicity [22, 25, 26]. The auxotrophy of the BCAA may be caused by NO treatment of *E. coli* under anaerobic conditions [22] and aerobic conditions [26]. NO forms dinitrosyl iron complex (DNIC) by reacting with ferrous iron of iron-sulfur clusters in IlvD in *E. coli*, and this causes auxotrophy in BCAA because of the lack of BCAA synthesis [25, 26]. Therefore, NO-exposed cell growth is restored through supplementation with the BCAA in *E. coli* [22].

Testing the effects of single amino acid supplementation on the NO-caused amino acid auxotrophy revealed glutamine (Gln) and glutamate (Glu) as major amino acids required for preventing amino acid auxotrophy. Gln and Glu serve as the main nitrogen metabolic sources for bacterial replication, and their biosynthesis and uptake are precisely regulated in bacterial response to availability and demand [27]. Recent research shows

that combinations of mutations in control of the nitrogen regulatory (*ntr*) system and high-affinity glutamine transporter operon (encoded by *glnQPH*) under mutation in glutamine biosynthesis (glutamine synthetase; GS, encoded by *glnA*) attenuate *S.* typhimurium virulence in mice [28]. The glutamine of cells also affects transcription of GS [29]. Various metabolic intermediates and assimilation of ammonia are sources, and many enzymes are required. During limited Nitrogen source, GS is used in the synthesis of glutamine, which leads to growth and replication [30, 31]. In *Salmonella*, there are three main paths for nitrogen metabolism: GS, glutamate synthase (GOGAT, encoded by *gltBD*), and glutamate dehydrogenase (GDH). The GS catalyze the glutamate to glutamine by using ATP, and the GOGAT catalyze the glutamine and 2-oxoglutarate to glutamate under high-affinity ammonium. The GDH, which catalyzes the 2-oxoglutarate to glutamate, is a low-affinity ammonium pathway enzyme [27, 32, 33]. For the adenylylation state of GS, three types of separable proteins are required [29, 34]. The first protein, PI via bifunctional adenylyltransferase enzyme; ATase is used to activate GS by catalyzing the removal of the adenylyl group [29, 34]. The second protein, GlnB-K-type PII, called an allosteric protein or regulatory protein for nitrogen assimilation (encoded by *glnB* and *glnK*) regulates GS activation or inactivation by two forms that exist as either uridylylated or deuridylylated, respectively [29, 35]. The ATase is regulated by the PII

protein [36]. GlnB directly stimulates NtrB (NR II; sensor histidine kinase) and NtrC (NR I; phosphorylated response regulator), two-component signal transduction systems in *E. coli* (provided to *glnL* and *glnG* in *Salmonella*, respectively) [29, 37]. In nitrogen deficiency, the phosphorylation state of NR I stimulates the expression of *glnA* [38]. Lastly, the nitrogen excess or deficiency status of bacteria is sensed by uridylyltransferase/uridylyl-removing enzyme (Utase; UT/UR) (encoded by *glnD*) [35].

In this study, using the genetics known for the biosynthesis for BCAA and Gln, I have tried to better understand the role of amino acid metabolism in *Salmonella* resistance to nitric oxide.

General introduction reference

1. Dandekar, T., et al., *Salmonella-how a metabolic generalist adopts an intracellular lifestyle during infection.* Front Cell Infect Microbiol, 2014. **4**: p. 191.
2. Vazquez-Torres, A. and F.C. Fang, *Oxygen-dependent anti-Salmonella activity of macrophages.* Trends Microbiol, 2001. **9**(1): p. 29-33.
3. Broz, P., M.B. Ohlson, and D.M. Monack, *Innate immune response to Salmonella typhimurium, a model enteric pathogen.* Gut Microbes, 2012. **3**(2): p. 62-70.
4. Song, J., et al., *A mouse model for the human pathogen Salmonella typhi.* Cell Host Microbe, 2010. **8**(4): p. 369-76.
5. Mathur, R., et al., *A mouse model of Salmonella typhi infection.* Cell, 2012. **151**(3): p. 590-602.
6. Popp, J., et al., *Role of host cell-derived amino acids in nutrition of intracellular Salmonella enterica.* Infect Immun, 2015. **83**(12): p. 4466-75.
7. Hensel, M., *Salmonella pathogenicity island 2.* Mol Microbiol, 2000. **36**(5): p. 1015-23.
8. Bang, I.S., et al., *Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp.* J Biol Chem, 2006. **281**(38): p. 28039-47.
9. Henard, C.A. and A. Vazquez-Torres, *Nitric oxide and salmonella*

pathogenesis. Front Microbiol, 2011. **2**: p. 84.

10. Calderon, P.F., et al., *The small RNA RyhB homologs from Salmonella typhimurium participate in the response to S-nitrosoglutathione-induced stress.* Biochem Biophys Res Commun, 2014. **450**(1): p. 641-5.
11. McLean, S., L.A. Bowman, and R.K. Poole, *Peroxynitrite stress is exacerbated by flavohaemoglobin-derived oxidative stress in Salmonella Typhimurium and is relieved by nitric oxide.* Microbiology, 2010. **156**(Pt 12): p. 3556-65.
12. Burton, N.A., et al., *Disparate impact of oxidative host defenses determines the fate of Salmonella during systemic infection in mice.* Cell Host Microbe, 2014. **15**(1): p. 72-83.
13. van der Heijden, J., et al., *Direct measurement of oxidative and nitrosative stress dynamics in Salmonella inside macrophages.* Proc Natl Acad Sci U S A, 2015. **112**(2): p. 560-5.
14. Fang, F.C., *Antimicrobial reactive oxygen and nitrogen species: concepts and controversies.* Nat Rev Microbiol, 2004. **2**(10): p. 820-32.
15. Husain, M., et al., *Redox sensor SsrB Cys203 enhances Salmonella fitness against nitric oxide generated in the host immune response to oral infection.* Proc Natl Acad Sci U S A, 2010. **107**(32): p. 14396-401.
16. Gardner, A.M., R.A. Helmick, and P.R. Gardner, *Flavorubredoxin, an*

inducible catalyst for nitric oxide reduction and detoxification in Escherichia coli. J Biol Chem, 2002. **277**(10): p. 8172-7.

17. Poole, R.K., *Nitric oxide and nitrosative stress tolerance in bacteria.* Biochem Soc Trans, 2005. **33**(Pt 1): p. 176-80.

18. Poock, S.R., et al., *Respiratory detoxification of nitric oxide by the cytochrome c nitrite reductase of Escherichia coli.* J Biol Chem, 2002. **277**(26): p. 23664-9.

19. Richardson, A.R., et al., *Multiple targets of nitric oxide in the tricarboxylic acid cycle of Salmonella enterica serovar typhimurium.* Cell Host Microbe, 2011. **10**(1): p. 33-43.

20. Alvarez, B. and R. Radi, *Peroxynitrite reactivity with amino acids and proteins.* Amino Acids, 2003. **25**(3-4): p. 295-311.

21. Ford, E., M.N. Hughes, and P. Wardman, *Kinetics of the reactions of nitrogen dioxide with glutathione, cysteine, and uric acid at physiological pH.* Free Radic Biol Med, 2002. **32**(12): p. 1314-23.

22. Ren, B., et al., *Nitric oxide-induced bacteriostasis and modification of iron-sulphur proteins in Escherichia coli.* Mol Microbiol, 2008. **70**(4): p. 953-64.

23. Rogers, P.A. and H. Ding, *L-cysteine-mediated destabilization of dinitrosyl iron complexes in proteins.* J Biol Chem, 2001. **276**(33): p. 30980-6.

24. Umbarger, H.E., *biosynthesis of the branched-chain amino acids In :*

Escherichia coli and Salmonella typhimurium: cellular and molecular biology. American Society for Microbiology Press, Wachington DC*Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 1996.

25. Duan, X., et al., *Reactivity of nitric oxide with the [4Fe-4S] cluster of dihydroxyacid dehydratase from Escherichia coli.* Biochem J, 2009. **417**(3): p. 783-9.

26. Hyduke, D.R., et al., *Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in Escherichia coli.* Proc Natl Acad Sci U S A, 2007. **104**(20): p. 8484-9.

27. Yan, D., *Protection of the glutamate pool concentration in enteric bacteria.* Proc Natl Acad Sci U S A, 2007. **104**(22): p. 9475-80.

28. Klose, K.E. and J.J. Mekalanos, *Simultaneous prevention of glutamine synthesis and high-affinity transport attenuates Salmonella typhimurium virulence.* Infect Immun, 1997. **65**(2): p. 587-96.

29. Leigh, J.A. and J.A. Dodsworth, *Nitrogen regulation in bacteria and archaea.* Annu Rev Microbiol, 2007. **61**: p. 349-77.

30. Reitzer, L., *Nitrogen assimilation and global regulation in Escherichia coli.* Annu Rev Microbiol, 2003. **57**: p. 155-76.

31. Brown, D.R., et al., *Nitrogen stress response and stringent*

response are coupled in Escherichia coli. Nat Commun, 2014. **5**: p. 4115.

32. Harper, C.J., et al., *Glutamate dehydrogenase and glutamine synthetase are regulated in response to nitrogen availability in Myocbacterium smegmatis.* BMC Microbiol, 2010. **10**: p. 138.

33. Vo, J., et al., *Mechanism for nitrogen isotope fractionation during ammonium assimilation by Escherichia coli K12.* Proc Natl Acad Sci U S A, 2013. **110**(21): p. 8696-701.

34. Stadtman, E.R., *The story of glutamine synthetase regulation.* J Biol Chem, 2001. **276**(48): p. 44357-64.

35. Ikeda, T.P., A.E. Shauger, and S. Kustu, *Salmonella typhimurium apparently perceives external nitrogen limitation as internal glutamine limitation.* J Mol Biol, 1996. **259**(4): p. 589-607.

36. Nolden, L., et al., *Sensing nitrogen limitation in Corynebacterium glutamicum: the role of glnK and glnD.* Mol Microbiol, 2001. **42**(5): p. 1281-95.

37. Hanau, R., et al., *Nucleotide sequence of the control regions for the glnA and glnL genes of Salmonella typhimurium.* J Bacteriol, 1983. **155**(1): p. 82-9.

38. van Heeswijk, W.C., et al., *An alternative PII protein in the regulation of glutamine synthetase in Escherichia coli.* Mol Microbiol, 1996. **21**(1): p. 133-46.

Chapter Ⅱ.

Nitrosative stress causes amino acid auxotrophy in *hmp* mutant *Salmonella* Typhimurium

국문초록

살모넬라 균의 *hmp* 돌연변이 균주에서 산화 스트레스에 의한 아미노산 영양요구성

박 윤 미

조선대학교 대학원

치의생명공학과

지도교수 : 방 일 수

세포독성의 산화질소는 다양한 박테리아의 분자에 손상을 주나 그 기작에 대해서는 아직 크게 알려지지 않았다. 우리는 살모넬라균에서 산화질소를 대사하는 효소인 flavohemoglobin Hmp를 결핍시킨 균에서 산화질소에 의해 아미노산 영양요구성이 나타나는 것을 보여주었다. 산화질소가 노출된 배양에서 아미노산 풀의 첨가는 Hmp 결핍 살모넬라를 정상 성장하게 하였으나 Cys 또는 BCAA인 Leu, Ile 또는 Val을 제외시킨 아미노산 풀에서는 성장회복이 감소하였다. 이 데이터에서 해독을 하지 못하는 경우의 산화질소는 아미노산을 제한한 숙주 환경에서 살모넬라의 분열에 필수인 아미노산의 생합성 경로에 중요한 효소를 억제시킬지 모른다.

A. Introduction

Virtually all animals produce nitric oxide (NO) and use it as a messenger molecule for intercellular signaling or a potent antimicrobial factor for inhibiting replication of pathogenic bacteria [1, 2]. For antimicrobial action, NO production of host animals largely depends on the inducible nitric oxide synthase (iNOS) of phagocytic cells generating high concentrations of NO [3]. As a lipophilic free radical, NO can freely diffuse across bacterial membrane and can be transformed chemically or biologically to NO-derived reactive nitrogen species (RNS) inside bacteria [4]. RNS are believed to confer cytotoxic activities to various bacterial targets including membrane lipids, nucleotide bases, thiols, metal centers, and protein tyrosines, causing abnormal bacterial metabolism and concomitant bacteriostasis [1]. However, mechanisms of metabolic alterations induced by NO and RNS have not been fully understood. Bacterial pathogens have successfully evolved to resist against nitrosative stresses produced by host animals. Intracellular pathogen, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) possesses four enzymes to detoxify NO. The flavohemoglobin Hmp, the flavorubredoxin NorV, the GSH-dependent formaldehyde dehydrogenase AdhC, and the periplasmic cytochrome *c* nitrite reductase NrfA can metabolize or detoxify NO *in vitro* [5-8]. In a mouse infection model, flavohemoglobin Hmp among these enzymes is the most important contributor for *Salmonella*

virulence in NO-producing mice and macrophages [5]. Hmp consumes almost all NO inside bacteria, and *hmp* mutant *S.* Typhimurium shows impaired growth under nitrosative stress conditions [5]. Although prominent roles of Hmp are demonstrated in NO metabolism and *Salmonella* pathogenesis [5, 9, 10], the reason for the growth defect of *hmp* mutant under nitrosative environments is not known. Using *hmp* mutant *Salmonella*, I have explored NO-induced bacteriostasis.

B. Materials and Methods

1. Bacterial strains and culture media

Salmonella Typhimurium strains used in this study are *S.* Typhimurium 14028S and its isogenic *hmp* mutant. For bacterial culture, Luria-Bertani (LB) broth (Difco, Detroit, MI, USA) or minimal E medium [11] supplemented with 0.2 % glucose (EG medium) was used. All chemicals, including BCAA, L-Ile, L-Leu, and L-Val, were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. S-nitrosoglutathione (GSNO) was used as an NO congener. It was synthesized according to the method reported previously by using the reaction of glutathione and acidified sodium nitrite [12].

2. Measurement of *Salmonella* susceptibility to nitrosative stress

Salmonella susceptibility to nitrosative stress was measured by observing bacterial growth in NO-producing cultures at 37℃ under three different oxygen tensions. *S.* Typhimurium grown overnight in LB broth were diluted in phosphate-buffered saline (PBS) to an optical density (OD_{600nm}) of 1.0. Then, the same amount of bacterial cells was inoculated into fresh EG media under different oxygen conditions. For a bioscreen, the same amount of bacterial cells (OD_{600nm} = 0.02) were inoculated into

wells of a microtiter plate containing EG liquid media in the presence or absence of GSNO. Bacterial growth was monitored by measuring the optical density (OD_{600nm}) of bacterial cultures in a Bioscreen C Microbiology Microplate reader (Labsystems, Helsinki) at 37℃ at 30-min intervals during 24hs.

C. Results

1. Amino acid supplementation restores growth of *hmp* mutant under nitrosative stress

As reported previously [5], growth of *hmp* mutant *S.* Typhimurium is severely impaired in NO-producing minimal E media compared to that of parental WT, *S.* Typhimurium 14028S (Fig. 1a). To examine the effect of nutrients to *Salmonella* replication under nitrosative stress conditions, I compared growth of *hmp* mutant in minimal E and in complex LB media containing GSNO, respectively. GSNO was synthesized by the reaction of glutathione and acidified sodium nitrite as described by [13]. As shown in Fig. 1b, growth of *hmp* mutant in minimal media was absolutely inhibited by GSNO, whereas, in LB media, growth was largely restored even at early growth-phases and reached the plateau phases of those cultured without GSNO, suggesting that the compounds in LB may rescue growth inhibition caused by nitrosative stress. Since one of the most known nutrients lacking in minimal media compared to LB media is amino acid, I examined the effect of casamino acid to bacterial replication in GSNO-containing minimal media. Supplementation of 1% casamino acid into minimal media restored growth of *hmp* mutant comparable to that cultured in LB media (Fig. 2a). Furthermore, supplementing the amino acid pool comprised 20 amino acids that fully restored the growth of *hmp* mutants, even in high concentrations of GSNO (Fig. 2b). These results clearly

demonstrate that nitrosative stresses, if not detoxified by NO metabolizing enzymes, can cause amino acid auxotrophy in *S.* Typhimurium.

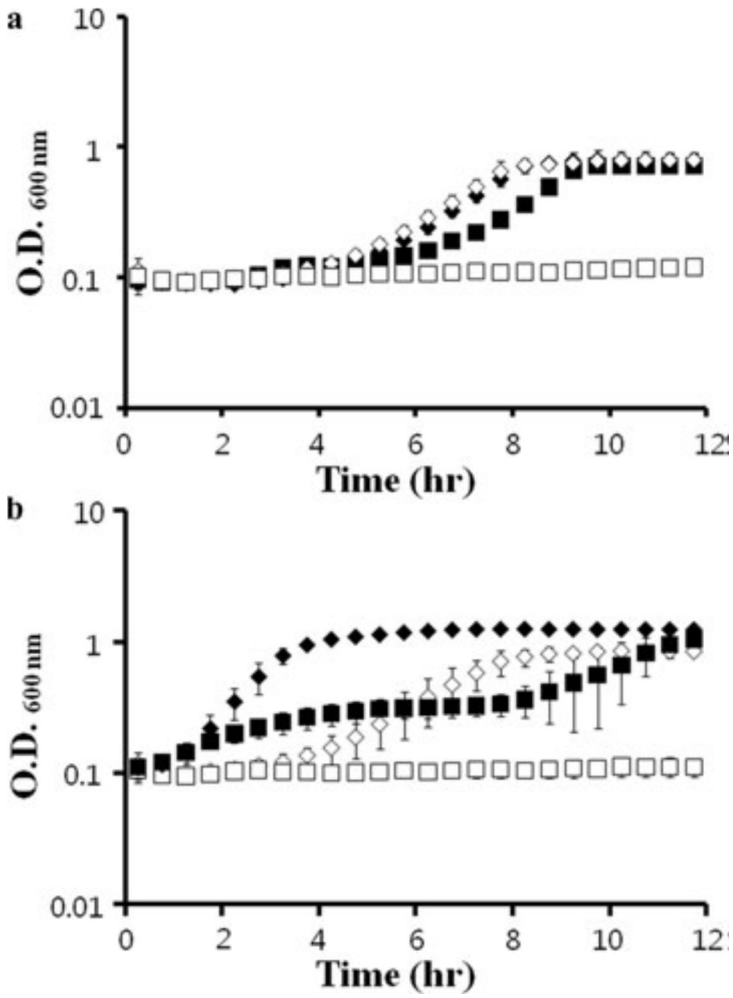


Figure 1. Comparison of *S.* Typhimurium growth in minimal and complex media under nitrosative stress conditions.

WT 14028S and its isogenic *hmp* mutant *S.* Typhimurium[5] were used throughout this study. Bacteria cultured overnight in LB media were diluted in PBS, then same amount of cell (OD_{600nm} = 0.02) were inoculated in microplates containing fresh media of minimal E or LB media. S-nitroso glutathione (GSNO; 500 μM) was added to the culture media as a NO congener. Growth kinetics were measured by determining the optical density (OD_{600nm}) at 37℃ with shaking on a Bioscreen C Microbiology Microplate reader (Growth Curves, Finland). **(a)** Growth of WT (*filled symbols*) and *hmp* mutant *S.* Typhimurium (*open symbols*) in minimal E media were compared in the presence (rectangles) and absence (*diamonds*) of GSNO. **(b)** *hmp* mutant was cultured in LB media (*filled symbols*) and minimal E media (*open symbols*) in the presence (*rectangles*) and absence (*diamonds*) of GSNO. Data shown are the means +/- SD from three independent experiments.

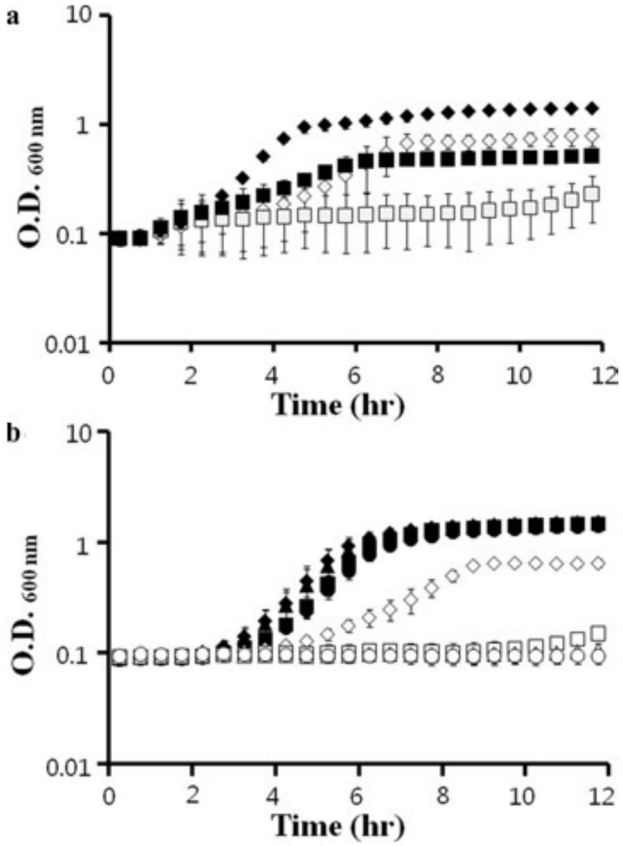


Figure 2. Casamino acid or amino acid pool restores growth of *hmp* mutant against nitrosative stress.

