A Thesis for the Degree of Master of Science

Identification and Characterization of Outer Membrane Vesicles – Associated Proteins in *Salmonella* Typhimurium Using Proteomic Profiling

Proteomic profiling을 통한 살모넬라 외막소포체 단백질 규명 및 특성분석

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ABSTRACT

Salmonella is one of major food-poisoning pathogen causing diverse health problems called salmonellosis in a wide range of animals as well as human all over the world. Outer membrane vesicles (OMV) are secreted in many Gram-negative bacteria including *Salmonella* and play diverse roles including bacterial survival, nutrient acquisition, resistance to antimicrobial agents and biofilm formation, while the biogenesis of vesicles formation and secretion is not fully understood yet.

In this study, OMV proteomic profiling was performed in order to understand OMV roles in *Salmonella* virulence and physiology. OMV were purified under two different culture conditions and their constituents were analyzed by LC-MS/MS. Proteins secreted via OMV in both conditions included enzymes related to carbon and lipid metabolisms, ribosomal proteins, transporters and even SPI-1 and 2 proteins. Interestingly, ribosomal proteins were abundant in the vesicles isolated from LB condition whereas transporters in those from acidic minimal medium and this culture-dependent difference in OMV protein constituents suggested the existence of specific mechanism in sorting proteins as cargo during vesiculation in response to environments.

Among a variety of proteins secreted via OMV specifically under acidic minimal condition, 3 proteins encoded by *hupA*, *glnH* and *phoN* genes were translocated into the macrophage cytosol. Moreover, mutant

strains lacking HupA, GlnH or PhoN were attenuated in survival inside macrophages. Immunoblotting verified the presence of these proteins in OMV isolated under acidic minimal medium mimicking intracellular environment. These results suggest that HupA, GlnH, and PhoN may play roles in *Salmonella* intracellular survival following secretion via OMV, although it cannot be ruled out that these proteins are also important for bacterial viability independent of OMV secretion.

Also, transport proteins contained in OMV were found to affect *Salmonella* growth after macrophages infection. *Salmonella* strains without 5 transport proteins including PstS, DppA, CysP, STM14_1918, or YcfM showed decreased growth while 6 strains lacking RbsB, PotD, PagL, YggE, MetQ, or YehZ showed increased growth. Although their specific roles in bacterial growth and stress resistance have to be revealed in further study, this result highlights the possibility of additory functions of transporters associated with OMV.

Keywords: OMV, *Salmonella* Typhimurium, Proteomics, Virulence

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I. INTRODUCTION

Salmonella is a Gram-negative, rod-shaped, and motile pathogen that causes salmonellosis through uptake of contaminated food or water. Up to date, 2,463 serotypes of *Salmonella* have been isolated and a serotype Typhimurium causing diarrhea, vomiting, abdominal pain in human has been widely used as an animal model for systemic infection study since it causes human typhoid like disease in mice [1], [2].

A large number of genes are required for virulence of *Salmonella* and during evolution *Salmonella* acquired various clusters of virulence- related genes including *Salmonella* pathogenicity islands (SPIs) [3]. Among pathogenicity islands, SPI-1 and SPI-2 are critical for virulence; SPI-1 is required for the early stages of infection such as invasion into host epithelial cells of the small intestine and SPI-2 has roles in the later stages of infection like systemic spread and proliferation inside the host cell [4].

Bacterial protein secretion systems play an essential role in infection process as efficient and timely delivery of bacterial proteins to the host cells is crucial for bacterial invasion and/or survival inside the host cells. Each of SPI-1 and SPI-2 encodes specialized secretion systems called Type 3 Secretion Systems (T3SSs) that translocate multiple effector proteins into host cells for successful *Salmonella* infection.

More than 30 effector proteins secreted by SPI-1 and SPI-2 T3SSs are known to be related to bacterial signal transduction, membrane-trafficking

and manipulation of immune responses in host cells.

Although *Salmonella* also possesses other secretion mechanisms like *sci*-encoded (*Salmonella enterica* centisome 7 genomic island) type VI secretion system and ZirT/ZirS pathway, T3SSs has been considered as major secretion systems for *Salmonella* pathogenicity [5].

Outer membrane vesicles are spherical structures of 10-300nm diameter derived from outer membrane of gram-negative bacteria and generally composed of phospholipids, endotoxin and pheriplasmic contents [6]. Recent studies have showed outer membrane vesicles (OMV) as one of virulence mechanisms in gram-negative bacteria and it have been demonstrated that OMV have diverse roles including protein secretion and delivery, bacterial survival, nutrient acquisition, biofilm formation, defense and resistance to various environmental stresses such as antimicrobial agents, bacteriophages. [7], [8], [9], [10].