Overnight cultures of *hmp* mutant cells were inoculated into fresh minimal E media containing 0 μM (*diamonds*), 1 mM (*rectangles*) or 3 mM (*circles*) GSNO. And cultures were supplemented with 1% casamino acid (*filled symbols*)in (**a**) or amino acid pool (*filled symbols* in **b**). Amino acid pool contains all 20 amino acids (2 mM each). Data shown are the means +/- SD from three independent experiments.

2. Cys and BCAA are required for NO resistance

To identify amino acids responsible for growth recovery of *hmp* mutants under nitrosative stress, I performed a drop-out experiment excluding each single amino acid from the amino acid pool (Fig. 3). When compared with control cultures without GSNO, dropping out each one of most amino acids from amino acid pool in GSNO containing cultures did not make significant changes of growth. However, exclusion of Cys or BCAAs Leu, Ile, or Val delayed the growth of *hmp* mutants. During 4-6 hr time periods of mid log-phase cultures in Fig. 3, in the absence of GSNO, optical density of cultures excluding Cys, Ile, Val, or Ile increased similar to that of cultures containing all amino acid (Cys, 0.45 to 1.08; Ile, 0.17 to 0.844; Leu, 0.15 to 0.71; Val, 0.14 to 0.68; all amino acids, 0.41 to 1.27), whereas in the presence of GSNO, it remained low while cultures containing all amino acids grow exponentially (Cys, 0.14 to 0.16; Ile, 0.09 to 0.17; Leu, 0.13 to 0.39; Val, 0.10 to 0.22; all amino acid, 0.25 to 1.01). Thiol residue of cysteine is well known to react with NO, and therefore acts as an antagonist for NO both *in vitro* and *in vivo* [14], indicating that Cys played a role as a general NO scavenger in this result. Among BCAAs, Ile and Val were more required than Leu for restoration of replication of *hmp* mutant *Salmonella* in NO-producing cultures. Unexpectedly, the addition of single or all three BCAAs alone hardly recovered the growth of *hmp* mutant in GSNO-containing culture

(data not shown). This suggests that unknown factors in the synthesis of other amino acids may also be susceptible to NO in *hmp* mutant. But the lack of these amino acids seems to be compensated by unknown roles of other amino acids provided in the amino acid pool, while BCAAs appears to be hardly substitutable by other amino acids. These results suggest that key enzymes for BCAA biosynthesis are vulnerable to NO, and that BCAA is centrally important for the replication of *S.* Typhimurium under nitrosative stress conditions.

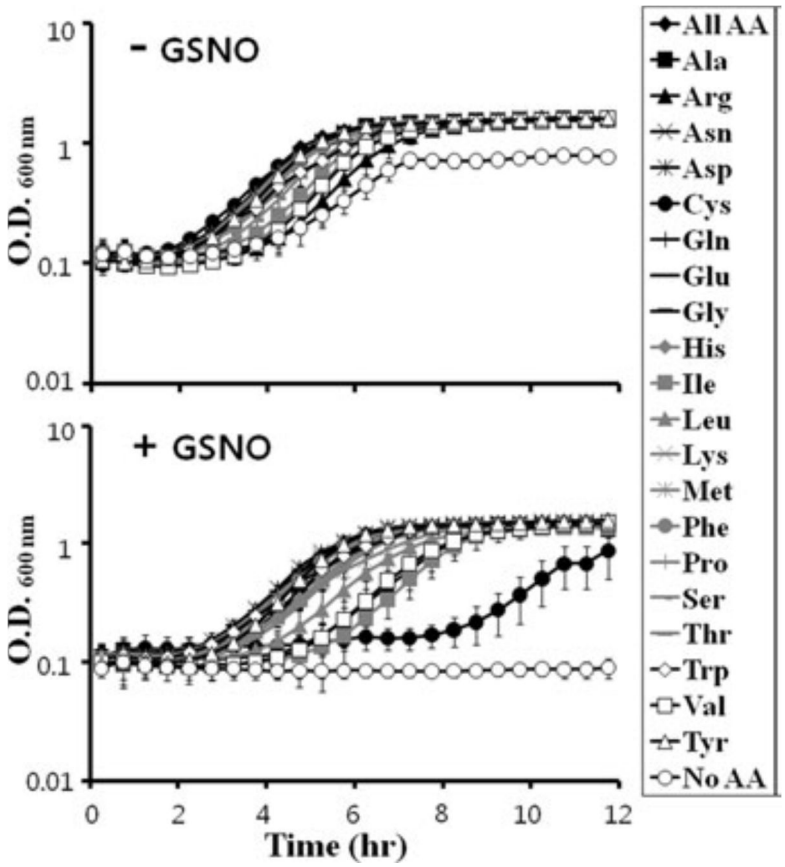


Figure 3. Cys and BCAA promotes RNS resistance of *hmp* mutant *Salmonella*.

Overnight cultures of *hmp* mutant S. Typhimurium were inoculated into fresh minimal E media containing no or 3 mM GSNO in the presence or absence of amino acid pool. To examine the role of each amino acid, amino acid pools subtracted each single amino acid (labeled) were supplemented to the media, respectively. Growth kinetics was measured by the methods as described in Fig. 1. Data shown are the means +/- SD from three independent experiments.

D. Discussion

BCAAs are essential amino acids for humans because the human body cannot synthesize them, whereas most bacteria can synthesize them from pyruvate. By a series of enzyme actions, pyruvate is catabolyzed to Leu and Val, and when combined with α-ketobutyrate, is catabolized to Ile. Among enzymes participating in BCAA synthesis, two enzymes, IlvD and LeuCD complex possess iron-sulfur clusters [15]. Considering iron-sulfur is a known RNS target, both enzymes seem to be the key enzyme explaining the reason of BCAA auxotrophy in *hmp* mutant under nitrosative conditions. IlvD is required for the synthesis of all three BCAAs, but LeuCD is needed for only Leu. As our results show dependence of all three BCAAs and a better effect of Ile and Val than Leu to the growth recovery of *hmp* mutants (Fig. 3), it seems that IlvD is the favorable target for NO. IlvD contains a [4Fe-4S] cluster essential for enzyme activity, and enzyme activity of purified IlvD from *Escherichia coli* is damaged by NO exposure [16, 17]. Since amino acid sequences of IlvD are 99% homologous between *S.* Typhimurium and *E. coli, S.* Typhimurium IlvD seems to be also susceptible to NO. There have been two recent reports about the role of BCAA related with IlvD activity in *E. coli* NO resistance *in vitro*. Ren *et al.* [17] showed that NO can irreversibly modify iron-sulfur cluster of IlvD only under anaerobic conditions, resulting in BCAA auxotrophy of *E. coli*. They also showed that switching cultures from anaerobic to aerobic conditions

can recover IlvD activity and dispense with BCAA in NO resistance. In contrast, Hyduke *et al.* [18] showed that NO can cause the BCAA auxotrophy in the presence of oxygen. Conflicting results of both studies in oxygen availability might result from different NO levels remaining inside bacterial cells between two studies. Since both studies used wild type *E. coli* expressing functional flavohemoglobin Hmp, BCAA auxotrophic phenotypes must be variable depending on both NO sources and induction conditions for Hmp expression that determine the intracellular NO level enough to modify the iron-sulfur cluster of IlvD. However, our data using *hmp* mutant *S.* Typhimurium performed in aerobic conditions imply the damage of IlvD by NO in the presence of oxygen. Unlike *E. coli*, many serovars of *S. enterica* including Typhimurium can cause systemic infection in animal hosts. After invasion to intestinal epithelial cells, *Salmonella* are engulfed by macrophages where virulent ones can survive and resist against a variety of antimicrobial factors including reactive oxygen species, antimicrobial peptides, as well as NO [1]. As a bacteriostatic agent, NO inhibits bacterial replication inside phagocytes [19]. Activated iNOS by bacterial LPS-induced IFN-γ continuously produces NO, thereby controlling *Salmonella* persistence while it travels in the animal body following phagocytosis [20]. In fact, *hmp* mutant *S.* Typhimurium lacking NO-metabolizing activity hardly persists during chronic infection in mouse [5]. In this study, I show that amino acids

can rescue NO-causing growth inhibition of *hmp* mutant, indicating that one of metabolic alterations of *Salmonella* caused by NO is amino acid auxotrophy. Thus, it opens up the possibility that amino acids available in an animal host can be used for reviving *Salmonella* persistence under nitrosative stress conditions. However, amino acids including BCAA in animal hosts may be insufficient for recovering amino acid auxotrophy caused by NO. Significant numbers of studies show that amino acid auxotrophs of many enteric pathogens are attenuated in experimental animal infection models. Aromatic amino acid auxotrophs of *S.* Typhi and *Neisseria gonorrhoeae* are a virulent [21-24]. And notably, various BCAA auxotrophic mutants of *Mycobacterium tuberculosis, Burkholderia pseudomallei,* and *S.* Typhimurium are also avirulent [25-27]. In conclusion, this study provides the first evidence that amino acid supplementation rescues replication arrest of *hmp* mutant *S.* Typhimurium caused by NO. It further implies that, without the NO-metabolizing activity of Hmp, *S.* Typhimurium hardly persists inside host animals that might provide insufficient levels of key amino acids such as BCAA for restoring bacterial replication against nitrosative stress. Thus, it is plausible that introduction of *hmp* mutation into vaccine candidate strains of *S.* Typhimurium tightly represses bacterial persistence, thereby supplying safer strains in actual applications.

E. References

1. Fang, F.C., *Antimicrobial reactive oxygen and nitrogen species: concepts and controversies.* Nat Rev Microbiol, 2004. **2**(10): p. 820-32.

2. Ignarro, L.J., *Nitric oxide: a unique endogenous signaling molecule in vascular biology.* Biosci Rep, 1999. **19**(2): p. 51-71.

3. MacMicking, J., Q.W. Xie, and C. Nathan, *Nitric oxide and macrophage function.* Annu Rev Immunol, 1997. **15**: p. 323-50.

4. Lundberg, J.O., et al., *Nitrate, bacteria and human health.* Nat Rev Microbiol, 2004. **2**(7): p. 593-602.

5. Bang, I.S., et al., *Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp.* J Biol Chem, 2006. **281**(38): p. 28039-47.

6. Gardner, A.M., R.A. Helmick, and P.R. Gardner, *Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in Escherichia coli.* J Biol Chem, 2002. **277**(10): p. 8172-7.

7. Liu, L., et al., *A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans.* Nature, 2001. **410**(6827): p. 490-4.

8. Poock, S.R., et al., *Respiratory detoxification of nitric oxide by the cytochrome c nitrite reductase of Escherichia coli.* J Biol Chem, 2002. **277**(26): p. 23664-9.

9. Laver, J.R., et al., *Bacterial nitric oxide detoxification prevents*

host cell S-nitrosothiol formation: a novel mechanism of bacterial pathogenesis. FASEB J, 2010. **24**(1): p. 286-95.

10. Barak, Y., et al., *Role of nitric oxide in Salmonella typhimurium-mediated cancer cell killing.* BMC Cancer, 2010. **10**: p. 146.
11. Vogel, H.J. and D.M. Bonner, *Acetylornithinase of Escherichia coli: partial purification and some properties.* J Biol Chem, 1956. **218**(1): p. 97-106.
12. T, H., *Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cystein and glutathione.* Tetrahedron Lett, 1985. **26**: p. 2013-2016.
13. De Groote, M.A., et al., *Genetic and redox determinants of nitric oxide cytotoxicity in a Salmonella typhimurium model.* Proc Natl Acad Sci U S A, 1995. **92**(14): p. 6399-403.
14. Ford, E., M.N. Hughes, and P. Wardman, *Kinetics of the reactions of nitrogen dioxide with glutathione, cysteine, and uric acid at physiological pH.* Free Radic Biol Med, 2002. **32**(12): p. 1314-23.
15. Umbarger, H.E., *biosynthesis of the branched-chain amino acids In : Escherichia coli and Salmonella typhimurium: cellular and molecular biology.* American Society for Microbiology Press, Wachington DC*Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 1996.
16. Flint, D.H., et al., *The role and properties of the iron-sulfur*

cluster in Escherichia coli dihydroxy-acid dehydratase. J Biol Chem, 1993. **268**(20): p. 14732-42.

17. Ren, B., et al., *Nitric oxide-induced bacteriostasis and modification of iron-sulphur proteins in Escherichia coli.* Mol Microbiol, 2008. **70**(4): p. 953-64.
18. Hyduke, D.R., et al., *Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in Escherichia coli.* Proc Natl Acad Sci U S A, 2007. **104**(20): p. 8484-9.
19. Vazquez-Torres, A., et al., *Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro.* J Exp Med, 2000. **192**(2): p. 227-36.
20. Monack, D.M., D.M. Bouley, and S. Falkow, *Salmonella typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization.* J Exp Med, 2004. **199**(2): p. 231-41.
21. Hoiseth, S.K. and B.A. Stocker, *Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines.* Nature, 1981. **291**(5812): p. 238-9.
22. Gunel-Ozcan, A., et al., *Salmonella typhimurium aroB mutants are*

attentuated in BALB/c mice. Microb Pathog, 1997. **23**(5): p. 311-6.

23. O'Callaghan, D., et al., *Immune responses in BALB/c mice following immunization with aromatic compound or purine-dependent Salmonella typhimurium strains.* Immunology, 1990. **69**(2): p. 184-9.

24. Chamberlain, L.M., et al., *Neisseria gonorrhoeae strain MS11 harbouring a mutation in gene aroA is attenuated and immunogenic.* Microb Pathog, 1993. **15**(1): p. 51-63.

25. Hondalus, M.K., et al., *Attenuation of and protection induced by a leucine auxotroph of Mycobacterium tuberculosis.* Infect Immun, 2000. **68**(5): p. 2888-98.

26. Atkins, T., et al., *A mutant of Burkholderia pseudomallei, auxotrophic in the branched chain amino acid biosynthetic pathway, is attenuated and protective in a murine model of melioidosis.* Infect Immun, 2002. **70**(9): p. 5290-4.

27. Leung, K.Y. and B.B. Finlay, *Intracellular replication is essential for the virulence of Salmonella typhimurium.* Proc Natl Acad Sci U S A, 1991. **88**(24): p. 11470-4.

ABSTRACT

Nitrosative stress causes amino acid auxotrophy in *hmp* mutant *Salmonella* Typhimurium

Park, Yoon-Mee

Advisor : Prof. Bang, Iel-Soo, PhD

Department of Dental Bioengineering,

Graduate School of Chosun University

Cytotoxic nitric oxide (NO) damages various bacterial macromolecules, resulting in abnormal metabolism by mechanisms largely unknown. I show that NO can cause amino acid auxotrophy in *Salmonella* Typhimurium lacking major NO-metabolizing enzyme, flavohemoglobin Hmp. In NO-producing cultures, supplementation with amino acid pool restores growth of Hmp-deficient *Salmonella* to normal growth phases, whereas excluding cystein (Cys) or BCAA (leucine [Lue], Isoluecine [Ile], or Valine [Val]) from amino acid pool reduces growth recovery. Data suggest that, without detoxification, NO might inactivate key enzymes in the biosynthesis pathway of amino acids essential for *Salmonella* replication in amino acid-limiting host environments.

Chapter Ⅲ.

Branched-chain amino acid supplementation promotes aerobic growth of *Salmonella* Typhimurium under nitrosative stress conditions

국문초록

곁가지 아미노산의 첨가는 산화스트레스가 주어진 조건에서 살모넬라 균의 호기성 성장을 촉진시킨다

박 윤 미

조선대학교 대학원

치의생명공학과

지도교수 : 방 일 수

산화질소는 박테리아에서 아미노산 생합성 경로의 iron-sulfur효소를 비활성화 시켜 아미노산 영양요구성을 일으킨다. 우리는 산화질소 대사를 하는 flavohemoglobin Hmp를 돌연변이 시킨 *Salmonella* Typhimurium와 branched-chain amino acids (BCAA)를 생합성 하는데 필수적이며 iron-sulphur효소를 가지고 있는 dihydroxy-acid dehydratase (IlvD)와 isopropylmalate isomerase (LeuCD)를 돌연변이화 시켰을 때 외부에서 추가 유입시켜 BCAA가 산소에 의존적으로 산화질소에 대한 저항성을 보여 주는 것을 확인하였다. BCAA의 첨가는 *S.* Typhimurium에서 산화질소 소비에 크게 영향을 주지 않아 산화질소 대사와 BCAA에 의한 산화질소 저항성이 독립적으로 이루어지는 판단하였다. BCAA가 추가됨에도 불구하고 산화질소 양이 변함없이 생성되는 RAW 264.7

대식세포 내에 감염된 *ilvD* 돌연변이 균주는 야생형균주와 비교하였을 때 BCAA첨가에 의해 세포 내 생존율이 증가되었다. 산화질소에 의해 야기된 *Salmonella*의 BCAA 영양요구성은 BCAA 생합성을 위한 iron-sulfur 효소를 비활성화 시키고 산소가 풍부한 환경에서의 추가된 BCAA를 박테리아가 이용하여 성장이 회복 될 수 있다.

A. Introduction

Nitric oxide (NO) produced in activated phagocytic cells of host animals has been implicated to target and inactivate various biomolecules of phagocytized bacteria, causing abnormalities in bacterial metabolism [1]. Therefore, NO and NO-mediated reactive nitrogen species (RNS) are considered to be potent innate immunity antimicrobials that limit the proliferation of invasive pathogenic bacteria. However, pathogenic bacteria have evolved to possess NO response systems that induce the expression of enzymes that detoxify NO into less toxic molecules [2]. For example, the transcriptional repressor NsrR, which regulates most of the NO-responsive genes in various bacteria, senses NO by interacting with its iron-sulfur cluster and releases the depressed transcription of target genes [3, 4]. Genes of the NsrR regulon, including the *hmp* gene encoding flavohemoglobin Hmp that detoxify NO inside bacteria, have major roles in bacterial defense against nitrosative stress both in vitro and *in vivo* [5, 6].

Because a broad spectrum of biomolecules, including proteins containing thiols and iron-sulfur clusters, react strongly with NO and RNS [1, 7], it is plausible that although bacteria have NO response and detoxification systems, they struggle to control their metabolism enough to sustain their life cycles under nitrosative stress conditions. However, there has been little evidence for specific NO targets in bacteria that

are important during interactions between bacterial pathogens and host animals. Recent studies have shown that NO can inactivate key enzymes of amino acid biosynthetic pathways and central metabolic pathways, causing starvation of some amino acids inside bacteria and consequent growth arrest [8, 9]. Several studies suggest that one of the major ways NO damages bacteria is the inhibition of branched-chain amino acid (BCAA) biosynthesis and that, at the same time, uptake of BCAA from surroundings may ameliorate the NO-caused growth inhibition [8, 10, 11].

Animals, including humans, are unable to synthesize BCAA, and thus, they must uptake BCAA in their diets. Maintaining and regulating BCAA levels in the human body are critically important in many diseases [12]. Accordingly, animal immunity largely relies on BCAA for proper function [13]. In contrast to animals, most bacteria are able to synthesize BCAA; therefore, NO-mediated inactivation of key bacterial enzymes for BCAA biosynthesis is an efficient and secure strategy for phagocytic cells to limit the proliferation of invading pathogens, without damaging host cells.

Most bacteria can synthesize BCAA, mainly from pyruvate and oxaloacetate, by the serial actions of enzymes[14]. In the BCAA biosynthesis pathway, dihydroxy-acid dehydratase (IlvD) is essential, and the iron-sulfur cluster in this enzyme has been demonstrated to be the target of NO. In *E. coli*, NO inactivates IlvD by forming the dinitrosyl

iron complex (DNIC) with the iron-sulfur cluster of IlvD, resulting in BCAA auxotophy [8, 15]. In our previous study with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), which lacks flavohemoglobin Hmp, NO induces amino acid auxotrophy. However, this auxotrophy can be overcome by supplying amino acid pools into the culture media, but only when BCAA are included in the pool, suggesting that iron-sulfur enzymes, including IlvD, in the BCAA biosynthetic pathway of *Salmonella* can also be a major target of NO [11]. In studies with *E. coli*, Ren *et al*. [10] reported that the DNIC of IlvD is irreversible only under anaerobic conditions; therefore, changing a bacterial culture from anaerobic to aerobic conditions reactivates IlvD activity and makes BCAA dispensable for the NO resistance of *E. coli* cultured aerobically. However, another study showed that the NO-mediated modification of IlvD was also irreversible under aerobic conditions because NO-exposed *E. coli* cells still needed BCAA supplementation for their growth [8]. Although it is well proven that DNIC formed of NO and the iron-sulfur cluster of IlvD with high affinity [15], the reversibility of this reaction and the concurrent BCAA requirement for bacterial growth under nitrosative stress conditions remains elusive in terms of oxygen availability.