Outer membrane vesicles (OMV) are secreted in many gram-negative bacteria both pathogenic species including *Escherichia coli*, *Shigella* spp. *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Vibrio* spp. , *Salmonella* spp. and nonpathogenic species [11]. Moreover, since it has firstly detected in *E. coli* that outer membrane vesicles (OMV) are existed in cell-free supernatant of lysine limiting culture condition that contains soluble LPS, OMV have been discovered under normal growth conditions and in infected host cells as well [7].

While the functions of outer membrane vesicles (OMV) are relatively well studied as above, process of vesicle formation and secretion is not fully understood yet.

So, it is important to know the compositions of proteins that are secreted through outer membrane vesicles under various conditions to understand the relevance of outer membrane vesicles with *Salmonella* virulence.

For these purposes, I purified outer membrane vesicles (OMV) from *Salmonella* grown under a standard laboratory condition (LB medium) and an acidic minimal medium mimicking the intracellular condition and analyze their constituents using a liquid chromatography-mass spectrometry-based proteomics method to find novel virulence related genes. In this study, proteins secreted via OMV were identified using proteomic analysis and Many studies have tried to figure out the physiological changes of bacteria within host cells and to find novel secreted factors through proteomic approaches [12],[13].

Among OMV associated proteins, HupA, GlnH and PhoN proteins were translocated into the host cell cytosol. Also, when these 3 proteins were deleted respectively, bacterial survival was decreased inside macrophages while there was no difference in growth rate *in vitro*. Therefore, these results showed that HupA, GlnH and PhoN proteins have roles in *Salmonella* survival inside host cells and they are secreted via OMV. Meanwhile, LC MS/MS-mediated proteomic profiling and survival assay in macrophages

showed that OMV delivered multiple transport proteins and some of them were important for Salmonella fitness within host cells.

Although the specific roles of these OMV-associated proteins in *Salmonella* virulence remain to be studied and it has to be determined whether their effects on *Salmonella* survival was attributable to delivery via OMV, the proteomic profiling on *Salmonella* OMV will give more insights into *Salmonella* virulence mechanisms and be utilized to understand OMV biogenesis in other bacteria as well as *Salmonella*.

II. MATERIALS AND METHODS

1. Bacterial strains and growth conditions

All strains used in this study were derived from strain *Salmonella enterica* subsp. enterica serovar Typhimurium 14028s (henceforth "*Salmonella*"). *Salmonella* strain was grown aerobically at 37˚C in Luria-Bertani broth (LB; 1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) or acidic minimal medium (MgM; 0.1 M Tris-Cl, 5 mM KCl, 7.5 mM, $(NH_4)_2SO$, 50 μM K_2SO_4, 1 mM KH_2PO_4, 0.2% glycerol, 0.1% casamino acid, 8 μM MgCl) [14] at adjusted pH 5.0 and pH 7.0 for OMV purification. To measure growth rates of each mutants under acidic minimal *in vitro* condition, overnight culture of each mutants in 3ml LB broth were washed and resuspended with fresh MgM (pH 5.0) broth and 1/100 were inoculated into the fresh MgM (pH 5.0) broth respectively then incubated aerobically at 37˚C. Appropriate antibiotic was used in the following concentration if necessary; ampicillin 50 μg/ml, chloramphenicol 25 μg/ml and kanamycin 50 μg/ml. All strains and plasmids used in this study are listed in Table 1.

2. Construction of bacterial strains and plasmids

Non-polar gene deletion was also generated by λ-red PCR-based gene

deletion method using pKD13 as a template then recombinant strains were selected by plating on LB plate containing kanamycin (50 μg/ml) and the homologues recombination site was checked by gel electrophoresis using diagnostic PCR with primer sets of each genes as listed in Table 2 [15].

Fusion of *cyaA′* gene to each chromosomal genes was performed in a similar strategy using λ-red PCR-based gene deletion method with some modification [15]. Briefly, insertions for *cyaA′* tagged strains were generated using pMini-Tn5-cycler [12] as a PCR template instead of using pKD13 as a template. DNA fragment containing *cyaA′* and kanamycin cassette was designed to be inserted just prior to the stop codon of each genes via homologues recombination and the chromosomal insertion was selected by plating on kanamycin containing LB plate (50 μg/ml).