Another reason for these conflicting observations might be due to differences of induction conditions for Hmp expression that largely contribute to determine NO level inside bacteria. In *E. coli*, the

intergenic region between divergently transcribed *hmp* and *glyA* genes seems to contain sequences for interacting with several regulator proteins that regulate *hmp* transcription. It has been shown that *hmp* transcription in *E. coli* is regulated, directly or indirectly, by the stationary-phase sigma factor RpoS, the integration host factor IHF, the stringent response modulator ppGpp, the methionine synthesis regulator MetR, the ferric uptake regulator Fur, and an oxygen-sensing regulator Fnr as well as the NO-sensitive regulator NsrR [3, 16-20]. As an ancient globin conserved in numerous bacteria and yeast, Hmp functions in a wide range of oxygen concentrations [21, 22]. By NO denitrosylase/dioxygenase activity detoxifying NO, Hmp protects central metabolisms of *E. coli* such as TCA cycle and respiration from nitrosative stress under both aerobic and anaerobic conditions [23, 24]. Under low oxygen conditions, alkylhydroperoxide reductase activity of Hmp can also protect *E. coli* from oxidative damages [25]. Also notably, Hmp expression can be induced under oxidative stress conditions, but its overexpression in the absence of NO can damage bacterial redox homeostasis[5, 26-28].

In this study, using a model strain of *S.* Typhimurium that lacks the flavohemoglobin Hmp and therefore minimize the NO metabolism and Hmp-mediated biochemical alterations inside *Salmonella*, I determined the effect of BCAA supplementation on *Salmonella* resistance to NO under three different oxygen conditions. Furthermore, to test the physiological roles

of the iron-sulfur cluster-containing enzymes in the *Salmonella* BCAA biosynthetic pathway, I constructed genetic mutants deficient in *ilvD* and *leuC/leuD*. Genes *leuC* and *leuD* encode the enzyme complex isopropylmalate isomerase (LeuCD). This complex contains an iron-sulfur cluster, which was shown in a study on *E. coli* to be vulnerable to reactive oxygen species, such as hydrogen peroxide [29], possibly being inactivated by NO to cause Leu auxotrophy. Using combinations of these gene mutations and an *hmp* mutation, I have tried to better understand the physiological roles of BCAA and the iron-sulfur enzymes essential for BCAA synthesis in protecting *Salmonella* from nitrosative stress.

B. Materials and Methods

1. Bacterial strains and culture media

Salmonella Typhimurium strains used in this study are listed in Table 1. *S.* Typhimurium 14028S was used as the wild-type (WT) parental strain. For *hmp* mutant, to avoid any artificial effect on regulatory sequences of the *glyA* gene that is transcribed divergently from the *hmp* gene, I employed an *hmp* ORF deletion mutant keeping intergenic sequences between *hmp* and *glyA* genes unmodified[30]. For bacterial culture, Luria-Bertani (LB) broth (Difco, Detroit, MI, USA) or minimal E medium [31] supplemented with 0.2 % glucose (EG medium) was used. To construct gene deletion mutation, I used the polymerase chain reaction (PCR)-mediated one-step gene mutation method using λ Red recombinase[32], with the DNA primers listed in Table 1. To construct genetic mutants with *ilvD* and *leuC/leuD* deletions, the primer pairs *ilvD-P1/ilvD-P2* and *leuCD*-P1/*leuCD*-P2 were used, respectively. Primer pairs, *ilvD*-Fw/*ilvD*-Rev and *leuCD*-Fw/*leuCD*-Rev, were used to confirm the mutation by PCR. All constructed mutations were transduced into a fresh WT strain with bacteriophage P22 HT105/1 *int*, and nonlysogenic colonies were selected for further studies by testing the sensitivity of each transductant to a lytic P22 variant H5. To combine gene mutations into a single strain, antibiotic cassettes in the host strains were removed using the FLP recombinase encoded by the pCP20 plasmid, and then other gene mutations were sequentially introduced by

P22 transduction, as described above. All chemicals, including BCAA, L-Ile, L-Leu, and L-Val, were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. S-nitrosoglutathione (GSNO) was used as an NO congener. It was synthesized according to the method reported previously by using the reaction of glutathione and acidified sodium nitrite [33].

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

Strains	Genotype	Source or References
IB1	*Salmonella enterica* serovar Typhimurium 14028s	ATCC
IB3	*hmp* ::KM	[30]
IB1334	*hmp* ::KM *ilvD* ::CM	This study
IB1339	*ilvD* ::KM	This study
IB1396	*leuCD* ::KM	This study
IB1397	*hmp* ::KM *leuCD* ::CM	This study
IB1406	*hmp ilvD*	This study
IB1407	*hmp ilvD leuCD* ::CM	This study
Plasmids	**Characteristics**	**Source or References**
pKD3	Plasmid carrying the FRT-Cm^r-FRT-cassette	[32]
pKD4	Plasmid carrying the FRT-Km^r-FRT-cassette	[32]
pTP223	Plasmid encoding IPTG-inducible Lamda red recombinase	[34]
pCP20	Ts(30℃) replicon plasmid encoding the Flp recombinase gene	[35]
DNA oligomers	**Sequence (5'-3')**	
ilvD-P1	cattcactcagtttgtgccgggtcacgttcatctgcgcgagtgtaggctggagctgcttc	
ilvD-P2	ctgcggttcgggatatcaatcgcaatagtgtcgccatcttcatatgaatatcctccttag	
ilvD-Fw	gctggttggatccggtaaat	
ilvD-Rev	ggaactgctgcagcaggat	
leuCD-P1	gagttcggcgtcgagctgtatgacctgaatcacccatatccatatgaatatcctccttag	
leuCD-P2	accactttaaagccgtaatcggtcaacgcccacggcgcgtgtgtaggctggagctgcttc	
leuCD-Fw	ccaacccaattgcgcagat	
leuCD-Rev	atggcgctaagcttatcag	

2. Measurement of *Salmonella* susceptibility to nitrosative stress

Salmonella susceptibility to nitrosative stress was measured by observing bacterial growth in NO-producing cultures at 37℃ under three different oxygen tensions. *S.* Typhimurium grown overnight in LB broth were diluted in phosphate-buffered saline (PBS) to an optical density(OD_{600nm}) of 1.0. Then, the same amount of bacterial cells was inoculated into fresh EG media under different oxygen conditions. To acquire a fully aerobic culture, aliquots (20 μl) of bacterial cultures were spotted onto the surface of solid EG media containing or lacking GSNO (500 μM) and then incubated for 16 h before measuring the growth intensity of the bacterial spotted area, which was estimated using the Image J program (National Institute of Health, USA). For a semi-aerobic culture, the same amount of bacterial cells (OD_{600nm} = 0.02) were inoculated into wells of a microtiter plate containing EG liquid media in the presence or absence of GSNO. Bacterial growth was monitored by measuring the optical density (OD_{600nm}) of bacterial cultures in a Bioscreen C Microbiology Microplate reader (Labsystems, Helsinki) at 37℃ at 30-min intervals. For an anaerobic culture, experiments with the same sets of cultures used for semi-aerobic culture were performed in an anaerobic chamber (Bactron I, Sheldon Manufacturing Inc.). The optical density of bacterial cultures was measured at 6-h intervals. Oxygen

concentration in the semi-aerobic cultures was measured using an oxygen sensor [ISO-OXY-2; World Precision Instruments (WPI) Inc., Santarosa, FL] connected to a free radical analyzer (TBR4100; WPI Inc.).

3. Measurement of the *Salmonella* NO consumption rate

The harvested *S.* Typhimurium cells grown with agitation in LB broth at 37℃ to OD_{600nm} ~ 1.0 were washed once with PBS and resuspended in 10 ml PBS. The consumption rate of NO was determined by measuring the remaining NO in the bacterial solution after adding a fast-releasing NO donor, ProliNONOate (2 μM; Cayman Chemical, Ann Arbor, MI) into the bacterial solution, while the bacterial solution was continuously stirred with a stir bar regulated by a magnetic stirrer. The concentration of NO in the solution was measured by using a NO-sensitive electrode with a 2-mm-diameter tip (ISO-NOP sensor; WPI Inc.) connected to a free radical analyzer (TBR4100; WPI Inc.). The data signal was obtained by using the Labchart program.

4. Measurement of intra-macrophage survival of *Salmonella* strains

To measure the rate of *S.* Typhimurium intra-macrophage replication, a murine macrophage-like cell line RAW 264.7 (ATCC TIB-7) was purchased from the American Type Culture Collection (ATCC, Rockville, MD). RAW 264.7

cells were grown in RPMI 1640 cell culture media (Hyclone, Logan, UT) containing 10 % heat-inactivated fetal bovine serum (Hyclone, Logan, UT) at 37℃, incubated with 5 % CO_2. Infection of macrophages with *S.* Typhimurium was performed as described previously [36]. Briefly, *S.* Typhimurium grown to the early stationary phase in LB were collected and, after washing with PBS, used to infect macrophage cells at a multiplicity of infection of 10. Extracellular bacteria were killed by washing with RPMI media containing gentamycin (10 μg/ml). To examine the effect of exogenous BCAA on *S.* Typhimurium survival, BCAA (2 mM each) were supplemented into the culture media at 30 min after bacterial infection. The surviving bacteria were harvested by using lysis buffer (0.05 % Triton X-100 in PBS) and enumerated by counting the colony-forming units (CFU) after incubation on LB agar plates. To activate macrophages, interferon (IFN)-γ (25 U/ml, Peprotech, Rocky Hill, NJ) was added to the culture media for 18 h before bacterial infection. To inhibit the inducible NO synthase (iNOS), *N*G-monomethyl-l-arginine (L-NMMA; 1 mM) was added to the media when bacteria were infected to the IFN-γ-primed macrophages.

5. Measurement of nitrite concentration

The nitrite that accumulated in the supernatants of culture media, which reflects the amount of NO expressed in *Salmonella*-infected

macrophages, was quantified by using the Griess reagent following the manufacturer's protocol (Sigma-Aldrich). Sodium nitrite was used as the standard for nitrite concentrations.

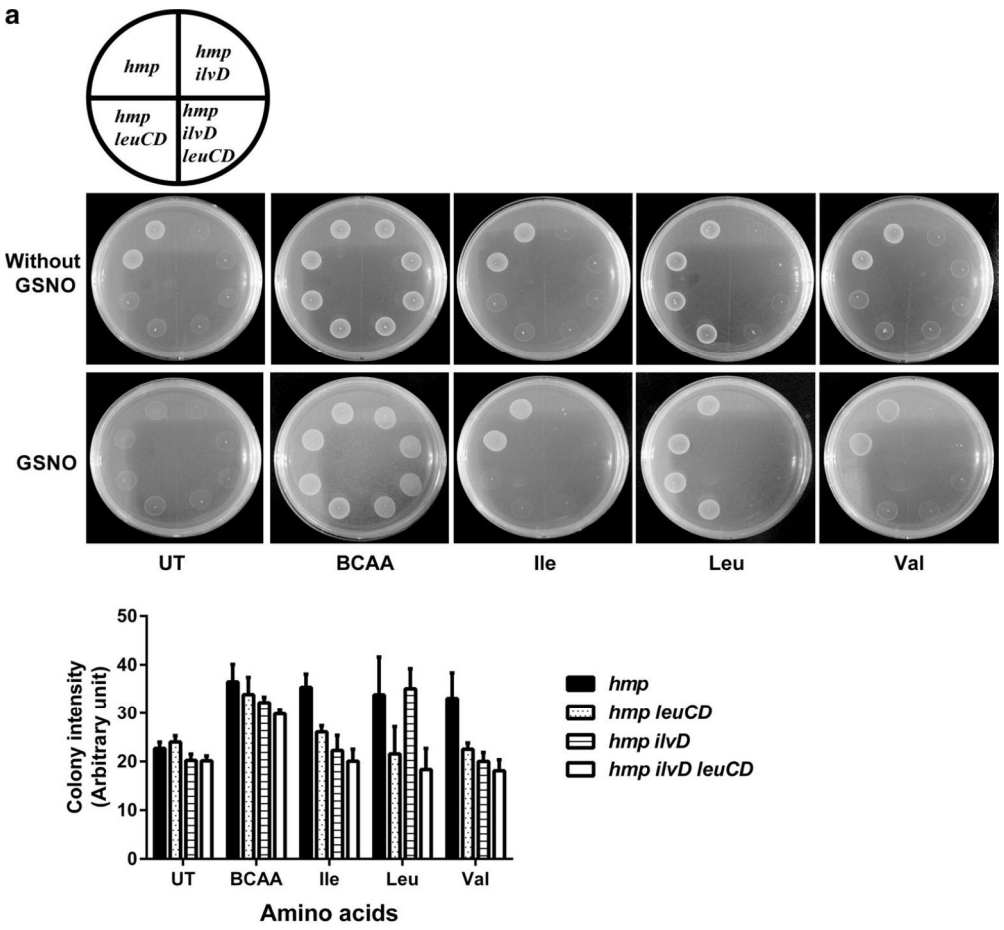
C. Results

1. BCAA supplementation recovers aerobic growth of *hmp* mutant *S.* Typhimurium in NO-producing cultures

The flavohemoglobin Hmp detoxifies most of the NO entering into *Salmonella*. Thus, *hmp* mutant *S.* Typhimurium is always hyper-susceptible to nitrosative stress, resulting in growth inhibition of this mutant in NO-containing cultures [5]. Supplementation with amino acid pools containing all amino acids recovered the NO resistance of the *hmp* mutant to a level comparable to that of WT cells, but excluding BCAA from the amino acid pools lessens the NO resistance of this mutant [11]. To test whether the exclusive supplementation of BCAA into the culture recovers the growth of *hmp* mutant under nitrosative stress conditions, I added all three BCAA together or each BCAA by itself (Leu, Ile, and Val) to the media containing GSNO and compared the *S.* Typhimurium growth. In aerobic cultures, the growth of *hmp* mutant was fully recovered on solid EG media containing all three BCAA and, to a lesser extent, media containing any of the BCAA, while growth was still inhibited on the media without any BCAA or on a control media supplemented with a combination of non-BCAA amino acids, His, Gly, and Pro (Fig. 1). In *E. coli* and *Salmonella*, the biosynthetic pathway of BCAA requires key enzymes containing an iron-sulfur cluster, IlvD and LeuCD. In the absence of LeuCD, *E. coli* cannot synthesize Leu, but in an *ilvD* mutant, none of the three BCAA are

synthesized because the product of the IlvD enzyme is required for both the Ile/Val and Leu synthetic pathways [14]. The *S.* Typhimurium mutants lacking *ilvD* and *leuCD* constructed in this study showed auxotrophy of all BCAA and Leu, respectively, and required those corresponding BCAA for their growth, confirming that enzymes encoded by these genes are essential for BCAA biosynthesis in *S.* Typhimurium (Fig. 1a; without GSNO). With the same sets of mutant *S.* Typhimurium, I further examined whether the BCAA-mediated increase in NO resistance still occurs with these mutants. In NO-producing media, strains containing an *ilvD* mutation required all three BCAA for their growth, and the *leuCD* mutant required Leu, demonstrating that supplementing BCAA into the culture can counterbalance the loss of theses enzymes under nitrosative stress conditions (Fig. 1a; GSNO). Next I examined whether BCAA supplementation can recover NO resistance in *hmp* mutant *S.* Typhimurium under less oxic environments. Under semi-aerobic conditions, with bacteria cultured in liquid media (up to 12 % oxygen level compared with that of aerobic culture), bacterial growth started to recover 12 h after the culture was started in BCAA-supplemented media containing the same (500 μM) or lower (250 μM) concentrations of GSNO used for aerobic cultures (Fig. 2a). However, under anaerobic conditions, BCAA supplementation did not promote bacterial growth at all in GSNO-containing cultures (Fig. 2b), whereas supplementation with the same concentrations of BCAA rescued auxotrophy

of mutant *S.* Typhimurium lacking IlvD and LeuCD in the absence of GSNO (data not shown). These results clearly show that the NO caused inhibition of *Salmonella* growth can be recovered by supplementing BCAA into the culture media in an oxygen-dependent manner. It suggests that NO-mediated modification of iron-sulfur cluster enzymes for BCAA biosynthesis may be irreversible in *Salmonella* under aerobic conditions, thereby requiring exogenous BCAA for their growth, and that, under anaerobic conditions, other biochemical factors in addition to BCAA are needed for promoting *Salmonella* growth in NO-producing cultures.



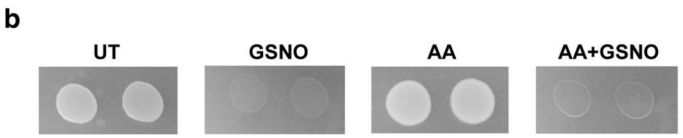


Figure 1. Effect of BCAA supplementation on the aerobic growth of *S.* Typhimurium.

(a) Aliquots of bacterial cultures containing the same amount of mutant *S.* Typhimurium strains [Δ*hmp* (IB3), Δ*hmp* Δ*ilvD* (IB1344), Δ*hmp* Δ*leuCD* (IB1397), Δ*hmp* Δ*ilvD* Δ*leuCD* (IB1407)] are spotted onto EG plates containing one of each BCAA, all three BCAA together, or no BCAA, in the presence or absence of GSNO (500 μM). After a 16-h incubation at 37℃, agar plates are photographed and colony intensities of each bacterial spot are measured by Image J. A *circle diagram* shows the divided region of each strain spotted in duplicates on the plate. Of three independent experiments, a representative picture set is shown, and the data [mean ± standard deviation (SD)] of colony intensities are graphed below. UT; untreated with amino acids. **(b)** The same experiments described in the legend for (a) are performed with the Δ*hmp* mutant *S.* Typhimurium (IB3), except using plates containing non-BCAA amino acids, His, Gly, and Pro (AA). A representative picture out of three independent experiments is shown. UT; untreated with amino acids and GSNO.

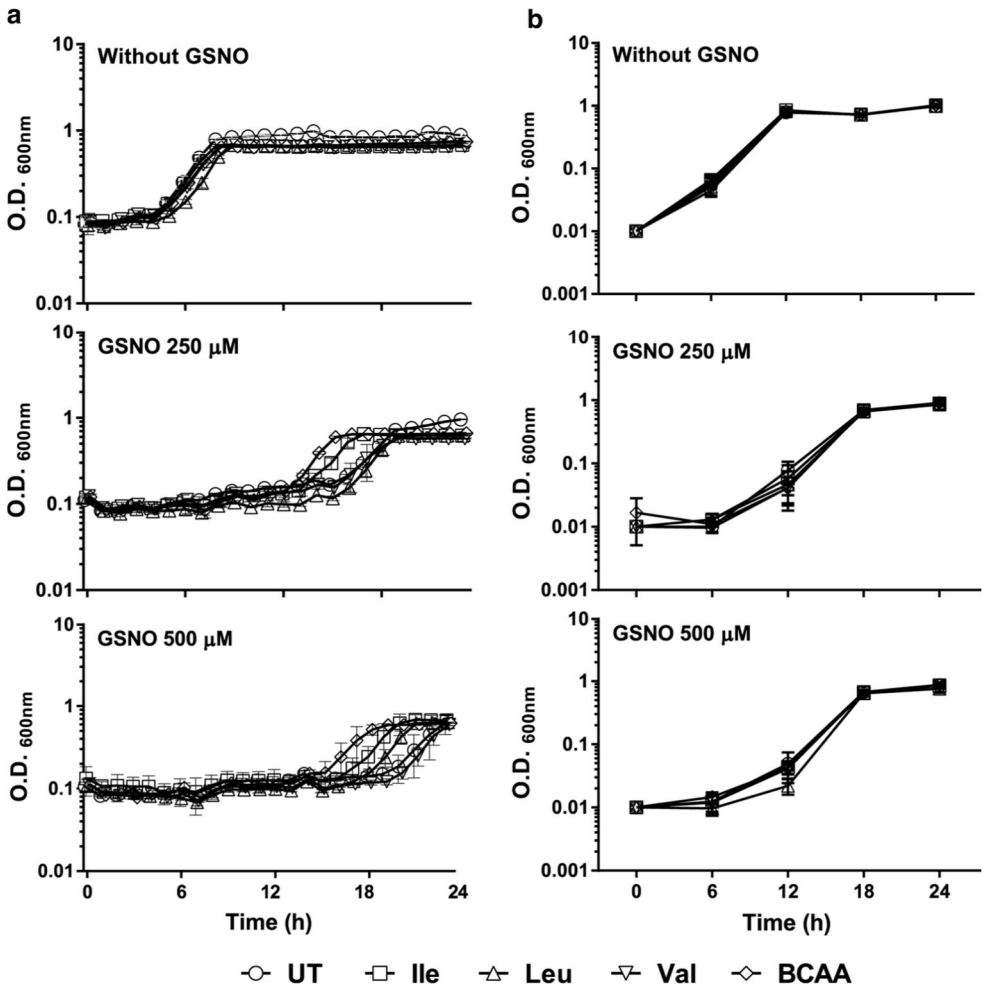


Figure 2. Effect of BCAA supplementation on the semi-aerobic and anaerobic growth of *S.* Typhimurium.