Plasmids for complementation of *hupA*, *glnH* and *phoN* mutants were constructed by cloning these genes into pACYC184 or pUHE21-2lacI^{q} vectors [16], [17]. Each gene was amplified by PCR using primers containing enzyme site of BamHI and HindIII then the resulted products of *hupA* and *phoN* were cloned between BamHI and HindIII sites of pACYC184 and that of glnH was cloned into pUHE21-2lacI^{q}.

Plasmids used for complementation were then confirmed by sequencing. HA tagged strains were constructed following the same way of *cyaA′* fusion construction using pKD13 as a PCR template and sequences of primers used

for strain construction are listed in table 2.

3. Purification of outer membrane vesicles (OMV)

In order to purify outer membrane vesicle both LB and acidic minimal medium (MgM, pH 5.0) were used. In detail, bacteria cultured in LB or MgM (pH 7.0) overnight were diluted into fresh LB or MgM (pH 5.0) at dilution ratios of 1:100 or 1:10 respectively. After 2 or 4 hours (LB or MgM respectively), each culture was centrifuged at 4℃, 10350 x g for 5 min to get rid of bacteria and the supernatant was then filtered by 0.22-μm filtration to remove residual bacteria. After that Stirred Ultrafiltration Cells (Model 8200, MILLIPORE) equipped with 100-kDa pore fiber membrane (Millipore # PLHK NMWL 100 kDa) was used to concentrate the supernatant to appropriate volume and to remove residual small secreted proteins simultaneously. The concentrates were then ultracentrifuged at 150,000 x g overnight at 4℃ (himac CP 100β, Hitachi, Japan) to pellet the outer membrane vesicles.

After ultracentrifugation, the supernatant was removed carefully and pellets were re-suspended with 1 ml of Tris-HCl (50 mM, pH 7.0) or HEPEs solution (40 mM HEPES, 3.4% NaCl, pH 7.4). Purified OMV were precipitated with 20% of trichloroacetic acid (TCA, Sigma Aldrich) according to a conventional method. Briefly, precipitated proteins were centrifuged at 14500 rpm for 10 min to spin down the precipitant and

supernatant was discarded. Then the precipitant was washed 2 times with 100% cold acetone with centrifugation as above and residual acetone was dried by incubating at 95˚C for 10 min.

Finally, precipitated proteins were resuspended in 1x Laemmli's SDS-sample buffer (GenDEPOT) and then separated on 10% polyacrylamide gel for LC-MS/MS proteomic analysis.

For the better OMV purification, OMV samples were re-ultracentrifuged with serial diluent of OptiPrep™ Density Gradient Medium (Sigma). Briefly, Optiprep (60% Iodixanol) was diluted in 1x HEPES solution in an appropriate volume to make serial dilution of Iodixanol (20 to 40% with 5% interval). Serial dilutions were layered over 2 ml of OMV samples in a tube and the serial density gradients were ultracentrifuged at 150,000 x g for 3 hours. Samples were divided into 9 fractions according to density after ultracentrifugation and then each fraction was separated by SDS-PAGE and stained using Power Stain™ Silver Stain Kit (ELPIS BIOTECH. INC) according to manufacturer's instruction.

4. LC-MS/MS analysis

Proteins seperated by SDS-PAGE were analyzed by LTQ linear ion trap LC-MS/MS (Finnigan). Briefly, OMV proteins were isolated from acrylamide gel and digested using trypsin and then masses of resultant peptides are measured by LC-MS/MS. Peptide analysis was performed using

MASCOT (version 2.2.04) against the *Salmonella* Typhimurium LT2 database.

5. Immunoblot analysis

HA-tagged *Salmonella* strains expressing GlnH-HA and PhoN-HA respectively were grown in acidic minimal media for 4 hours. Bacteria cell pellets of each strain were collected by centrifugation for total cell lysates and OMV samples were purified from bacterial cultures of each strain as described above.

All samples were resuspended in 1x SDS buffer and loaded on 10% polyacrylamide gel. The resultant SDS-PAGE gel was transferred to PVDF membrane (Millipore) and then HA tagged proteins and DnaK protein were detected using anti-HA (Sigma) and anti-DnaK (Stressgen) antibodies respectively with the concentration suggested.

Following primary antibody treatment, the membrane was then treated with horseradish peroxidase-linked anti-mouse IgG antibody as a secondary antibody and the immunoblot signal was detected by WEST-ZOL^{R} (plus) Western Blot Detection System (iNtRON Biotecnology) according to protocol.