The Δ*hmp* mutant *S.* Typhimurium (IB3) are cultured in EG media containing or lacking GSNO. Each single or all BCAA are supplemented into the media. For semi-aerobic cultures (**a**), bacteria are cultured with agitation in a microtiter pate and the optical density (OD_{600nm}) of bacteria is measured using the Bioscreen C microplate reader for 24 h at 37℃. For anaerobic cultures (**b**), bacterial growth, cultured in an anaerobic chamber, is monitored by measuring the optical density of culture aliquots taken at timed intervals. Data shown are the mean ± SD from three independent cultures.

2. BCAA supplementation does not change the NO-metabolizing activity of *S.* Typhimurium

The BCAA-mediated growth recovery of *hmp* mutant *S.* Typhimurium in NO-producing cultures may be the result of a reduction in NO and RNS levels due to the activation of a Hmp-independent NO metabolism. To test this possibility, I measured the NO consumption rate of *hmp* mutant and WT *S.* Typhimurium in the presence or absence of BCAA. As shown in Fig. 3, after NO was injected into bacterial cultures, WT *S.* Typhimurium consumes all of the NO in less than 2 min, whereas concentrations of the remaining NO were similar in solutions containing the *hmp* mutant and the buffer control, regardless of BCAA supplementation. Moreover, BCAA supplementation did not change the NO consumption rate of WT, either. This result clearly indicates that the effect of bacterial culture supplementation with BCAA is not related to the NO metabolism of *S.* Typhimurium, suggesting that exogenous BCAA are utilized for compensating for the NO-induced loss of BCAA in *Salmonella*.

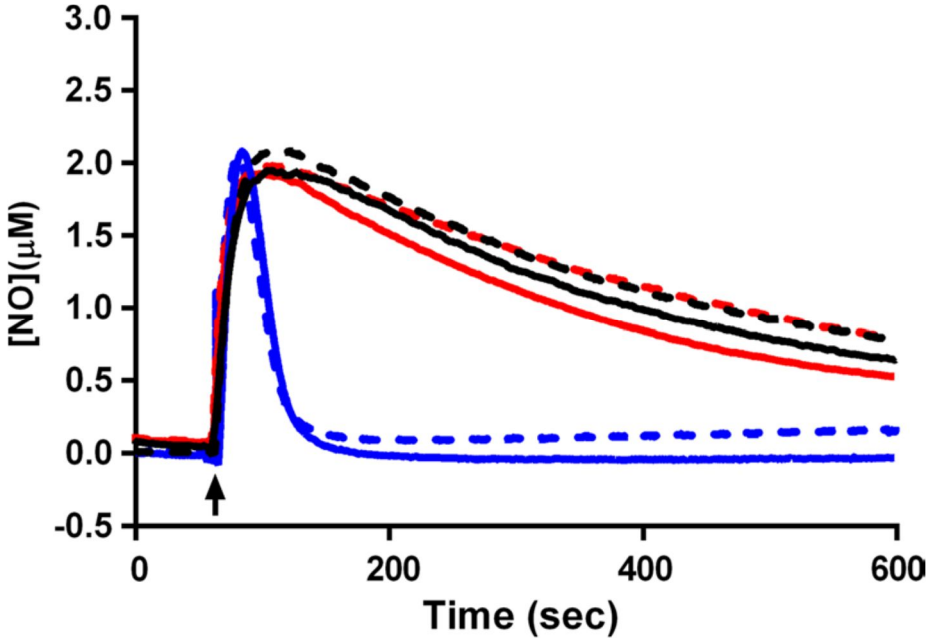


Figure 3. Comparison of the NO consumption rate of *S.* Typhimurium in the presence or absence of BCAA supplementation.

Bacterial solutions for measuring the NO consumption rate are prepared in PBS buffer (*black*) by adding the same amount of WT (IB1; *blue*) and Δ*hmp* mutant (IB3; *red*) *S.* Typhimurium strains cultured to log phase (OD_{600nm} ~ 0.5) in the presence (*dashed lines*) or absence (*solid lines*) of BCAA. The NO consumption of *S.* Typhimurium strains is recorded by measuring the remaining NO in bacterial solutions and in the control PBS buffer after addition of ProliNONOate (2 μM) as indicated by the *arrow*. A NO-sensitive electrode connected to a free radical analyzer is employed to measure NO levels, as described in the "Methods" section. Data are representative of three independent experiments.

3. *ilvD* mutant *S.* Typhimurium in RAW 264.7 macrophages is auxotrophic for BCAA

Our data show that BCAA supplementation can rescue auxotrophy of mutant *S.* Typhimurium lacking IlvD and LeuCD. To investigate whether this finding also applies to *S.* Typhimurium inside of macrophages, I measured the survival rate of *S.* Typhimurium strains after infection of RAW 264.7 macrophages supplemented with low or high concentrations of BCAA. In macrophages cultured in the low-BCAA-containing RPMI media, which contains 0.38 mM Ile, 0.38 mM Leu, and 0.17 mM Val as provided by manufacturer, the *leuCD* mutant and WT *S.* Typhimurium replicated similarly. However, the replication of the *ilvD* mutant showed a distinct difference in the presence or absence of additional BCAA supplementation. In the low-BCAA media, the replication of *ilvD* mutant decreased up to 1000-fold in inactivated macrophages, and above ten-fold in IFN-γ-primed macrophages, compared with that of WT *S.* Typhimurium (Fig. 4a; low BCAA). In contrast, in the high-BCAA-containing media, which was supplemented with an additional amount of BCAA (2 mM each), the *ilvD* mutant replicated similarly to the WT in both naive and IFN-γ-primed macrophages (Fig. 4a; high BCAA). Of note, macrophage NO production, measured as the amount of nitrite accumulated in the media, was not considerably affected by changing the concentrations of BCAA supplemented into the cell media (Fig. 4b). Collectively, these results demonstrate that, in the low-BCAA media,

the concentration of BCAA in macrophages is enough to grow the *leuCD* mutant, but not the *ilvD* mutant at WT levels. However, increasing the BCAA amount can successfully promote the growth of *ilvD* mutant to levels comparable with that of WT. These results suggest that the BCAA auxotrophy of *Salmonella* caused by inactivation of IlvD and LeuCD can be rescued by supplying sufficient BCAA into macrophages.

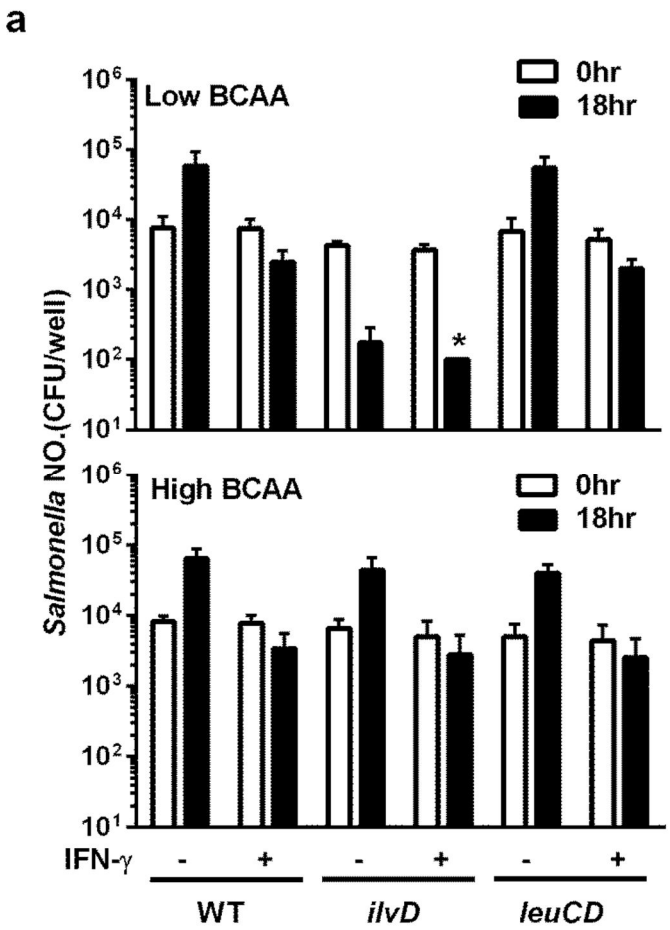


Figure 4. Effect of BCAA supplementation on the intra-macrophage survival of *S.* Typhimurium.

(a) IFN-γ-primed or naive (UT) RAW 264.7 cells cultured in low- or high-BCAA-containing media are infected with WT (IB1), Δ*ilvD* mutant (IB1339), and Δ*leuCD* mutant (IB1396) *S.* Typhimurium strains, and the number of intracellular *Salmonella* per well after infection for 18 h is determined by counting *Salmonella* CFU. Data shown are the mean CFU of *Salmonella* plated from triplicate wells for each experiment from five independent experiments. *Asterisk* ≤100 CFU. **(b)** Concentrations of nitrite produced in the supernatants of macrophages infected with *S.* Typhimurium. Data shown are the mean ± SD of the same five experiments as performed for (a).

4. BCAA supplementation cause little effect on the replication of *hmp* mutant *S.* Typhimurium in NO-producing RAW 264.7 macrophages

I next examined the effect of BCAA supplementation on the replication of *hmp* mutant *S.* Typhimurium in RAW 264.7 macrophages. The replication of *hmp* mutant *S.* Typhimurium was almost nullified in IFN-γ-primed macrophages, while it was significantly recovered by treating macrophages with an iNOS inhibitor, L-NMMA, confirming that there is the NO-mediated growth inhibition of *hmp* mutant in macrophages (Fig. 5a). However, increasing the BCAA amount in macrophage media caused little effect to the replication of *hmp* mutant in both inactivated and IFN-γ-primed macrophages (Fig. 5a). There was no considerable change in NO production between *hmp* mutant infected macrophages cultured in media containing low and high BCAA, respectively (Fig. 5b). These results indicate that BCAA supplementation can hardly promote the replication of *hmp* mutant *Salmonella* inside the NO-producing macrophages.

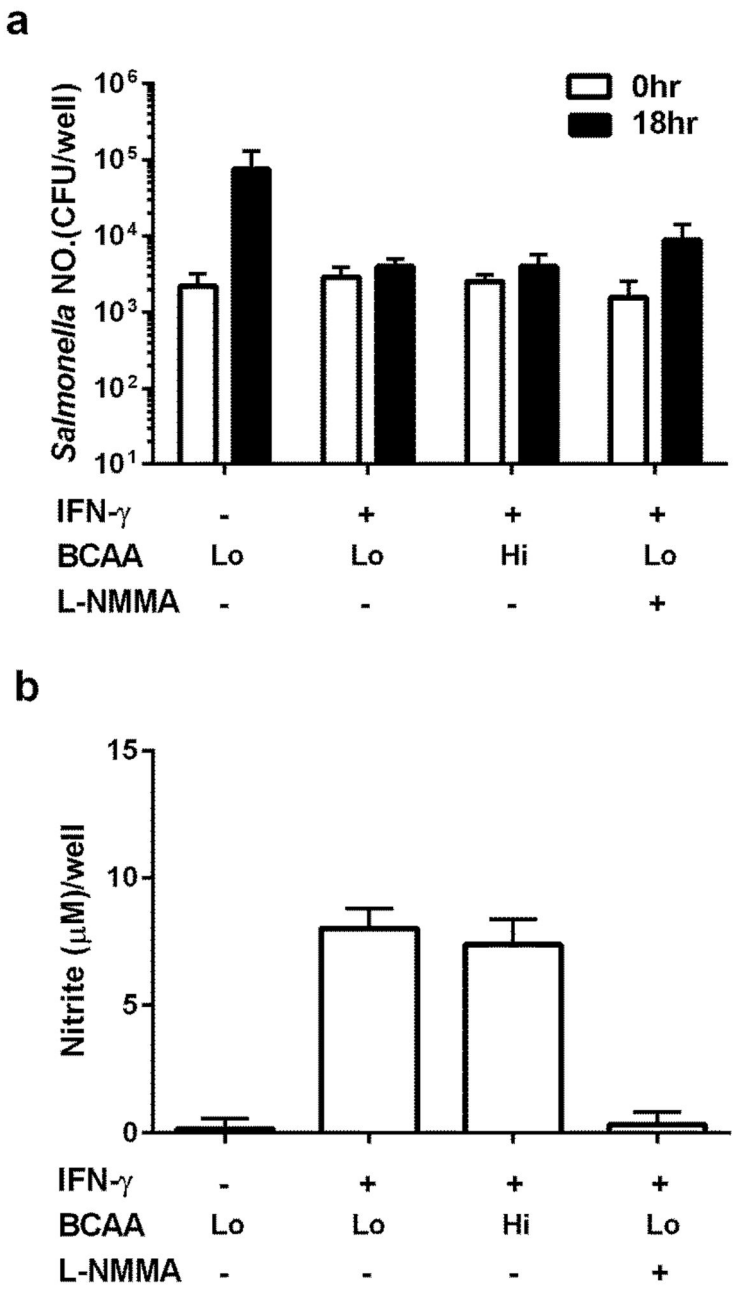


Figure 5. Effect of BCAA supplementation on the intramacrophage survival of *hmp* mutant *S.* Typhimurium.

(a) RAW 264.7 macrophages cultured in low- (Lo) or high-BCAA (Hi)-containing media are infected with the Δ*hmp* mutant *S.* Typhimurium (IB3). L-NMMA (1 mM) added to media for IFN-γ-primed macrophages to inhibit iNOS. The number of intracellular bacteria is determined as described in Fig. 4. Data are the mean CFU of *Salmonella* plated from triplicate wells for each experiment from three independent experiments. **(b)** Concentrations of nitrite produced in experiments performed for (a) are shown as the mean ± SD.

D. Discussion

In order to replicate inside the phagocytes of host animals, intracellular enteric pathogen *S.* Typhimurium must deal with the biostatic agents NO and RNS [37]. In this study, I demonstrated that exogenous BCAA supplementation can rescue the NO-induced growth arrest under nitrosative stress conditions and can promote intramacrophage survival of BCAA auxotrophic *S.* Typhimurium. This finding is partly consistent with previous in vitro *E. coli* studies showing that NO inactivates IlvD to cause BCAA auxotrophy, thereby requiring BCAA supplementation in NO-producing cultures to recover bacterial growth [8, 10]. Given that almost identical homology of iron-sulfur enzymes IlvD and LeuCD between *E. coli* and *Salmonella*, it seems likely that NO also targets these enzymes in *S.* Typhimurium to cause BCAA auxotrophy. Our findings with mutant *S.* Typhimurium strains lacking these enzymes also support this idea. Gene mutations deleting IlvD and LeuCD caused BCAA and Leu auxotrophy, respectively, in *S.* Typhimurium, and these were recoverable with corresponding BCAA and Leu supplementation. These phenotypes were the same as that of *E. coli*, and they remained unchanged, regardless of the oxygen concentration. Surprisingly, under nitrosative stress conditions, these findings were reproducible only in cultures containing oxygen (Figs. 4, 5). In contrast to the previous finding that BCAA supplementation rescues *E. coli* from the NO-caused growth arrest in

anaerobic cultures [10], our results showed that BCAA has no effect on the anaerobic growth of *S.* Typhimurium in NO-producing cultures. Transcription of *ilvC* encoding the ketol-acid reductoisomerase in the BCAA biosynthetic pathway is known to be induced by NO exposure in aerobic and anaerobic cultures of *S.* Typhimurium and *E. coli*, respectively [9, 38], implying a reduction in intracellular levels of BCAA under both conditions. However, our results propose that, in addition to BCAA, there are other factors required for recovering the anaerobic growth of *S.* Typhimurium under nitrosative stress conditions. In fact, supplementation with all amino acids was able to recover the growth of *S.* Typhimurium in NO-producing anaerobic cultures (data not shown), suggesting that there may be other amino acid auxotrophies that must be overcome for *Salmonella* growth. This difference under nitrosative stress conditions distinguishes the anaerobic metabolism of *S.* Typhimurium from that of *E. coli*, and its details and mechanisms require further investigation.

In aerobic environments, the NO metabolism of *S.* Typhimurium largely depends on the NsrR regulon expressing more than 20 genes in response to NO[6]. Among them, the flavohemoglobin Hmp consumes the majority of NO. The hybrid cluster protein Hcp also plays a minor role in NO metabolism inside *S.* Typhimurium. The effect of amino acids on bacterial NO metabolism or on bacterial gene regulation for NO metabolism has not been

investigated. This study demonstrated that BCAA supplementation into bacterial culture has no effect on the NO-consuming activity of *S.* Typhimurium (Fig. 3), suggesting little relation of BCAA to bacterial NO metabolism. Therefore, the BCAA-promoted *Salmonella* growth in NO-producing cultures results from the compensation for the NO-exposed *Salmonella* deficit in BCAA rather than from a regulation of the NO metabolism itself.

Previous studies have shown that IlvD activity is required for the virulence of two intracellular pathogens *Mycobacterium tuberculosis* and *Aspergillus fumigatus* [39, 40]. The loss of *ilvD* mutant *S.* Typhimurium intra-macrophage survival shown in this study is consistent with the findings in those pathogens and adds evidence in the importance of IlvD activity for bacterial virulence. However, this study also showed that changing the cell media from low-BCAA- to high-BCAA-containing media recovered the survival rate in macrophages of this mutant to levels comparable to that of WT (Fig. 4). This finding suggests that supplementing sufficient BCAA could counterbalance the loss of IlvD activity in intracellular pathogens. Because BCAA are essential amino acids in all animals, including humans, the concentration of BCAA inside host animals is dependent on their diet. Although it is unknown how much BCAA is maintained in the host animals used for bacterial virulence studies, some analytical studies with human fluids suggest that the

amount of BCAA inside a host would not be limiting compared with amount of other essential amino acids, such as Met and Trp [41, 42]. Therefore, it is possible that a loss of IlvD activity in pathogenic bacteria, either by gene mutation or through NO, would not be detrimental to their replication inside hosts where the concentration of BCAA is maintained at levels sufficient for bacterial needs.

Notably, I found that BCAA supplementation had little effect on the intra-macrophage survival of WT *S.* Typhimurium (Fig. 4). This result is consistent with the previous finding that, although dietary restriction of all three BCAA or any single BCAA results in a higher mortality of mice to *S.* Typhimurium infections, bactericidal activities of macrophages from control and BCAA-restricted groups of mice are not significantly different from one another [43]. Moreover, I showed that changing the concentrations of exogenous BCAA did not affect the NO production in macrophages. This finding is in line with the prediction, based on a molecular systems analysis for metabolic immuno modulators of macrophage, that regulation by BCAA in NO production is improbable [44]. Taking into account our results that BCAA supplementation promotes the replication of *ilvD* mutant *S.* Typhimurium in NO-producing cultures and inside macrophages, I propose that BCAA supplementation in diets may improve the general immunity of animal hosts, but at the same time, it may also invalidate one of the important antimicrobial targets for the NO produced

inside phagocytes.

The flavohemoglobin Hmp protects many bacteria including *Salmonella* from nitrosative stress by consuming most of NO entering into bacteria that otherwise damages various macromolecules to cause bacterial growth arrest in vitro and the reduction in bacterial survival in animal hosts. Amino acid auxotrophy has been proved as a phenotype of *hmp* mutant *S.* Typhimurium cultured under nitrosative conditions [11]. In this study, I show that the aerobic growth of *hmp* mutant in NO-producing cultures can be recovered by BCAA supplementation (Figs. 4, 5). However, during infection of NO-producing RAW 264.7 macrophages, the replication of *hmp* mutant *S.* Typhimurium could not be promoted by increasing exogenous BCAA amount which is enough for rescuing the BCAA auxotrophy of *ilvD* mutant in both inactivated and IFN-γ-primed macrophages (Figs. 7, 8). Thus, in this RAW 264.7 macrophage infection model, the physiological relevance of the NO-mediated BCAA auxotrophy of *hmp* mutant remains uncertain. Given that the attenuation of *hmp* mutant *S.* Typhimurium for the intra-macrophage survival is observed mostly in primary macrophages isolated from mice [5, 45], future study using the appropriate mouse infection model is required to expand understandings about physiological roles of BCAA in *Salmonella* pathogenesis under nitrosative stress conditions.

In summary, I have presented new evidence that BCAA supplementation can promote the aerobic growth of *S.* Typhimurium under nitrosative stress

conditions. It is likely that exogenous BCAA does not change bacterial NO metabolism but rather compensates for a deficiency in BCAA caused by the NO-induced inactivation of key enzymes essential for the BCAA biosynthetic pathway in *Salmonella*.

E. References

1. Fang, F.C., *Antimicrobial reactive oxygen and nitrogen species: concepts and controversies.* Nat Rev Microbiol, 2004. **2**(10): p. 820-32.

2. Prior, K., et al., *All stressed out. Salmonella pathogenesis and reactive nitrogen species.* Adv Microb Physiol, 2009. **56**: p. 1-28.

3. Rodionov, D.A., et al., *Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks.* PLoS Comput Biol, 2005. **1**(5): p. e55.

4. Tucker, N.P., et al., *There's NO stopping NsrR, a global regulator of the bacterial NO stress response.* Trends Microbiol, 2010. **18**(4): p. 149-56.

5. Bang, I.S., et al., *Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp.* J Biol Chem, 2006. **281**(38): p. 28039-47.

6. Karlinsey, J.E., et al., *The NsrR regulon in nitrosative stress resistance of Salmonella enterica serovar Typhimurium.* Mol Microbiol, 2012. **85**(6): p. 1179-93.

7. Laver, J.R., et al., *Nitrosothiols in bacterial pathogens and pathogenesis.* Antioxid Redox Signal, 2013. **18**(3): p. 309-22.

8. Hyduke, D.R., et al., *Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial*

target in Escherichia coli. Proc Natl Acad Sci U S A, 2007. **104**(20): p. 8484-9.

9. Richardson, A.R., et al., *Multiple targets of nitric oxide in the tricarboxylic acid cycle of Salmonella enterica serovar typhimurium.* Cell Host Microbe, 2011. **10**(1): p. 33-43.

10. Ren, B., et al., *Nitric oxide-induced bacteriostasis and modification of iron-sulphur proteins in Escherichia coli.* Mol Microbiol, 2008. **70**(4): p. 953-64.

11. Park, Y.M., et al., *Nitrosative stress causes amino acid auxotrophy in hmp mutant Salmonella Typhimurium.* Microbiol Immunol, 2011. **55**(10): p. 743-7.

12. Burrage, L.C., et al., *Branched-chain amino acid metabolism: from rare Mendelian diseases to more common disorders.* Hum Mol Genet, 2014. **23**(R1): p. R1-8.

13. Calder, P.C., *Branched-chain amino acids and immunity.* J Nutr, 2006. **136**(1 Suppl): p. 288S-93S.

14. Umbarger, H.E., *biosynthesis of the branched-chain amino acids In : Escherichia coli and Salmonella typhimurium: cellular and molecular biology.* American Society for Microbiology Press, Wachington DC*Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 1996.

15. Duan, X., et al., *Reactivity of nitric oxide with the [4Fe-4S]*

cluster of dihydroxyacid dehydratase from Escherichia coli. Biochem J, 2009. **417**(3): p. 783-9.

16. Membrillo-Hernandez, J., G.M. Cook, and R.K. Poole, *Roles of RpoS (sigmaS), IHF and ppGpp in the expression of the hmp gene encoding the flavohemoglobin (Hmp) of Escherichia coli K-12.* Mol Gen Genet, 1997. **254**(5): p. 599-603.

17. Membrillo-Hernandez, J., et al., *A novel mechanism for upregulation of the Escherichia coli K-12 hmp (flavohaemoglobin) gene by the 'NO releaser', S-nitrosoglutathione: nitrosation of homocysteine and modulation of MetR binding to the glyA-hmp intergenic region.* Mol Microbiol, 1998. **29**(4): p. 1101-12.

18. Poole, R.K., et al., *Nitric oxide, nitrite, and Fnr regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12.* J Bacteriol, 1996. **178**(18): p. 5487-92.

19. Bodenmiller, D.M. and S. Spiro, *The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator.* J Bacteriol, 2006. **188**(3): p. 874-81.

20. Hernandez-Urzua, E., et al., *Multiple regulators of the Flavohaemoglobin (hmp) gene of Salmonella enterica serovar Typhimurium include RamA, a transcriptional regulator conferring the multidrug resistance phenotype.* Arch Microbiol, 2007. **187**(1): p. 67-77.

21. Forrester, M.T. and M.W. Foster, *Protection from nitrosative stress: a central role for microbial flavohemoglobin.* Free Radic Biol Med, 2012. **52**(9): p. 1620-33.

22. Hausladen, A. and J.S. Stamler, *Is the flavohemoglobin a nitric oxide dioxygenase?* Free Radic Biol Med, 2012. **53**(5): p. 1209-10; author reply 1211-2.

23. Gardner, P.R., G. Costantino, and A.L. Salzman, *Constitutive and adaptive detoxification of nitric oxide in Escherichia coli. Role of nitric-oxide dioxygenase in the protection of aconitase.* J Biol Chem, 1998. **273**(41): p. 26528-33.

24. Stevanin, T.M., et al., *Flavohemoglobin Hmp affords inducible protection for Escherichia coli respiration, catalyzed by cytochromes bo' or bd, from nitric oxide.* J Biol Chem, 2000. **275**(46): p. 35868-75.

25. Bonamore, A., et al., *Escherichia coli flavohemoglobin is an efficient alkylhydroperoxide reductase.* J Biol Chem, 2003. **278**(25): p. 22272-7.

26. Membrillo-Hernandez, J., et al., *Paraquat regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12 is SoxRS independent but modulated by sigma S.* J Bacteriol, 1997. **179**(10): p. 3164-70.

27. McLean, S., L.A. Bowman, and R.K. Poole, *Peroxynitrite stress is*

exacerbated by flavohaemoglobin-derived oxidative stress in Salmonella Typhimurium and is relieved by nitric oxide. Microbiology, 2010. **156**(Pt 12): p. 3556-65.

28. Gilberthorpe, N.J., et al., *NsrR: a key regulator circumventing Salmonella enterica serovar Typhimurium oxidative and nitrosative stress in vitro and in IFN-gamma-stimulated J774.2 macrophages.* Microbiology, 2007. **153**(Pt 6): p. 1756-71.

29. Jang, S. and J.A. Imlay, *Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes.* J Biol Chem, 2007. **282**(2): p. 929-37.

30. Crawford, M.J. and D.E. Goldberg, *Role for the Salmonella flavohemoglobin in protection from nitric oxide.* J Biol Chem, 1998. **273**(20): p. 12543-7.

31. Vogel, H.J. and D.M. Bonner, *Acetylornithinase of Escherichia coli: partial purification and some properties.* J Biol Chem, 1956. **218**(1): p. 97-106.

32. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.

33. T, H., *Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cystein and glutathione.* Tetrahedron Lett, 1985. **26**: p. 2013-2016.

34. Poteete, A.R. and A.C. Fenton, *Lambda red-dependent growth and recombination of phage P22.* Virology, 1984. **134**(1): p. 161-7.

35. Cherepanov, P.P. and W. Wackernagel, *Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant.* Gene, 1995. **158**(1): p. 9-14.

36. Vazquez-Torres, A., et al., *Analysis of nitric oxide-dependent antimicrobial actions in macrophages and mice.* Methods Enzymol, 2008. **437**: p. 521-38.

37. Vazquez-Torres, A., et al., *Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro.* J Exp Med, 2000. **192**(2): p. 227-36.

38. Justino, M.C., et al., *New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide.* J Biol Chem, 2005. **280**(4): p. 2636-43.

39. Singh, V., et al., *Downregulation of Rv0189c, encoding a dihydroxyacid dehydratase, affects growth of Mycobacterium tuberculosis in vitro and in mice.* Microbiology, 2011. **157**(Pt 1): p. 38-46.

40. Oliver, J.D., et al., *The Aspergillus fumigatus dihydroxyacid*

dehydratase Ilv3A/IlvC is required for full virulence. PLoS One, 2012. **7**(9): p. e43559.

41. Lewis, A.M., C. Waterhouse, and L.S. Jacobs, *Whole-blood and plasma amino acid analysis: gas-liquid and cation-exchange chromatography compared.* Clin Chem, 1980. **26**(2): p. 271-6.

42. Le Boucher, J., et al., *Amino acid determination in biological fluids by automated ion-exchange chromatography: performance of Hitachi L-8500A.* Clin Chem, 1997. **43**(8 Pt 1): p. 1421-8.

43. Petro, T.M. and J.K. Bhattacharjee, *Effect of dietary essential amino acid limitations upon the susceptibility to Salmonella typhimurium and the effect upon humoral and cellular immune responses in mice.* Infect Immun, 1981. **32**(1): p. 251-9.

44. Bordbar, A., et al., *Model-driven multi-omic data analysis elucidates metabolic immunomodulators of macrophage activation.* Mol Syst Biol, 2012. **8**: p. 558.

45. McCollister, B.D., et al., *N(2)0(3) enhances the nitrosative potential of IFNgamma-primed macrophages in response to Salmonella.* Immunobiology, 2007. **212**(9-10): p. 759-69.

ABSTRACT

Branched-chain amino acid supplementation promotes aerobic growth of *Salmonella* Typhimurium under nitrosative stress conditions

Park, Yoon-Mee

Advisor : Prof. Bang, Iel-Soo, PhD

Department of Dental Bioengineering,

Graduate School of Chosun University

Nitric oxide (NO) inactivates iron-sulfur enzymes in bacterial amino acid biosynthetic pathways, causing amino acid auxotrophy. I demonstrate that exogenous supplementation with branched-chain amino acids (BCAA) can restore the NO resistance of *hmp* mutant *Salmonella* Typhimurium lacking principal NO-metabolizing enzyme flavohemoglobin, and of mutants further lacking iron-sulfur enzymes dihydroxy-acid dehydratase (IlvD) and isopropylmalate isomerase (LeuCD) that are essential for BCAA biosynthesis, in an oxygen-dependent manner. BCAA supplementation did not affect the NO consumption rate of *S.* Typhimurium, suggesting the BCAA-

promoted NO resistance independent of NO metabolism. BCAA supplementation also induced intracellular survival of *ilvD* and *leuCD* mutants at wild-type levels inside RAW 264.7 macrophages that produce constant amounts of NO regardless of varied supplemental BCAA concentrations. Our results suggest that the NO-induced BCAA auxotrophy of *Salmonella*, due to inactivation of iron-sulfur enzymes for BCAA biosynthesis, could be rescued by bacterial taking up exogenous BCAA available in oxic environments.

Chapter Ⅳ.

Nitric oxide represses the transcription of the glutamine synthetase gene *glnA*, which causes glutamine auxotrophy in *Salmonella* Typhimurium

국문초록

글루타민 합성효소인 *glnA*의 발현은 산화질소에 의해 억제되고 살모넬라에서 글라타민 영양요구를 유발

박 윤 미

조선대학교 대학원

치의생명공학과

지도교수 : 방 일 수

산화질소는 다양한 박테리아의 분자에 영향을 주어 비정상적인 대사와 성장을 보여준다. 하지만 박테리아 에서의 산화질소 타겟과 산화스트레스로부터 피하기 위한 전략은 아직 알려지지 않았다. 이전 논문에서 우리는 살모넬라균의 *hmp* 돌연변이균주에서 산화질소는 아미노산 영양요구성을 나타내고 박테리아 내부로 유입된 산화질소를 거의 대사할 수 있음을 보여주었다. 이 논문에서는 외부에서 첨가하는 glutamine과 glutamate가 산화질소가 노출된 배지에서 자라는 살모넬라의 *hmp* 돌연변이 균주의 성장을 최대로 성장시킴을 확인하였다. Gln과 Glu의 생합성은 밀접한 관련이 있기 때문에 우리는 Gln과 Glu 생합성에 관련되어 있는 유전자를 결핍시킨 *hmp* 돌연변이 균주의 성장을 통해서 Gln이 그 들의 성장에 필요함을 확인하였다.

오직 L-Gln만이 박테리아 성장을 회복시켰는데 이 결과는 박테리아 세포벽 합성과 성장회복과는 관계가 적음을 보여준다. 야생형균주와 *hmp* 돌연변이 균주의 산화질소 소비율에서 Gln의 첨가는 영향을 주지 않았는데 이 결과는 Gln에 의한 산화질소 저항성은 산화질소 대사와는 독립적으로 이루어짐을 알 수 있다. 이 결과들로 세포독성의 산화질소는 살모넬라의 Gln 레벨유지를 손상시켜, 그 결과로 Gln의 영양요구성을 가져올 수 있으나 주변의 Gln을 흡수함으로써 회복될 수 있을 것이라 여겨진다.

A. Introduction

Salmonella is a representative gastrointestinal pathogen that infects animal hosts through contamination of food or water. Proliferation of *Salmonella enterica* serovar Typhimurium can be restricted by nitric oxide (NO) produced in host cells as a component of innate immune responses, as NO and NO-derived reactive nitrogen species (RNS) targets multiple bacterial macromolecules, causing abnormal metabolism [1]. Against nitrosative stress, enteric pathogens including *Salmonella* have evolved to conserve the NO-metabolizing enzyme flavohemoglobin Hmp that detoxifies almost all NO entering into pathogens. So, the growth of *hmp* mutant *S.* Typhimurium lacking flavohemoglobin is delayed in media containing NO [2]. In previous studies, I have found that, without detoxification by flavohemoglobin, NO and RNS can cause amino acid auxotrophy in *S.* Typhimurium that can be recovered by supplying exogenous amino acids [3, 4]. In this study, testing the effects of single amino acid supplementation on the NO-caused amino acid auxotrophy revealed glutamine (Gln) and glutamate (Glu) as major amino acids required for rescuing amino acid auxotrophy. Gln and Glu serve as the main nitrogen metabolic sources for bacterial replication, and their biosynthesis and uptake are precisely regulated in bacterial response to availability and demand [5]. To further confirm the function of Gln and Glu in *Salmonella* resistance against nitrosative stress, I constructed mutant *S.*

Typhimurium lacking genes for the biosynthesis and interconversion of Gln and Glu and the regulation of their expression, then tested their replication in cultures containing Gln or Glu in the present or absent of NO. Further, I analyzed the effect of these amino acids on NO-metabolism and the effect of NO on the regulation of gene expression for Gln/Glu biosynthesis in *S.* Typhimurium. Data presented here show that Gln is needed for *Salmonella* replication under NO-producing conditions.

B. Materials and Methods

1. Bacterial strains, growth conditions, and chemicals

Salmonella enterica serover Typhimurium strains used in this study are shown in Table 1. *S.* Typhimurium 14028s was used as the wild-type (WT) parental strain. The bacterial cultures were grown overnight in Luria-Bertani (LB) broth (Difco, Detroit, MI) or minimal E media (1.66 mM $MgSO_4$, 9.5 mM citric acid monohydrate, 57 mM K_2HPO_4, 16.7 mM $NaNH_3PO_4$) [6], supplemented with 0.2% glucose (0.2% E.glucose) in the presence of kanamycin (50 μg/ml; KM), chloramphenicol (50 μg/ml; CM), or ampicillin (100 μg/ml; AP), and incubated at 37℃ with 220 rpm shaking. All amino acids purchased from Sigma-Aldrich (St. Louis, MO) were used at 2 mM concentration in the media. S-nitrosoglutathione (GSNO) was used as an NO donor, synthesized from the reaction of glutathione and acidified sodium nitrite as described previously [7].

Table 1. Bacterial strains, plasmids used in this study

Strains	Genotype	Source or References
IB1	*Salmonella enterica* serovar Typhimurium 14028s	ATCC
IB3	*hmp* ::KM	[8]
IB423	*glnA*-lacZ ::KM	[9]
IB1077	*glnA* :: CM *hmp* :: KM	This study
IB1090	*hmp* :: KM *gltB* :: CM	This study
IB1091	*hmp* :: KM *glnB* :: CM	This study
IB1092	*hmp* :: KM *glnD* :: CM	This study
IB1093	*hmp* :: KM *gltD* :: CM	This study
IB1110	*hmp* :: KM *glnK* :: CM	This study
IB1165	*hmp* :: KM *glnG* :: CM	This study
IB1166	*hmp* :: KM *glnL* :: CM	This study
IB1920	*hmp* :: KM *glnA*-lacZ ::KM	This study
IB1951	*hmp* *glnL* :: CM *glnA*-lacZ ::KM	This study
IB1952	*hmp* *glnG* :: CM *glnA*-lacZ ::KM	This study
plasmids	**Characteristics**	**Source or References**
pKD3	Plasmid carrying the FRT-Cm^r-FRT cassette	[10]
pKD4	Plasmid carrying the FRT-Km^r-FRT cassette	[10]
pTP233	Plasmid encoding IPTG-inducible Lamda red recombinase	[11]
pCP20	Temperate-sensitive(30℃) replicoation plasmid encoding the recombinase gene	[10]

Table 2. Primer sequences and used in this study

Primer	Sequence (5'-3')
Mutant construction	
glnA-P1	acgagatcttcaattctgctggccgtgtcgccgtaaaaatgtgtaggctggagctgcttc
glnA-P2	accccgcacccggtagagtttgagctgtactacagcgtttcatatgaatatcctccttag
glnA-FW	gtggaaactttcagcccatc
glnA-Rev	cacattggtgcaacagaacg
glnB-P1	acctagccacaaggtagtgcgtaccgccatacacgcgctggtgtaggctggagctgcttc
glnB-P2	ctgcacgtgctcggtgatcagaccgcgcagatgctcttcgcatatgaatatcctccttag
glnB-FW	gcgaatgcgaggtaagcgta
glnB-Rev	ctggcgcatgccggtgatatc
glnB-P1	ctggccgaggtaggtattaccggcatgacggtcactgaagcatatgaatatcctccttag
glnB-P2	gtcgtcttcttcgccagtacggatacgaataacgcgggcggtgtaggctggagctgcttc
glnB-Fw	ccgcacggaattctataagc
glnB-Rev	attgtcgacctgagatgggtt
glnD-P1	cagcggcatttgtgctaacagcttattgaagcccgaacccatatgaatatcctccttag
glnD-P2	ccagcagaccaggttgatcgagcgcgatcaattccatgaagtgtaggctggagctgcttc
glnD-Fw	cgtgactggagtgaacgagc
glnD-Rev	cgcaccgtcgacaacctggtt
glnD-P1	ctactgcgagtggaaatgtccggtacataactacatcccggtgtaggctggagctgcttc
glnD-P2	ataattcgtccctgcgagtccagttccacgctgtgtttcgcatatgaatatcctccttag
glnD-FW	atctgcgcggtctgatcacc
glnD-Rev	caccataagcttctctggcttc
glnK-P1	gggactgaccgtcaccgaagtgaaagggtttggccgtcaggtgtaggctggagctgcttc
glnK-P2	atatcgatcacctcttccagttggtcgtcggcgatagccacatatgaatatcctccttag
glnK-Fw	atatccattcagaagagtcc

glnK-Rev	acgttcttggcgcgaattcat
glnL-P1	cgacaggctacgcaaccttgtcgatcgtttgctggggccgcatatgaatatcctccttag
glnL-P2	gaaggcggtacgggtacgcagcgtaatttcgccgccttccgtgtaggctggagctgcttc
glnL-Fw	ctgagcacatccccaggagc
glnL-Rev	ggtacaggtcgaccctgcc
glnG-P1	gatttgattgaatccgaactgtttggccatgagaaaggcg catatgaatatcctccttag
glnG-P2	ccacgcctaattcacgggcggcgacctgcaaaaaatggcgtgtaggctggagctgcttc
glnG-Fw	acccgatggtcagcggtcg
glnG-Rev	gtgtggctgtcgaccgctc
RT-PCR	
glnA-Fw	cagcgcaatatacgcatcga
glnA-Rev	cggtctggacgggattaaga
gltB-Fw	cagcaggaaaccctgtcgat
gltB-Rev	ttcgacaggctgcatacgta
gltD-Fw	cgttgatccgccgaagaaac
gltD-Rev	atacgcccttcgttagccag

2. Construction of *S.* Typhimurium

S. Typhimurium mutants were constructed using the PCR-mediated gene mutation method of the λ Red recombinase system [10]. All primers used for the mutations in *Salmonella* are listed in Table 2. For mutant strain construction, kanamycin and chloramphenicol resistance cassettes were used from pKD4 and pKD3, respectively. All deletion mutations constructed were transduced into a fresh WT 14028s *Salmonella* strain background with bacteriophage P22 HT105/1 *int*., and nonlysogenic colonies were selected for further studies by testing the sensitivity of each transductant to a lytic P22 variant H5. PCR was performed with a primer pair to confirm the insertion of the antibiotic resistance site. To combine gene mutations with another strain, CM and KM antibiotic cassettes in the host strains were removed using FLP recombinase encoded by the pCP20 plasmid. Additional gene mutations were then progressively introduced by P22 transduction, as described above.