6. Gentamicin protection assay

To examine the survival of non-polar deletion strains inside macrophages,

standard gentamycin protection assay was performed. In detail, bacteria were diluted in DMEM medium containing 10% FBS and subsequently added on confluent Raw 264.7 macrophage-like cells grown in 24-well plates at 37℃ with 5% CO_2 at a multiplicity of infection (MOI) of 100 which corresponds to $5x10^7$ CFU /well. 30 min post-infection, DMEM was removed and 500 μl of fresh DMEM containing gentamicin at a final concentration of 100 μg/ml was added to each well and incubated for 1 hour to kill remaining extracellular bacteria. For overnight infection (15 hour post-infection) DMEM containing gentamicin (20 μg/ml final concentration) was newely added after three time washing with PBS. Cells were gently washed three times with PBS and disrupted with 500 μl of 1% Triton X-100 (Sigma Chemical) for 20 minutes after 1.5 hour and indicated time points respectively. Then, the number of intracellular bacteria was determined as CFU / well by plating the lysate on LB agar plate after serial dilution.

7. cAMP assay

Translocation of OMV proteins into the macrophage cytosol was tested using *Salmonella* producing CyaA'-tagged proteins. RAW264.7 macrophage-like cells were seeded on 24 well plate at 5.0 x 10^5 cells/well and incubated overnight to form monolayer. DMEM medium was changed to fresh DMEM medium and incubated at 37℃ with 5% CO_2 and after 1hour each *cyaA'* fusion strains were infected to the cell monolayer at a

multiplicity of infection (MOI) of 100 which corresponds to $5x10^7$ colony forming unit (CFU)/well. After 30 min infection, DMEM was removed and replaced with 500 μl of fresh DMEM containing gentamicin at a final concentration of 100 μg/ml or 20 μg/ml as the same way described in gentamicin protection assay above. After 16 hours of infection cells were washed with PBS by 3 times and lysed with 0.1 M HCl for 20 minutes at 37℃ with 5% CO_2 without shaking. Macrophage cell lysate was centrifuged at 5000 x g for 2 minutes to obtain the macrophage cytosol and 100 μl of supernatant was used for cAMP assay. Concentration of cyclic AMP (cAMP) was examined using Direct cAMP ELISA kit (catalog # ADI-900-066) according to manufacturer's instructions. All DMEM used here contains 10% of FBS as a nutrient supplement.

Table 1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Genotype	Reference or source
***Salmonella* Typhimurium strains**		
14028s	WT *S. enterica* serovar Typhimurium	ATCC
YC0855	*hupA:: cyaA'*	This study
YC0861	*glnH:: cyaA'*	This study
YC0852	*phoN:: cyaA'*	This study
YC0638	*sseJ:: cyaA'*	[10]
JB0001	*ΔhupA*	This study
JB0002	*ΔglnH*	This study
JB0003	*ΔphoN*	This study
JB0005	GlnH-HA	This study
JB0006	PhoN-HA	This study
JB0007	*ΔhupA*/ pACYC184::*hupA*	This study
JB0008	*ΔglnH*/ pUHE21-2lacI^{q}::*glnH*	This study
JB0009	*ΔphoN*/ pACYC184::*phoN*	This study
YC0892	*pstS::kan*	This study
YC0903	*dppA::kan*	This study
YC0906	*cysP::kan*	This study
YC0907	STM14_1918*::kan*	This study
YC0909	*ycfM::kan*	This study
YC0893	*rbsB::kan*	This study
YC0902	*potD::kan*	This study
YC0910	*pagL::kan*	This study
YC0911	*yggE::kan*	This study
YC0915	*metQ::kan*	This study
YC0916	*yehZ::kan*	This study
Escherichia coli		
DH5α	F-supE44 *hsd*R17 *rec*A1 *gyrA*96 thi-1 *rel*A1	[18]

Plasmids		
pKD13	Ap^R FRT Km^R FRT PS1 PS4 oriR6Kψ	[15]
pKD46	Ap^R P_{BAD}-*gam-beta-exo* *oriR101*	[15]
pCP20	*repA101*^{ts}	[15]
pMini-Tn5-cycler	pCRScript + mini-Tn5-cycler	[12]
pACYC184	Cloning vector, Cm^R Tet^R p15A *ori*	[16]
pUHE21-2*lacI*^{q}	rep_{pMB1} Ap^R *lacI*^{q}	[17]

Table 2. Primers used for strain construction

Primers	DNA sequence from 5′ to 3′
hupA-cyaAF1	GCG TTT GTT TCT GGT AAA GCT CTG AAA GAC GCA GTT AAG CTG TCT CTT ATA CAC ATC TCA
hupA-cyaAR1	ATG AGC CCC TTC GAT AAA ACT GTT CAC AGT TAT GCG TCC TGT CTC TTA TAC ACA TCT GGT
glnH-cyaAF1	AAC GAA ATC TAC AAA AAA TGG TTC GGT ACA GAA CCT AAA CTG TCT CTT ATA CAC ATC TCA
glnH-cyaAR1	GTC CTC ACT ACC GGG CCA CAA GCC AAC ATC AGG TCG TTC TGT CTC TTA TAC ACA TCT GGT
phoN-cyaAF1	AAT TTA TTG AGT AAA GAA GAT CAC CCC AAA CTT AAT TAC CTG TCT CTT ATA CAC ATC TCA
phoN-cyaAR1	TCA TTT GCT GTG GCC AGT TTG CGG GAA GAC TTT CAC CTC TGT CTC TTA TAC ACA TCT GGT
Red-hupA-F	CGA TGC TTA GCA AGC GAT AAA CAC ATT GTA AGG ATA ACT TTG TAG GCT GGA GCT GCT TCG
Red-hupA-R	CTT TAC CAG AAA CAA ACG CCG GTA CGT TAG CGG CGG CGA TAT TCC GGG GAT CCG TCG ACC
Red-Di-hupA-F2	GCA ATG ACA CCA GAA AAC GTG AT
Red-Di-hupA-R2	TTC GTA TCT TTA CGC CAG ATA CG
Red-glnH-F	TTA AAA GTT TCA CTG GCT GCA CTT ACC CTG GCT TTT GCG GTG TAG GCT GGA GCT GCT TCG
Red-glnH-R	CTC ACT ACC GGG CCA CAA GCC AAC ATC AGG TCG TTT TAT TAT TCC GGG GAT CCG TCG ACC

Red-Di-glnH-F2	CTA AAT CAG TGC CCC AAA ACG GT
Red-Di-glnH-R2	GTC ATT TTA GCG CCC TCA AGC AA
Red-phoN-F	TTA CAT CTG TTT ATT ATT GCC TGA TCC GGA GTG AGT CTT TTG TAG GCT GGA GCT GCT TCG
Red-phoN-R	TTT CAT TTG CTG TGG CCA GTT TGC GGG AAG ACT TTC ACC TAT TCC GGG GAT CCG TCG ACC
Red-Di-phoN-F2	TCG GGG ACA AAA GTT AAA CAA AAT GC
Red-Di-phoN-R2	ATT CGC TGT GGA TAG TCA GTG AG
glnH-HA-Red-F	TAA CGA AAT CTA CAA AAA ATG GTT CGG TAC AGA ACC TAA ATA TCC GTA TGA TGT TCC TGA TTA TGC TAG CCT CTA ATG TAG GCT GGA GCT GCT TCG
glnH-HA-Red-R	GCG TCC TCA CTA CCG GGC CAC AAG CCA ACA TCA GGT CGT T AT TCC GGG GAT CCG TCG ACC
phoN-HA-Red-F	TAA TTT ATT GAG TAA AGA AGA TCA CCC CAA ACT TAA TTA CTA TCC GTA TGA TGT TCC TGA TTA TGC TAG CCT CTG ATG TAG GCT GGA GCT GCT TCG
phoN-HA-Red-R	TTT CAT TTG CTG TGG CCA GTT TGC GGG AAG ACT TTC ACC TAT TCC GGG GAT CCG TCG ACC
hupA-BamHI-F2	GAA ACG CTG GAT CCC GCT ATT GA
hupA-HindIII-R	AAA ACT GTT CAA GCT TAT GCG TCT TAC
glnH-BamHI-F	TAT TGT GCA CAG GAT CCT TTC ACG