3. Disk diffusion assay

To measure the effect of GSNO on glutamine, the disk diffusion method was performed by placing cotton discs (paper disc; 6 mm, Toyo Roshi Kaisha, Ltd) on the 1.5% minimal E-glucose agar plate. The bacterial suspension (100 μl) at an optical density (OD_{600nm}) of 0.01 was inoculated on E-glucose agar plates. The agar plates with inoculated cells were

dried for 20 min. The cotton discs were placed on the plates using forceps. Then, 15 μl of 500 mM GSNO was applied via droplets onto the cotton discs. The agar pates were incubated at 37℃ in an ambient air incubator for 16 h. The zone radius of GSNO inhibition was measured with a pair of calipers.

4. Measurement of NO consumption rate

The cultured *Salmonella* cells in LB were resuspended in 10 ml fresh minimal E.glucose (0.2%) media at 37℃ until log phase (OD_{600nm} ~0.8) and washed once with PBS. NO consumption rate was measured by detecting the remaining NO in bacteria after supplementing the bacteria solution with 2 μM ProliNONOate (Cayman Chemical, Ann Arbor, MI). The bacterial solution was continuously stirred with a magnetic stirring bar. The data signal of NO concentration was measured using an NO electrode [ISO-NOP sensor; World Precision Instruments (WPI) Inc, Sarasota, USA] attached to a free radical analyzer (TBR4100; WPI Inc.), and the program used was LabChart (A Instruments, Colorado, Springs, CO, USA). Data are expressed as μM of NO.

5. Measurement of Growth Kinetics

Overnight *Salmonella* cells in LB broth were diluted in PBS at an OD_{600nm} of 0.1, and then inoculated in a honeycomb microplate containing fresh

minimal 0.2% E.glucose media with the presence or absence of GSNO and amino acids. The growth kinetics were measured by determining the OD_{600nm} with a Bioscreen C Microbiology Microplate Reader (Transgalactic Ltd, Finland) every 30 min at 37℃.

6. Quantification of glutamine-associated gene transcription by real-time reverse transcription PCR (RT-PCR)

Bacteria were grown in 15 ml 0.2% E-glucose at 37℃ until an OD_{600nm} of ~0.5, and then treated with 1 mM of GSNO for 1 h at 37℃ with shaking. Gene transcription was stopped by adding a 1:5 ratio of ice-cold phenol/ethanol (5% phenol/95% EtOH, v/v) solution for 20 min in ice. Harvested cells were obtained by centrifugation for 10 min at 4,000 rpm. The precipitated cells were incubated with 10 mg/ml lysozyme (Sigma-Aldrich, St. Louis, Mo, USA) in TE buffer [10 mM Tris-HCl, 1 mM EDTA (ethylene diamine tetra-acetic acid); pH 8.0] at 37℃ and vortexed every 10 min for 30 min. Total RNA was extracted by the RNeasy® Plus mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. To measure the gene transcription levels of glutamine-associated genes, RT-PCR was performed using a TOPreal™ One-step RT qPCR kit [SYBR Green *with low ROX* (Enzynomics, Daejeon, Korea)] according to the manufacturer's instruction. The *rpoD* gene was used as a housekeeping gene control for the normalization of gene expression. DNA sequences of

the primer pairs used in this study are as listed in Table 2.

7. β-galactosidase assay

The overnight cultured *Salmonella* cells in LB were inoculated in fresh minimal E.glucose (0.2%) media, and then cultured at 37℃ until log phase (OD_{600nm} ~0.5). The metabolism of precipitated cell samples was stopped by transferring samples to ice for 20 min prior to analysis. For measurement, 100 μl of samples were placed in 900 μl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol); cells were permeabilized by adding 30 μl chloroform and 10 μl 0.1% SDS, and vortexed for 10s. These samples were incubated in a 37℃ water bath for 5 min, and 200 μl of ONPG (4 mg/ml Orthonitrophenyl-β-D-galactose in 0.1 M potassium phosphate buffer) was added. The reaction was stopped by adding 500 μl of 1 M Na_2CO_3. β-galactosidase values represent the average of three experiments.

C. Results

1. Supplemetation with Gln or Glu recovers the growth of *hmp* mutant *S.* Typhimurium in NO-producing cultures

Supplementation with casamino acids (CAA) into culture media greatly reduced the GSNO inhibition zone of WT *S.* Typhimurium (Fig. 1a-b). This CAA effect was also observed in *hmp* mutant *S.* Typhimurium lacking the major NO-metabolizing enzyme flavohemoglobin Hmp, as observed in the previous study using the liquid culture (Fig. 1 c-d [4]). These results suggest that nitrosative stress may cause deficiency in some amino acids in *S.* Typhimurium. I selected the liquid culture of *hmp* mutant *S.* Typhimurium as a model system for further study, because the phenotype for NO resistance was greatest and more specific to the NO-induced bacterial damages. To examine whether any single amino acid is responsible for the growth recovery under nirosative stress conditions, I tested the effects of supplementation with a single amino acid on the growth of *hmp* mutant in GSNO-containing cultures. As shown in Fig 2, out of 20 amino acids tested, supplementations with glutamine (Gln), glutamate (Glu), or cysteine (Cys) were able to fully recover the *Salmonella* growth in GSNO-containing cultures. Cys has been known as a general NO scavenger because the thiol residue of Cys readily reacts with NO [12]. Our results newly determined that supplementation of Gln or Glu

play major roles in rescue of the *Salmonella* growth under nitrosative conditions, suggesting that NO may cause an interruption to the homeostasis of Gln and Glu levels in *Salmonella*.

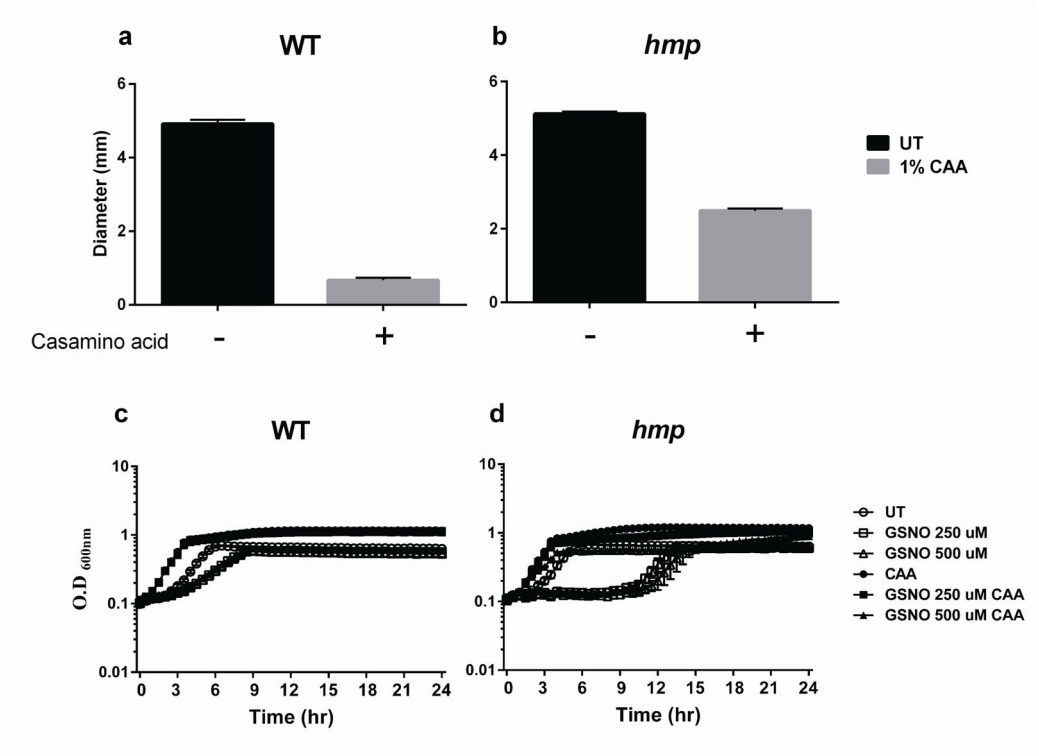


Figure 1. The effect of casamino acid (CAA) supplementation on *S.* Typhimurium resistance against NO.

The susceptibility of WT and *hmp* mutant of *S.* Typhimurium to NO was measured **(a-b)** by paper disc diffusion assays and **(c-d)** by monitoring bacterial growth curve. For disc diffusion assays, overnight bacterial cultures were diluted and spread onto minimal E-glucose agar plates (0.2% glucose) supplemented with (*black bar*) or without (*gray bar*) 1% CAA. Then, 15 μl of GSNO (500 mM) was applied via droplets onto each paper disc. The inhibition zone was measured after 16 h of growth at 37℃ and expressed as the mean diameter ± standard deviation (SD) of three independent experiments. The bacterial growth curves of WT and *hmp* mutant of *Salmonella* were measured in minimal E.glucose media (*circle*) supplemented with (*open symbols*) or without (*filled symbols*) 1% CAA, in the presence of 250 μM (*rectangles*) or 500 μM (*triangles*) GSNO. The optical density of bacterial cultures was measured on a Bioscreen C Microbiology Microplate Reader at 37℃ for 24 h. Data shown are the mean O.D. ± SD from three independent cultures. UT, untreated

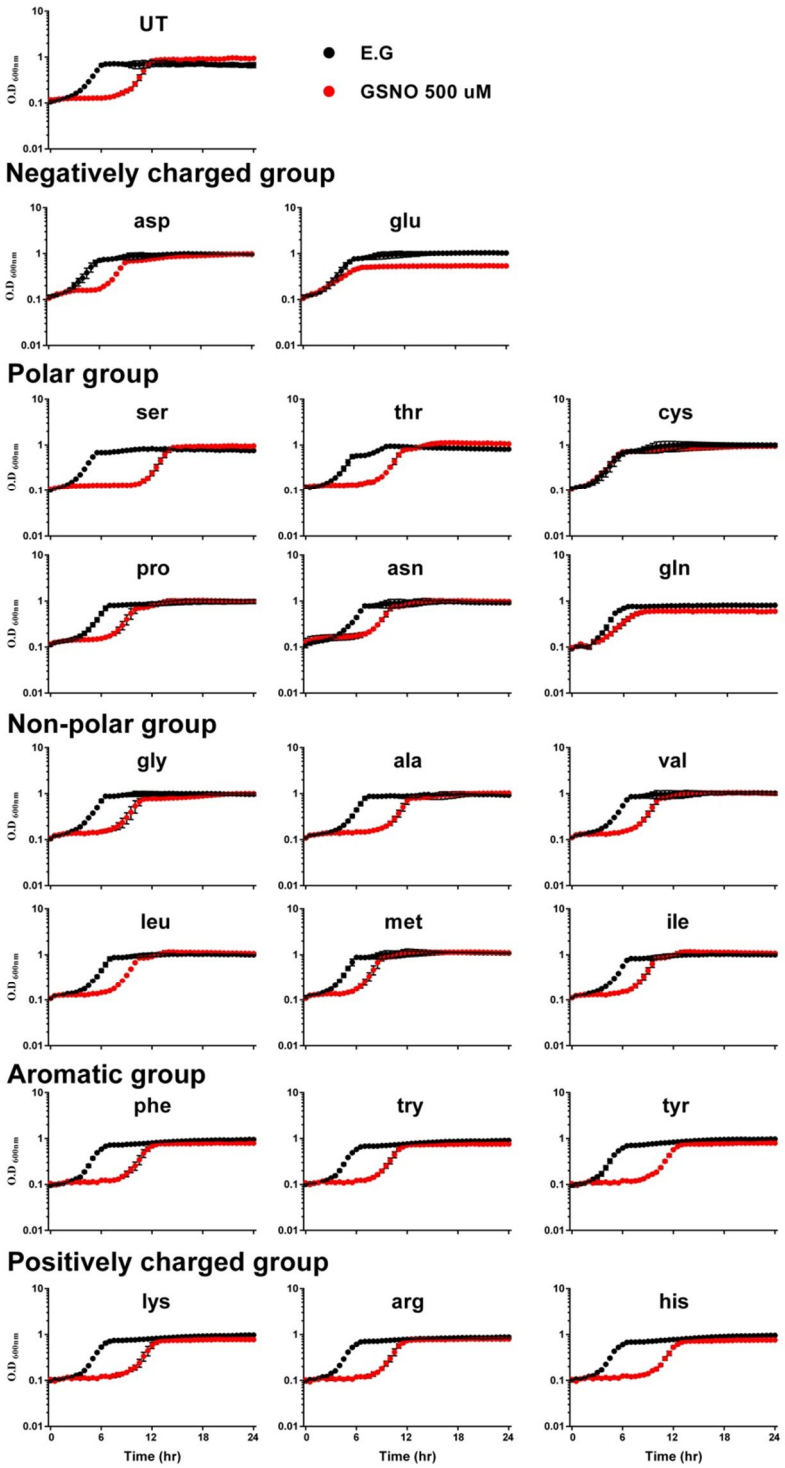


Figure 2. The effect of amino acid supplementation on the growth of *S.* Typhimurium under nitrosative stress.

The *hmp* mutant of *S.* Typhimurium was cultured in minimal E.glucose media supplemented with or without each single amino acid (2 mM), in the absence (*black symbols*) or presence of (*red symbols*) 500 μM GSNO. Bacterial growth was monitored as described previously. UT, untreated

2. Conversion of Glu to Gln is required for *Salmonella* growth in NO-producing cultures

Gln and Glu are main sources for bacterial nitrogen metabolism [13]. In bacterial nitrogen assimilation, the exogenous nitrogen source ammonia is converted to Glu and Gln by glutamate dehydrogenase (GDH) and glutamine synthetase (GS), respectively. Glu and Gln can also be interconverted by GS and glutamate synthetase (GOGAT) (Fig. 3a). Thus, it is plausible that the requirement of Glu or Gln for the *Salmonella* growth in NO-producing cultures could be the result of an NO-induced interruption to these conversion cycles for either Glu or Gln. To examine which conversion reaction is responsible, the growth of *hmp* mutants *S.* Typhimurium further lacking genes encoding GS (*glnA*) and GOGAT (*gltB*/*gltD*) was compared in GSNO-containing cultures supplemented with Gln or Glu (Fig. 3c). In the absence of GSNO, the growth of all mutants was fully recovered by supplementation with Gln and Glu. In GSNO-containing cultures, Gln-supplemented *Salmonella* strains could grow with a normal growth rate, while in Glu-supplemented cultures, all mutants showed long lag phases. This result clearly indicates that Gln rather than Glu is required for recovering *Salmonella* growth in NO-producing cultures, and suggests that the Glu-supplemented growth recovery may relate to the reactions converting Glu to Gln.

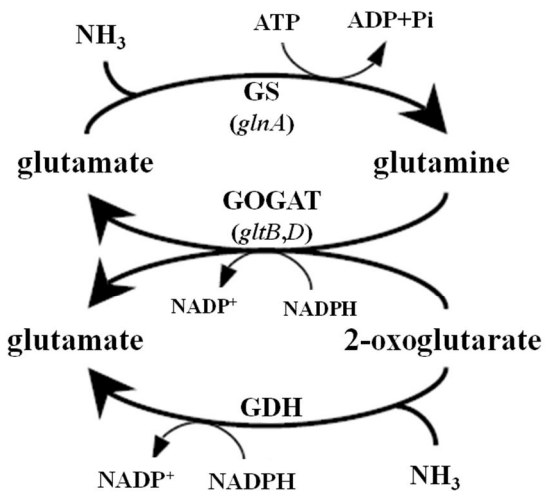
To confirm the Gln-dependence to *Salmonella* NO resistance, I further tested the growth of *hmp* mutants *S.* Typhimurium that were also deficient in P_{II} (GlnB), a P_{II} paralog (GlnK), and UTase (GlnD). The concerted regulatory function of PII and UTase is central for bacterial sensing of the intracellular availability of nitrogen sources and for regulating the GS activity and the NRI/NRII two-component signal transduction system that can coordinate the transcription of genes including *glnA* for bacterial nitrogen homeostasis (Fig. 3b, [13]). Supplementation with Glu failed to recover the growth of *glnB*, *glnK*, and *glnD* mutants in GSNO-containing cultures, while Gln supplementation fully recovered bacterial growth (Fig. 3c). This result indicates that the Glu-supplemented growth of *Salmonella* strictly requires the PII/UTase-mediated signal transduction system, suggesting the importance of GS regulation under nitrosative conditions. It further suggests that, in the presence of NO, the regulated activities of corresponding enzymes for the conversion of Glu to Gln may largely contribute to the *Salmonella* replication.

Of note, supplementation with D-stereoisomer of Gln did not lead to the growth recovery of the *hmp* mutant and WT *Salmonella* in both solid and liquid cultures (Fig. 4), suggesting that the Gln-mediated NO resistance is not related to the bacterial cell wall synthesis.

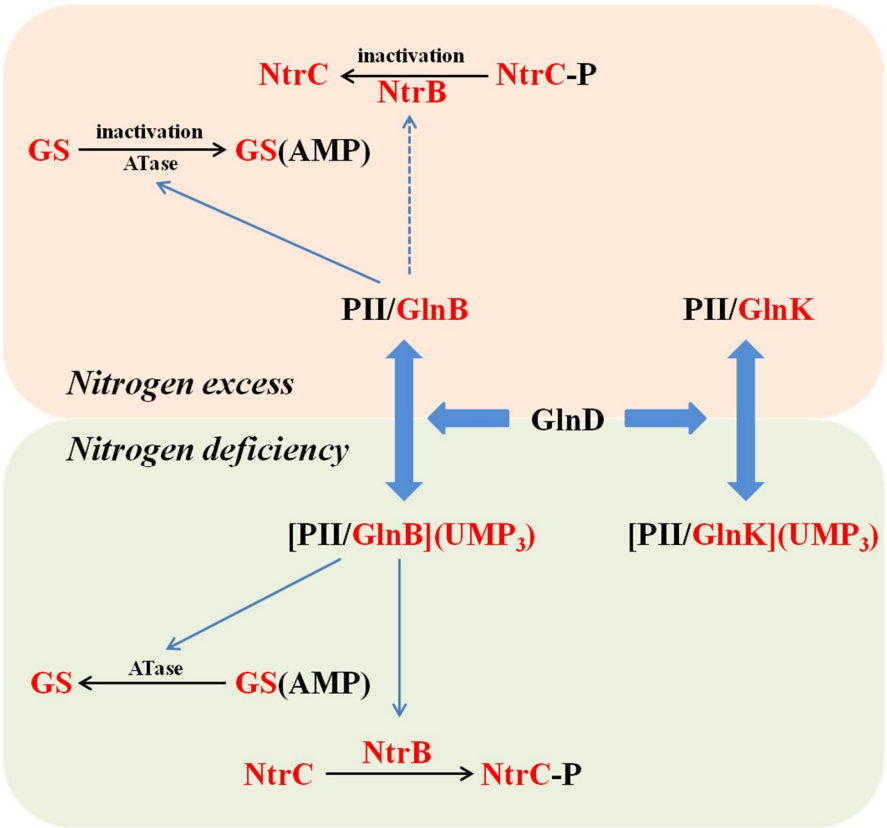
Taken together, the results demonstrate that *Salmonella* replication requires Gln under nitrosative stress conditions, suggesting that NO may

cause a Gln deficiency in *Salmonella*.

a



b



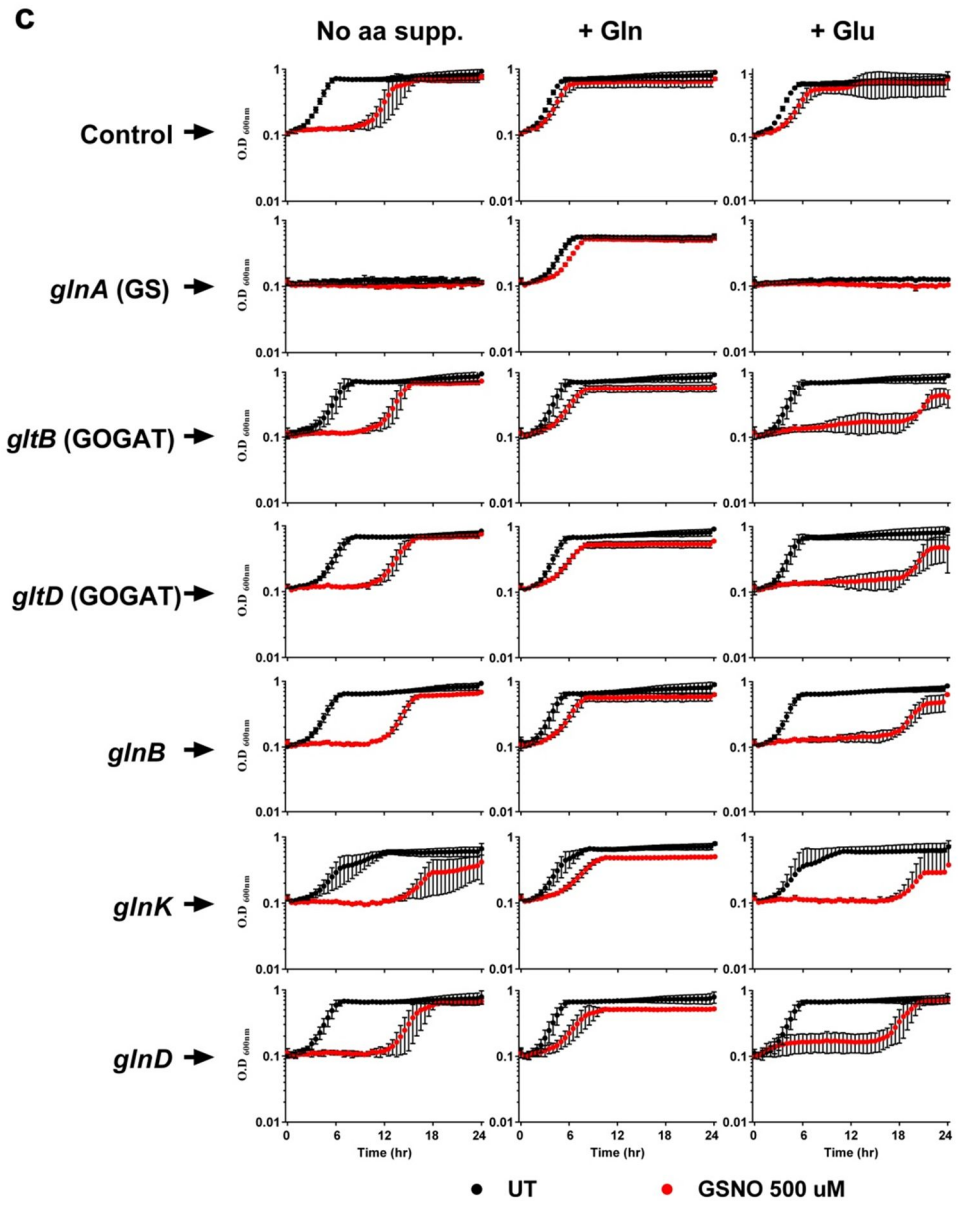


Figure 3. Conversion of Glu to Gln is required for the growth of *S.* Typhimurium under nitrosative stress.