glnH-HindIII-R	CGG GCC ACA AGC TTA CAT CAG GT
phoN-BamHI-F2	ATG ACT GAT GGA TCC TAT CGG GGT
phoN-HindIII-R	AGT TTG CGG GAA GCT TTT CAC CTT
pACYC-Di-F1	CAA GAG ATT ACG CGC AGA CC
pUHE-Di-R3	GGT CAT TAC TGG ATC TAT CAA CA
HA-Di-R	GCT AGC ATA ATC AGG AAC ATC ATA
Red-pstS-F	TCA AAC AAC GAT TTA CCG AAA CCG TGC AGG AGA CAT TAT GGT GTA GGC TGG AGC TGC TTC
Red-pstS-R	TCC GCC AAT TCG TTA ATT AAT ACA GCG CCT TAC CGT TGC TTT CCG GGG ATC CGT CGA CCT
Red-dppA-F	AGG GCA AAA CAA CAT ACA TCA CAA TTG GAG CAG AAG AAT GGT GTA GGC TGG AGC TGC TTC
Red-dppA-R	CGC AGC GCT CTT TTA ATT ATT CGA CAG AGA CGT TTT CGA ATT CCG GGG ATC CGT CGA CCT
Red-cysP-F	GAC CAT AAG AAA GTC ATT AAA TTT ATA AGG GTG CGC AAT GGT GTA GGC TGG AGC TGC TTC
Red-cysP-R	GCA AGC ATT ACT TAC GCC CCG CCG CCA ACA GTT TGT CCA GTT CCG GGG ATC CGT CGA CCT
Red-STM14_1918-F	GAA GCA GCT ATA CAC GTT TTT ATC AAA GGG AGT CGT CAT GGT GTA GGC TGG AGC TGC TTC
Red-STM14_1918-R	CCC CTC CAT ACC CTA TTT ACA GAG CAA TGC GAA TAA CAT CTT CCG GGG ATC CGT CGA CCT

Red-ycfM-F	GTC AGG CTT TAT CTT TAT TTG TGA GGA GTG AAT TTT GAT GGT GTA GGC TGG AGC TGC TTC
Red-ycfM-R	CCG CAC CTT TAC CTG ACC AGA TTA TCT CGC CAG TTT GCA CTT CCG GGG ATC CGT CGA CCT
Red-rbsB-F	CAA AAA GCA GTA ACA ACG ACT ACA GGA CAT CTG AAA TAT GGT GTA GGC TGG AGC TGC TTC
Red-rbsB-R	TGA CCC GTG TCG TTT ATT ACT GCT TGA TGA CCA GTT TCA GTT CCG GGG ATC CGT CGA CCT
Red-potD-F	CAA GGG CCG ATA AGG CCC GTA ATT CAG GGG ACG TTA AAT GGT GTA GGC TGG AGC TGC TTC
Red-potD-R	AGG CGC TTT GGC TGA ATT AGC GTC CTG CTT TTA GCT TCT GTT CCG GGG ATC CGT CGA CCT
Red-pagL-F	GGT TAA AAA TAA CTA TTG ACA TTG AAA TGG TGG TGG AGT GGT GTA GGC TGG AGC TGC TTC
Red-pagL-R	TAA TTG TTA TTC AAC TTC AGA AAT TAT AAC TAA TTG AAG CTT CCG GGG ATC CGT CGA CCT
Red-yggE-F	TAT ATC AGG GCA ATG ACT GCA ATA TGG AGG AGA GAC TGT GGT GTA GGC TGG AGC TGC TTC
Red-yggE-R	CCC AGG CCG GGT GAG ATT ACT GCG TGC TGG CGG CCG TTG TTT CCG GGG ATC CGT CGA CCT
Red-metQ-F	GTT AAA CAC AAC ACA AAT ACT CAT TAA GGA AAT AAG CAT AAG CAT GGT GTA GGC TGG AGC TGC TTC
Red-metQ-R	GGA ATC AGC AGC CTA AAA CTT ACC AGC CTT TCA CCG CGC CGC CTT CCG GGG ATC CGT CGA CCT
Red-yehZ-F	TCT CTG AAA AAG GCC GTA AAA GGA TGA GGA AAG CAT GGT GTA GGC TGG AGC TGC TTC
Red-yehZ-R	CAG CAT CAC TCA CAG ATT ACT TCA CCC ACC CTT TTT GTC GTT CCG GGG ATC CGT CGA CCT

III. RESULTS

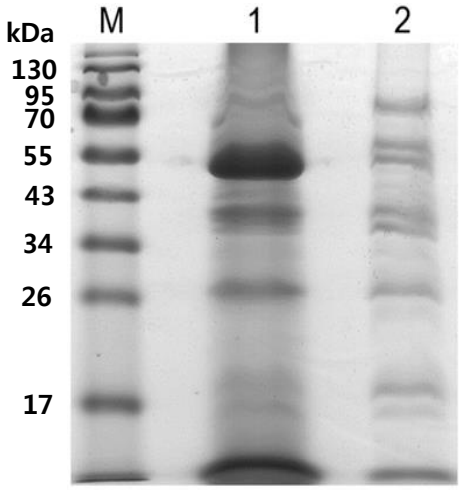
1. Protein amounts and compositions of outer membrane vesicles are different depending on culture conditions.

After purifying outer membrane vesicles from same volume of LB and acidic minimal (MgM pH 5.0) culture, total protein concentration of each sample was determined by BCA (bicinchoninic acid) method. As a result, the amount of proteins from LB culture was about 5 times more than that from MgM pH 5.0 culture (Data not shown).

Also, SDS-PAGE showed the dissimilarity of protein compositions (figure 1A) and this culture dependent difference was well observed by silver-staining of gel followed after gradient ultracentrifugation (figure 1B).

This result demonstrated that OMV yield and their constituents are different depending on the culture environments.

A


kDa
M
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2
130
95
70
55
43
34
26
17
M : marker
1: LB
2: MgM (pH 5.0)

B

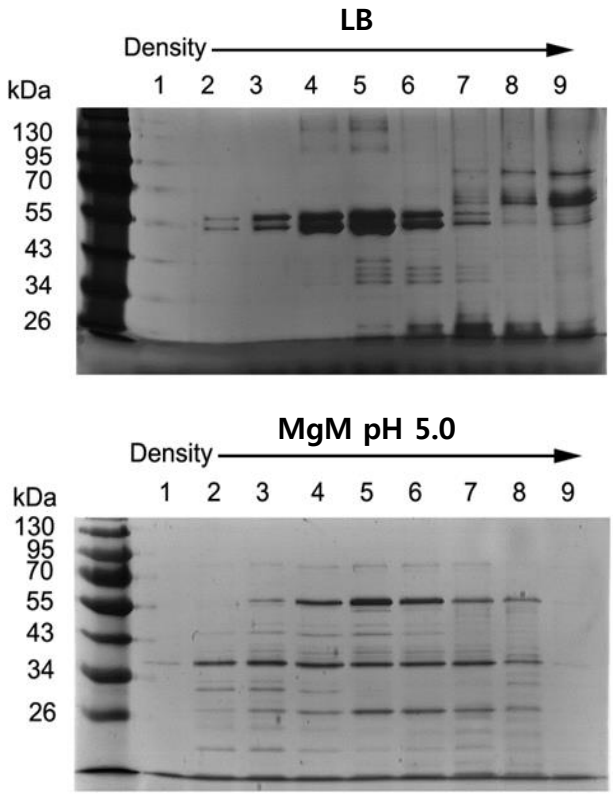
LB
Density
kDa
1 2 3 4 5 6 7 8 9
130
95
70
55
43
34
26
MgM pH 5.0
Density
kDa
1 2 3 4 5 6 7 8 9
130
95
70
55
43
34
26

Figure 1. Difference in OMV-associated protein amount and composition according to culture conditions. (A) Protein concentration of purified outer membrane vesicles from equal volume of LB and acidic minimal condition was determined by BCA assay. 40 μg and 20 μg of proteins from LB and MgM (pH 5.0) culture respectively were separated by SDS-PAGE and then stained by coomassie blue. (B) To observe culture dependent differences in greater detail, samples were separated by density gradient ultracentrifugation. Each portion of 9 fractions after density gradient ultracentrifugation was loaded on SDS-PAGE and resulted gels were stained using silver staining method as described in materials and methods.

2. LC-MS/MS results showed diverse protein constituents in outer membrane vesicles.

OMV proteins separated by SDS-PAGE (figure 1A) were analyzed by LC-MS/MS. Detected proteins were categorized in 15 groups according to predicted functions and lists commonly abundant in both conditions were enzymes, translation, transport and virulence. (figure 2A).

What is more ribosomal proteins related to translation were abundant in vesicles isolated from LB condition and transporters in those from acidic minimal medium. In addition, among the virulence related proteins, SPI-1 proteins were detected only in LB condition while SPI-2 proteins were in acidic minimal condition.

A

	Common	LB	MgM
• cell adhesion	-	-	1
• conjugation	1	-	-
• enzyme	11	2	4
• Flagella	6	-	-
• heat shock protein	1	-	-
• lipid A biosynthesis	-	1	-
• lipoprotein	5	-	-
• outer membrane protein	-	-	1
• phage	2	-	-
• Scaffolding protein	1	-	-
• transcription	4	-	3
• translation	12	11	2
• transport	12	2	16
• unknown function	19	6	11
• virulence	7	6	2
sum	81	28	40