(a) The three main enzymes for bacterial nitrogen metabolism: glutamine synthetase (GS, encoded by *glnA*), glutamate synthase (GOGAT, encoded by *gltB/D*), and glutamate dehydrogenase (GDH). Glutamate and glutamine can be synthesized by the action of these enzymes [5]. **(b)** The enzymes in response to nitrogen stress: bifunctional adenylyltransferase enzyme (ATase), regulatory protein for nitrogen assimilation (GlnB-K-type PII, encoded by *glnB* and *glnK*), and Uridylyltransferase/Uridylyl-removing enzyme (Utase;UT/UR encoded by *glnD*) [14]. **(c)** The growths of *hmp* mutant of *S.* Typhimurium and of mutants lacking additional genes for the interconversion of Gln and Glu were monitored in cultures of minimal E.glucose media supplemented with or without Gln or Glu (2 mM), in the absence (*black symbols*) or presence of (*red symbols*) 500 μM GSNO.

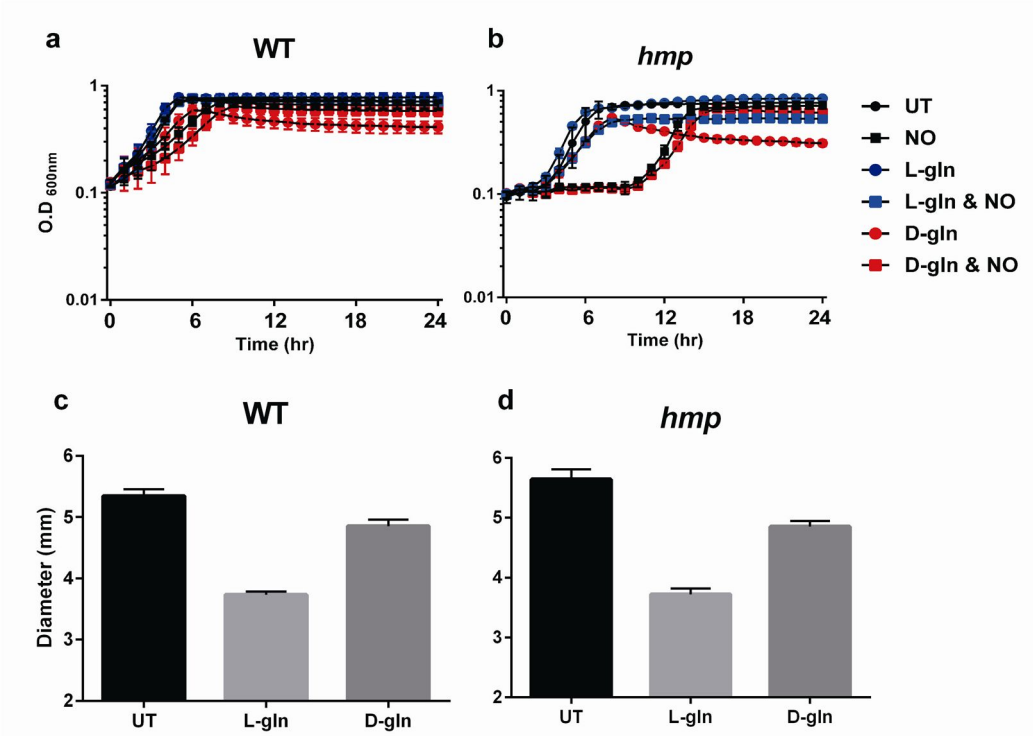


Figure 4. L-Gln but not D-Gln is required for the growth of *S.* Typhimurium under nitrosative stress.

(a-b) The growth of WT and *hmp* mutant of *S.* Typhimurium was monitored in cultures of minimal E.glucose media supplemented with either L-Gln or D-Gln (2 mM each), in the presence or absence of GSNO. **(c-d)** For disc diffusion assays, the overnight cells of WT and *hmp* mutant of *S.* Typhimurium were diluted and spread onto minimal E.glucose agar plates supplemented L-gln or D-gln. Then, 15 μl of GSNO (500 mM) was applied via droplets onto each paper disc. The inhibition zone was measured after 16 h of growth at 37℃, and expressed as the mean diameter±standard deviation (SD) of three independent experiments.

3. Gln supplementation does not change the NO consumption rate of *S.* Typhimurium

Bacterial NO resistance is largely dependent on the denitrosylase and NO reductase activity of the flavohemoglobin Hmp [15, 16]. In *S.* Typhimurium, the *hmp* gene included in NsrR regulon mostly contribute to the NO metabolism and subsequent NO resistance [2, 17]. To examine whether Gln supplementation can affect NO metabolism of *Salmonella*, I measured the NO consumption rate of WT and *hmp* mutant *S.* Typhimurium cultured in the presence and absence of exogenous Gln (Fig. 5). As observed in our previous study [3], WT *Salmonella* rapidly consumed all of NO in 30 seconds while the NO consumption rate of *hmp* mutant paralleled that of buffer control. However, supplementation of Gln to the culture did not lead to any change in the NO consumption rate of both WT and *hmp* mutant *S.* Typhimurium, demonstrating that Gln supplementation causes no alteration in NO metabolism itself.

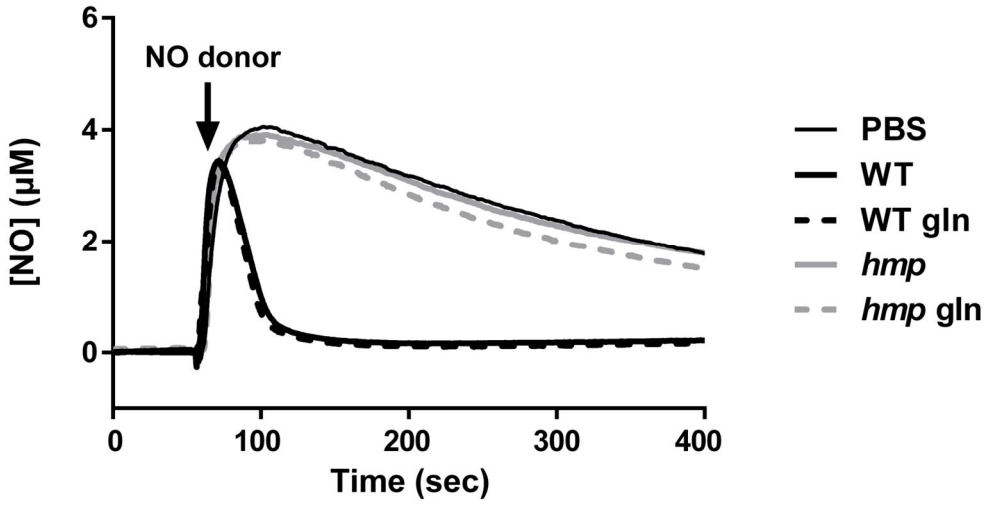


Figure 5. Comparison of the NO consumption rate of *S.* Typhimurium in the presence or absence of Gln supplementation.

The same amount of *Salmonella* cultures were grown until mid-log phase (OD_{600nm} ~0.8) in minimal E.glucose media supplemented with (*dotted*) or without (*solid*) Gln, diluted in PBS buffer. The NO consumption of WT (*black*) and *hmp* (*gray*) mutant of *Salmonella* strains was recorded with an NO-sensitive electrode connected to a free radical analyzer after addition of the fast-releasing NO donor, ProliNONOate (2 μM), as indicated by the *arrow*. PBS buffer (*thin black*) was used as a control. Results of a representative of three independent experiments are shown.

4. *glnA* transcription is repressed under nitrosative conditions in an NRI/NRII-dependent manner

To determine if NO can affect the regulation on GS expression, a quantitative real-time PCR analysis and the β-galactosidase assay using a merodiploid *glnA*-lacZ operon fusion were employed to monitor *glnA* transcription. As shown in Fig. 6a, *glnA*, *gltB*, and *gltD* transcription were repressed 53, 20, and 22 fold by exposure to GSNO, respectively, and in Fig.6c, *glnA*-lacZ activity was also decreased 2.4 fold in GSNO-containing cultures as compared with cultures without GSNO in *hmp* mutant. Notably, taking into consideration that β-galactosidase activity results from the cumulative levels of the enzyme expressed from lacZ fusion in the cell, reduction of the *glnA*-lacZ expression in GSNO-containing cultures that reached to the same optical density (14h) as cultures without GSNO (6h) practically shows the persistent repression of *glnA* transcription under nitrosative conditions.

Next, to examine the role of regulators for *glnA* transcription under nitrosative conditions, I measured *glnA* transcription in *hmp* mutants lacking *glnB*, *glnD*, *glnL* (encoding NRI), or *glnG* (encoding NRII) cultured in the presence and absence of GSNO. In *glnB* and *glnD* mutants, *glnA* mRNA levels were also decreased by exposure to GSNO; however, these changes were abolished in *glnL* and *glnG* mutants (Fig. 6b). The *glnA*-lacZ

expression in *glnL* and *glnG* mutants was not considerably changed, either (Fig. 6c). The results demonstrate that NO can repress *glnA* transcription in which NO-induced alterations in the activity of the NRI/NRII two-component signal transduction system play important roles.

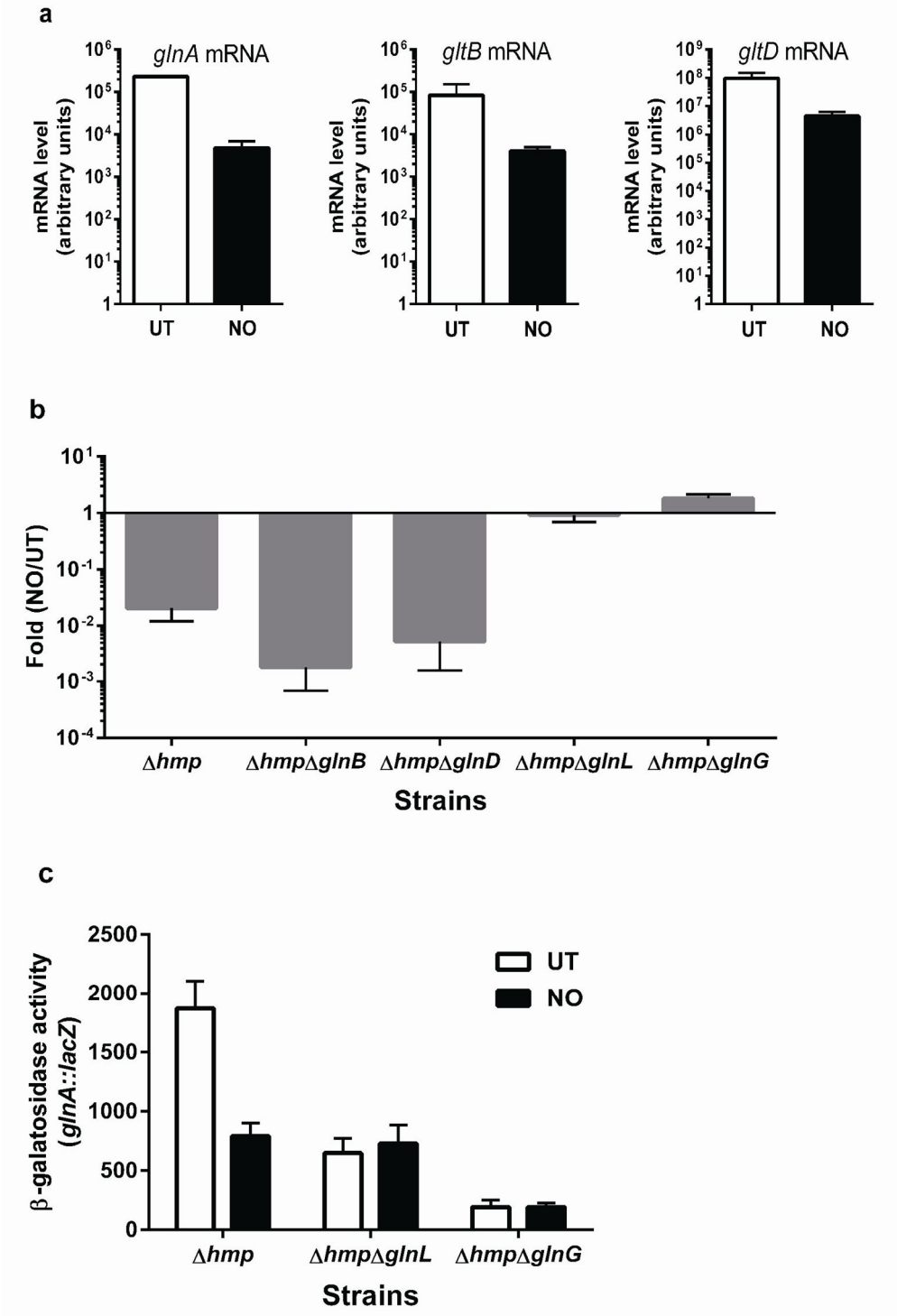


Figure 6. The effect of NO on the expression of *glnA*, *gltB*, and *gltD*.

For RT-PCR assays, *S.* Typhimurium grown until log phase (OD_{600nm} ~0.5) were untreated (*white bar*) or treated (*black bar*) with 1 mM GSNO for 1 h in minimal E.glucose media. Total RNA was purified before and after treatment with GSNO (1 mM). RNA was isolated and mRNA levels of *glnA*, *gltB* and *gltD* were measured by RT-PCR analysis. **(a)** mRNA levels of *glnA*, *gltB* and *gltD* were measured in *hmp* mutant *S.* Typhimurium. **(b)** The expression of *glnA* in the *hmp*, *hmp glnB*, *hmp glnD*, *hmp glnL*, and *hmp glnG* mutants *S.* Typhimurium. The fold induction rates of NO treated with UT are shown. The housekeeping gene *rpoD* was used for the normalization of transcript levels. **(c)** Expression of *glnA*::lacZ fusions. Strains with mutations in *hmp*, *hmp glnL*, and *hmp glnG* including *glnA*::lacZ fusions were assayed for β-galactosidase activity using growth cells until log phase (OD_{600nm} ~0.5), with (*black bar*) or without (*white bar*) 250 μM GSNO. Data are representative of three independent experiments.

D. Discussion

ROS and RNS produced by host phagocytes can inhibit the proliferation of *Salmonella* as innate immune response. To survive and replicate in the host, *Salmonella* Typhimurium must be resistant to nitric oxide. Therefore, I confirm the importance of amino acids for replication under nitrosative stress [2, 18].

ROS, including superoxide and hydrogen peroxide, cause bactericidal action in bacteria within 6hrs. In ROS detoxification, *salmonella* uses enzymes such as superoxide dismutases (SODs), catalases, and peroxidases [2, 19, 20]. In SOD mutant not for detoxification, reveals amino acid auxotrophy in *Escherichia coli* and yeast [21, 22], ostensibly auxotrophies for branched chain, sulfur-containing, and aromatic amino acids in *E. coli* [23]. ROS and RNS have similar metabolisms; for example, they are made in phagocytes, attack the iron-sulfur clusters, and reduce NADPH as substrate [18]. However, most bacterial metabolic alterations caused by NO remain poorly understood. Therefore, we expected that RNS would induce amino acid auxotrophy and deficiency.

Our data shows that Flavohemoglobin Hmp mutant was recovered by adding glutamine and glutamine, even if treated with nitric oxide (Fig. 2). In previous studies, *S.* Typhmurium is known to be prototrophic for all amino acids, but *salmonella* have NO-inducible methionine (M) and lysine (K)

auxotrophy because they cannot synthesize M or K under nitrosative stress [24]. The $ONOO^-$ and nitric oxide directly react to sulfur residue of cysteine *in vitro* and *in vivo* [12, 25]. Supplementation of cysteine is effective for growth of *E. coli* in aerobic conditions may help in repairing the NO-modified iron-sulfur cluster proteins [26, 27]. Moreover, in my previous study, BCAA (Branched-chain amino acid) supplementation promoted growth of *hmp* mutant in *Salmonella* Typhimurium under nitrosative stress conditions [3, 4]. The auxotrophy of amino acid mutant strains attenuated intracellular proliferation in epithelial HeLa cells and macrophage-like RAW 264.7 cells, and there was recovery of replication by supplementation with amino acids [28]. The mutant strain of glutamine biosynthesis and transporter in *S.* Typhimurium attenuated intracellular survival in mic e[29]. Thus, it seems that amino acids are important for intracellular survival and replication in the host.

In this study, the transcription of *glnA*, *gltB*, and *gltD* gene was depressed under nitrosative stress (Fig. 6a), but the transcription of *glnA* in *hmp glnL* and *hmp glnG* mutants was no different between UT and NO treated (Fig. 6b). These results shows that down-expression of *glnA* in NO-exposed glutamine-associated gene mutants was affected by the GlnL/GlnG two-component system in *hmp* background mutant.

In general, previously studies have shown that expression of glutamine-associated genes involved in glutamine biosynthesis and transporter and

nitrogen regulation is down-regulated in RNS (nitrite, nitrate, and NO) other bacteria as shown in Table 3. In *E. coli*, *Salmonella enterica* Serovar Typhimurium, expression of *glnA* gene decreased more than when untreated with NO [30, 31], but this level was increased in *Desulfovibrio vulgaris* [32]. Moreover, in *Shewanella oneidensis* [33], *Salmonella* Typhimurium [31] showed low expression of *gltB* and *gltD*, similar to our data (Fig. 6a). These results show that RNS affect related glutamine expression in several bacteria.

In summary, amino acid supplementation can promote the growth of *S.* Typhimurium under nitrosative stress conditions, indicating that NO can cause amino acid auxotrophy. Among 20 amino acids tested, supplementation with Gln or Glu majorly contributed to preventing the NO-mediated amino acid auxotrophy of *S.* Typhimurium. Mutation studies with genes for interconversion between Gln and Glu revealed that Gln rather than Glu is required. NO caused a drastic reduction in the transcription of the *glnA* gene encoding glutamine synthetase. Moreover, Gln supplementation did not change the NO-metabolizing activity of *S.* Typhimurium. These results demonstrate that NO can target the biosynthetic pathway for Gln, resulting in auxotrophy for Gln, and suggest that *S.* typhimurium can overcome Gln auxotrophy by taking up Gln from surroundings.

Table 3. Glutamine associated gene expression of other bacteria in RNS

bacteria	gene	function	substrate	Concentration/ treated Time	fold [a] (treatment/control)	Journal
Mycobacterium tuberculosis	Rv2563	Active transport of glutamine	nitrate	5 mM	0.23	[34]
Desulfovibrio vulgaris	DVU0105(*glnQ*)	Glutamine ABC transporter; ATP-binding protein	nitrite	2.5 mM/90min	0.59	[32]
	DVU0106(*glnP*)	Glutamine ABC transporter; permease protein			0.62	
	DVU0107(*glnH*)	Glutamine ABC transporter; periplasmic glutamine-binding protein			0.39	
	DVU3392(*glnA*)	Glutamine synthetase type I			2.8	
Shewanella oneidensis	S00761(*glnB*1)	nitrogen regulatory protein P-II 1	nitrate	5 mM/210min	-0.799	[33]
	S01324(*gltD*)	glutamate synthase, small subunit			-1.499	
	S01325(*gltB*)	glutamate synthase, large subunit			-3.489	
	S03760(*glnE*)	glutamate-ammonia-ligase adenylyltransferase			-2.825	
Escherichia coli	*gltP*	Proton glutamate symport protein	NO	50 uM /15min	-3.3	[35]
	glnS	Glutamine tRNA synthetase			-5.7	

Escherichia coli	*glnE*	adenylylating enzyme for glutamine synthetase	DeaNO	8 uM/5min	1.58	[30]
	glnG	nitrogen regulation protein NR(I)			-1.35	
	glnL	nitrogen regulator II, NRII			-2.57	
	glnA	Glutamine synthetase type			-2.04	
	gltP	glutamate-aspartate symport protein			1.48	
Porphyromonas gingivalis	PG2033	*gltD* glutamate synthase beta subunit (glutamate synthase small subunit)	DeaNO	0.36 uM /15min	-2.28	[36]
					expresstion value [b]	
Salmonella enterica Serovar Typhimurium	*glnD*	Uridylyltransferas	sperNO	250 uM /20min	321	[31]
	glnK	hypothetical protein			150	
	glnS	Glutamine tRNA synthetase			102	
	glnQ	glutamine high-affinity transperter			82	
	glnP	glutamine high-affinity transperter			82	
	glnH	glutamine high-affinity transperter			82	
	glnB	regulatory protein(P-II) for nitrogen assimilation by glutamine synthetase (ATase)			16	
	glnE	adenylylating enzyme for glutamine synthetase			7	

glnG	nitrogen regulation protein NR(I)	2
glnL	nitrogen regulator II, NRII	2
glnA	Glutamine synthetase	2
gltB	glutamate synthase, large subunit	6
gltD	glutamate synthase, small subunit	6

a The fold of expression value at addition of treated substrate(nitrate, nitrite, NO) to cultures contrast to controls without treated addition.

b The absolute gene expression shows as TPM (Transcript Per Million) values. If it's close by, at the 1000 value is high expression and 10 value is no expression. The expression data have been compiled into SalCom, a compendium of *Salmonella* transcriptomic data that can be interrogated at http://tinyurl.com/HintonLabSalCom[31].

E. References

1. Fang, F.C., *Antimicrobial reactive oxygen and nitrogen species: concepts and controversies.* Nat Rev Microbiol, 2004. **2**(10): p. 820-32.

2. Bang, I.S., et al., *Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp.* J Biol Chem, 2006. **281**(38): p. 28039-47.

3. Park, Y.M., et al., *Branched-chain amino acid supplementation promotes aerobic growth of Salmonella Typhimurium under nitrosative stress conditions.* Arch Microbiol, 2015. **197**(10): p. 1117-27.

4. Park, Y.M., et al., *Nitrosative stress causes amino acid auxotrophy in hmp mutant Salmonella Typhimurium.* Microbiol Immunol, 2011. **55**(10): p. 743-7.

5. Yan, D., *Protection of the glutamate pool concentration in enteric bacteria.* Proc Natl Acad Sci U S A, 2007. **104**(22): p. 9475-80.

6. Vogel, H.J. and D.M. Bonner, *Acetylornithinase of Escherichia coli: partial purification and some properties.* J Biol Chem, 1956. **218**(1): p. 97-106.

7. Bang, I.S., et al., *Alternative sigma factor interactions in Salmonella: sigma and sigma promote antioxidant defences by enhancing sigma levels.* Mol Microbiol, 2005. **56**(3): p. 811-23.

8. Crawford, M.J. and D.E. Goldberg, *Role for the Salmonella*

flavohemoglobin in protection from nitric oxide. J Biol Chem, 1998. **273**(20): p. 12543-7.

9. Ikeda, T.P., A.E. Shauger, and S. Kustu, *Salmonella typhimurium apparently perceives external nitrogen limitation as internal glutamine limitation.* J Mol Biol, 1996. **259**(4): p. 589-607.
10. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.
11. Poteete, A.R. and A.C. Fenton, *Lambda red-dependent growth and recombination of phage P22.* Virology, 1984. **134**(1): p. 161-7.
12. Ford, E., M.N. Hughes, and P. Wardman, *Kinetics of the reactions of nitrogen dioxide with glutathione, cysteine, and uric acid at physiological pH.* Free Radic Biol Med, 2002. **32**(12): p. 1314-23.
13. van Heeswijk, W.C., H.V. Westerhoff, and F.C. Boogerd, *Nitrogen assimilation in Escherichia coli: putting molecular data into a systems perspective.* Microbiol Mol Biol Rev, 2013. **77**(4): p. 628-95.
14. Leigh, J.A. and J.A. Dodsworth, *Nitrogen regulation in bacteria and archaea.* Annu Rev Microbiol, 2007. **61**: p. 349-77.
15. Hausladen, A. and J.S. Stamler, *Is the flavohemoglobin a nitric oxide dioxygenase?* Free Radic Biol Med, 2012. **53**(5): p. 1209-10; author reply 1211-2.
16. Forrester, M.T. and M.W. Foster, *Protection from nitrosative stress:*

a central role for microbial flavohemoglobin. Free Radic Biol Med, 2012. **52**(9): p. 1620-33.

17. Karlinsey, J.E., et al., *The NsrR regulon in nitrosative stress resistance of Salmonella enterica serovar Typhimurium.* Mol Microbiol, 2012. **85**(6): p. 1179-93.
18. Vazquez-Torres, A. and F.C. Fang, *Oxygen-dependent anti-Salmonella activity of macrophages.* Trends Microbiol, 2001. **9**(1): p. 29-33.
19. McLean, S., L.A. Bowman, and R.K. Poole, *Peroxynitrite stress is exacerbated by flavohaemoglobin-derived oxidative stress in Salmonella Typhimurium and is relieved by nitric oxide.* Microbiology, 2010. **156**(Pt 12): p. 3556-65.
20. van der Heijden, J., et al., *Direct measurement of oxidative and nitrosative stress dynamics in Salmonella inside macrophages.* Proc Natl Acad Sci U S A, 2015. **112**(2): p. 560-5.
21. Carlioz, A. and D. Touati, *Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life?* EMBO J, 1986. **5**(3): p. 623-30.
22. Bilinski, T., et al., *Physiological suppression of superoxide dismutase deficiency in yeast Saccharomyces cerevisiae.* Acta Microbiol Pol, 1993. **42**(1): p. 101-4.
23. Benov, L. and I. Fridovich, *Why superoxide imposes an aromatic amino acid auxotrophy on Escherichia coli. The transketolase*

connection. J Biol Chem, 1999. **274**(7): p. 4202-6.

24. Richardson, A.R., et al., *Multiple targets of nitric oxide in the tricarboxylic acid cycle of Salmonella enterica serovar typhimurium.* Cell Host Microbe, 2011. **10**(1): p. 33-43.

25. Alvarez, B. and R. Radi, *Peroxynitrite reactivity with amino acids and proteins.* Amino Acids, 2003. **25**(3-4): p. 295-311.

26. Ren, B., et al., *Nitric oxide-induced bacteriostasis and modification of iron-sulphur proteins in Escherichia coli.* Mol Microbiol, 2008. **70**(4): p. 953-64.

27. Rogers, P.A. and H. Ding, *L-cysteine-mediated destabilization of dinitrosyl iron complexes in proteins.* J Biol Chem, 2001. **276**(33): p. 30980-6.

28. Popp, J., et al., *Role of host cell-derived amino acids in nutrition of intracellular Salmonella enterica.* Infect Immun, 2015. **83**(12): p. 4466-75.

29. Klose, K.E. and J.J. Mekalanos, *Simultaneous prevention of glutamine synthesis and high-affinity transport attenuates Salmonella typhimurium virulence.* Infect Immun, 1997. **65**(2): p. 587-96.

30. Hyduke, D.R., et al., *Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in Escherichia coli.* Proc Natl Acad Sci U S A, 2007. **104**(20):

p. 8484-9.

31. Kroger, C., et al., *An infection-relevant transcriptomic compendium for Salmonella enterica Serovar Typhimurium.* Cell Host Microbe, 2013. **14**(6): p. 683-95.

32. He, Q., et al., *Energetic consequences of nitrite stress in Desulfovibrio vulgaris Hildenborough, inferred from global transcriptional analysis.* Appl Environ Microbiol, 2006. **72**(6): p. 4370-81.

33. Beliaev, A.S., et al., *Global transcriptome analysis of Shewanella oneidensis MR-1 exposed to different terminal electron acceptors.* J Bacteriol, 2005. **187**(20): p. 7138-45.

34. Cunningham-Bussel, A., T. Zhang, and C.F. Nathan, *Nitrite produced by Mycobacterium tuberculosis in human macrophages in physiologic oxygen impacts bacterial ATP consumption and gene expression.* Proc Natl Acad Sci U S A, 2013. **110**(45): p. E4256-65.

35. Justino, M.C., et al., *New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide.* J Biol Chem, 2005. **280**(4): p. 2636-43.

36. Boutrin, M.C., et al., *Nitric oxide stress resistance in Porphyromonas gingivalis is mediated by a putative hydroxylamine reductase.* J Bacteriol, 2012. **194**(6): p. 1582-92.

ABSTRACT

Nitric oxide represses the transcription of the glutamine synthetase gene *glnA*, which causes glutamine auxotrophy in *Salmonella* Typhimurium

Park, Yoon-Mee

Advisor : Prof. Bang, Iel-Soo, PhD

Department of Dental Bioengineering,

Graduate School of Chosun University

Nitric oxide (NO) damages various bacterial macromolecules, resulting in abnormal metabolism and subsequent growth arrest. But, the NO targets and strategies for avoiding nitrosative stress in bacteria are still not largely understood. In previous studies, we have shown that NO can cause auxotrophy for amino acids in *hmp* mutant *S.* Typhimurium that can hardly metabolize NO entering into bacteria. This study presents that exogenous supplementation with glutamine (Gln) or glutamate (Glu), major amino acids for nitrogen sources, can fully restore the replication of *hmp* mutant *S.* Typhimurium in NO-producing cultures. Because reactions for the

biosynthesis of Gln and Glu are interrelated, we analyzed the effect of supplementation with Gln or Glu on the growth of *hmp* mutants further lacking genes for the biosynthesis of Gln and/or Glu, and found that *S.* Typhimurium required Gln for their growth in the presence of NO. Only L-form of Gln could restore bacterial growth, suggesting that this growth recovery may have less relation with bacterial cell wall biosynthesis requiring D-Gln. The Gln supplementation did not affect the NO consumption rate of *S.* Typhimurium, indicating that the Gln-promoted NO resistance is independent of NO metabolism itself. Upon NO exposure, the transcription of glnA encoding glutamine synthetase was respressed in a way depending on the NRI/NRII two component regulatory system, suggesting that NO can disturb signal transduction system of Salmonella sensing nitrogen availability. These results suggest that cytotoxic NO may impair reactions for maintaining levels of Gln in *S.* Typhimurium, resulting in auxotrophy for Gln, but which can be overcome by bacterial taking up Gln from surroundings.

Chapter Ⅴ.

General conclusion

Nitric oxide (NO) damages various bacterial macromolecules, resulting in abnormal metabolism and subsequent growth arrest. Therefore, NO can restrict the proliferation of bacterial pathogens such as *Salmonella enterica* serovar Typhimurium, and further impair that of mutant *S.* Typhimurium lacking the major NO-metabolyzing enzyme flavohemoglobin Hmp. Using *hmp* mutant *Salmonella* that experience severe nitrosative stress when exposed to NO, This study have found that NO can induce amino acid auxotrophy in *Salmonella*. In analysis to identify major amino acids responsible for NO-induced auxotrophy, two different approaches have been taken when supplementing amino acids into the culture media. One is a drop-out analysis that supplement with amino acid pool excluding each single amino acid from the amino acid pool, the other is the exclusive supplementation with only single amino acid. In the drop-out analysis, BCAA are revealed as the most required, but in the analysis of single amino acid supplementation, Gln shows the most effective.

As summarized in Fig. 1, flavohemoglobin Hmp detoxifies most of NO entering into *Salmonella*. But, NO still can cause bacteriostasis in WT *Salmonella* expressing Flavohemoglobin, suggesting that NO and NO-mediated RNS can damage bacterial targets. In fact, bacterial respiration is inhibitted by NO-mediated inactivation of proteins in electron transport chains. This study has presented more evidences of other NO-mediated

alterations in *Salmonella* metabolism. Results in this study shows that iron-sulfur cluster enzymes for BCAA biosynthesis (IlvD and LeuCD) may be vulnerable to nitrosative stress, causing BCAA auxotrophy in *Salmonella*, and also shows that NO may perturb the NRI/NRII-dependent signal transduction system for *Salmonella* nitrogen sensing through unknown mechanism, causing the repression of *glnA* transcription and subsequent Gln auxotrophy (Fig. 1). During infection in phagocytic cells that produces NO in high concentration, *Salmonella* is expected to encounter to varied nutrient conditions in the cell whose roles in *Salmonella* pathogenesis remain incompletely understood. This study provides evidences that amino acid supplementation rescues the NO-caused growth arrest of *S.* Typhimurium, implying that the persistent *Salmonella* infection may be dependent on the availability of host amino acids that can compensate for the NO-induced auxotrophy.

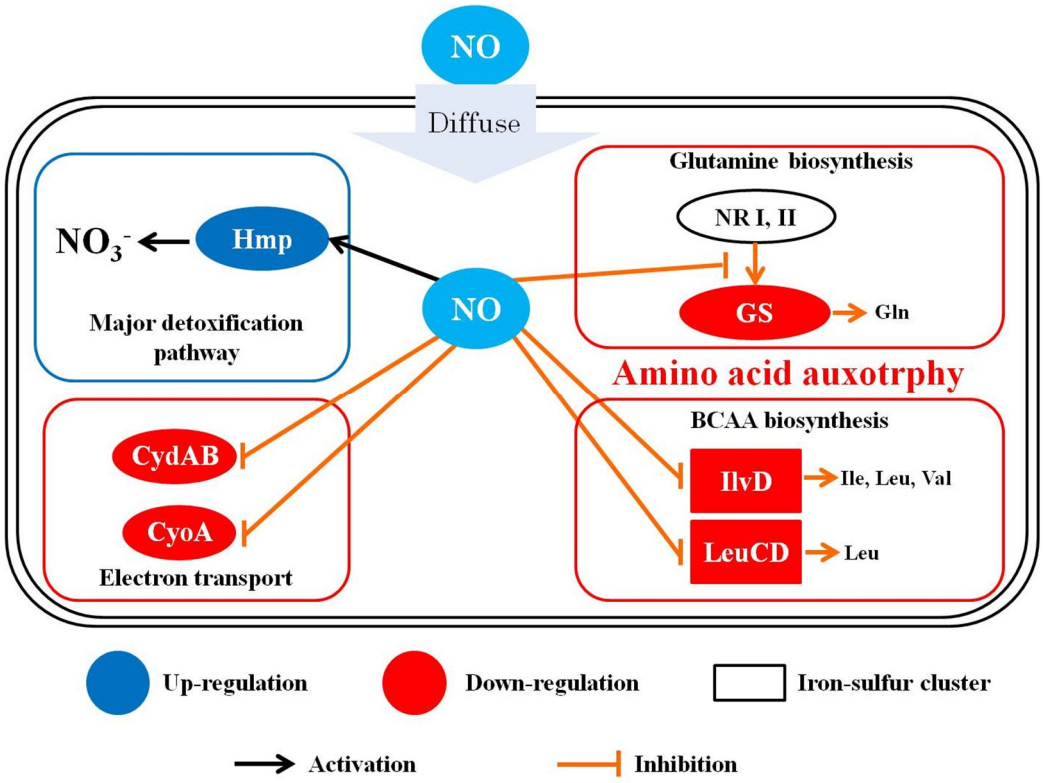


Figure 1. The NO-mediated modification of metabolism in *S.* Typhimurium.

CyoA : cytochrome bo oxidase, CydAB : cytochrome bd oxidase,

GS : glutamine synthetase; GlnA, Hmp : flavohemoglobin Hmp,

IlvD : dihydroxyacid dehydratase , LeuCD : isopropylmalate isomerase,

NR I : phosphorylated response regulator; NtrC ,

NR II : histidine kinase; NtrB

General abstract

산화질소는 숙주의 대식세포에서 발생되어 병원균의 성장을 억제시키는 항균성 요소로 작용한다. 세균의 증식은 산화질소과 산화질소로부터 만들어지는 활성질소종에 의해 조절되나 대개의 세균은 flavohemoglobin Hmp을 이용하여 산화질소를 대사시켜 생존하게 된다. 하지만, 여전히 산화질소에 의한 세균 대사 변화는 여전히 잘 알려지지 않았다. 이 연구에서, 산화스트레스에 의한 아미노산 영양요구성에 BCAA (곁가지 사슬 아미노산; 류신, 이소류신 그리고 발린)와 글루타민이 살모넬라의 증식에 중요하다는 것에 대하여 연구하였다.

살모넬라 Hmp 돌연변이 균주는 산화질소 스트레스가 주어졌을 때 성장이 지연되나 카사미노산이나 아미노산 풀이 포함된 배지에서 회복되었다. 아미노산 풀에서 각각의 아미노산을 제외시킨 drop-out 실험 방법을 통해 BCAA가 산화스트레스가 주어진 *hmp* 돌연변이 균주의 성장회복에 필요하다는 것을 확인하였다. BCAA가 합성되기 위해 LeuCD와 IlvD가 필요로 하는데 이들은 산화질소에 타겟이 되는 Fe-S 클러스터를 포함한다. 그러므로 산화질소에 의해 효소가 비활성 되어 산화질소에 의존적인 BCAA 영양요구성을 야기시킨다.

각각의 BCAA가 첨가되면 *hmp* 돌연변이 균주가 증식되었으나 BCAA첨가에도 불구하고 산소가 결핍된 환경에서는 성장하지 못하였다. 산화질소 meter를 통해 산화질소 소비율을 측정한 실험을 보면 BCAA의 첨가는 산화질소를 거의 대사하지 못하는 것을 확인하였다. 세포 내에서의 살모넬라 증식을 확인하기 위해 RAW264.7에 살모넬라를 감염시켰고 *ilvD* 돌연변이 균주의 증식이

증가되는 것을 확인하였다.

한 종류의 아미노산 첨가가 산화질소에 노출된 *hmp* 돌연변이 균주의 성장에 미치는 영향을 알아보기 위해 산화질소가 처리된 배지에 아미노산을 첨가하여 그 성장을 확인하였다. 글루타민과 글루탐산이 첨가되면 산화스트레스가 주어지더라도 *hmp* 돌연변이 균주의 증식을 회복시킬 수 있다는 것을 확인하였다. 그리고 *hmp* 돌연변이 균주에 글루타민과 글루탐산 생합성과 관련된 유전자 돌연변이시켜 글루타민과 글루탐산가 처리되었을 때의 성장을 확인하였다. 산화질소가 노출된 배지에서의 살모넬라의 성장 증식에는 글루탐산보다 글루타민을 필요로 한 것을 확인하였다. 아미노산은 자연상에 L-/D- form 두 가지 형태로 존재하는데 보통 생물학적 대사에는 L-form의 아미노산이 이용되며 산화스트레스에 노출된 살모넬라의 증식에는 L-form의 글루타민 만이 성장에 영향을 주었다. 산화질소 소비율을 보면 산화질소는 글루타민에 의해 대사되지 않았으며 이것은 글루타민이 산화질소 대사에 영향을 주지 못함을 의미한다. 글루타민을 합성하는 *glnA*의 전사는 산화스트레스에 의해 저하되고 NRI, NRII 두 개의 component 시스템에 의해 조절되었다. 현재까지 결과를 종합하면, BCAA와 글루타민은 살모넬라의 산화스트레스에 의한 아미노산 영양요구성을 해결하는 역할을 한다.