B

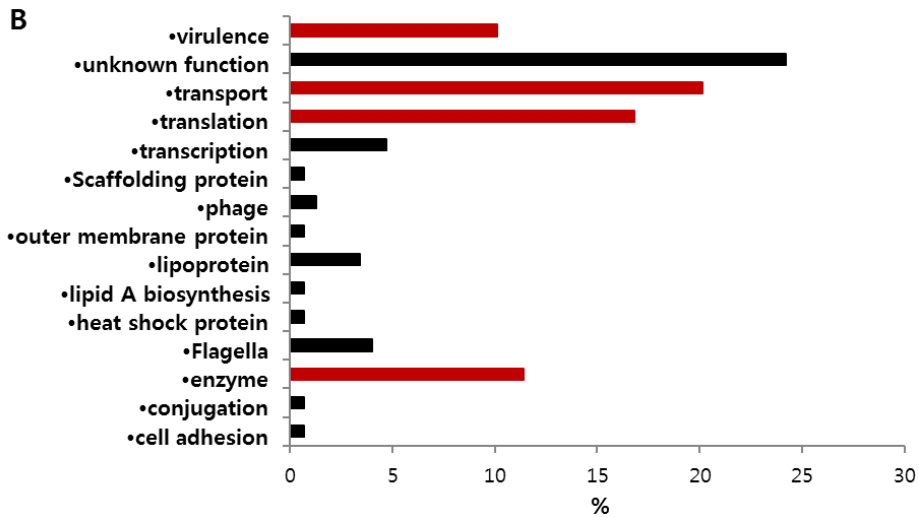

Figure 2. Protein constituents in outer membrane vesicles.

(A) Category of proteins analyzed by LC-MS/MS was shown in table.

(B) Percent rate of each list was displayed as a bar graph.

3. HupA, GlnH and PhoN are translocated to the macrophage cytosol.

Since *Salmonella* resides inside the modified phagosome called SCV (***S****almonella-****C****ontaining* ***V****acuole*) after it is taken up by macrophages, it is necessary to translocate effector factors across the SCV into the host cytosol using specialized apparatuses such as SPI-2 T3SS for efficient infection [19] and it was reported that OMV also deliver virulence factors into the host cytosol [10]. In order to find new proteins translocated into the macrophage cytosol via OMV, 28 proteins identified in MgM-derived OMV were chosen as candidates to check their translocation using *cyaA'* fusion. CyaA' is an adenylate cyclase activity domain of *Bordetella pertussis* which is inactivated in bacterial cell. [20]. Because the activity of CyaA' is totally dependent on animal cell ubiquitous calmodulin, translocation of *cyaA'* fusion proteins to animal cells cytosol can be determined by measuring the cAMP levels of macrophage [21]. Most of their predicted location is periplasmic space or cytosol according to PSORTb v3.0.2 program suggesting that they are enclosed within OMV and delivered into the host cytosol and cAMP assay revealed that proteins encode by *hupA, glnH* and *phoN* respectively showed relatively high levels of cAMP at 16 hours post-infection to RAW 264.7 cell line among 28 fusions tested. SseJ is a known virulence factor secreted into the host cytosol and sseJ- *cyaA'* fusion strain was used as a positive control [22]. (Figure3)

Result of cAMP assay indicated that HupA, GlnH and PhoN proteins were

secreted via OMV and then translocate into the host cell cytosol although their functions inside the host cell still remain to be revealed.

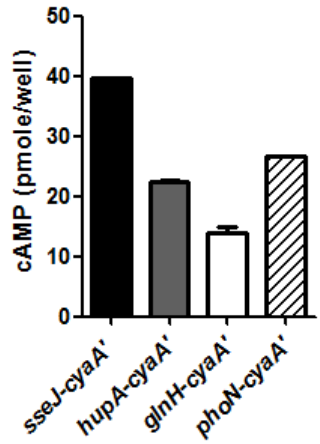

Figure 3. CyaA' fusions of HupA, GlnH and phoN proteins showed high cAMP levels. cAMP levels were determined by 28 fusion strains at 16 hour post-infection of RAW 264.7. Among 28 strains, *hupA- cyaA', glnH-cyaA', phoN- cyaA'* fusion strains showed high cAMP levels. This cAMP assay result indicates the translocation of HupA, GlnH and PhoN into macrophage cytosol.

4. GlnH and PhoN proteins were secreted via outer membrane vesicle under *in vitro* condition.

To clarify the secretion of HupA, GlnH and PhoN proteins through outer membrane vesicles, each strain was tagged with HA and used to purify outer membrane vesicles respectively. Then the presence of HA tagging proteins in OMV were detected using anti-HA antibody. GlnH and PhoN proteins were detected by western blot both in OMV and cell pellet while the cytosolic protein DnaK was not found in purified OMV samples. This result showed that GlnH and PhoN are proteins specifically secreted via OMV under mimicking intracellular condition.

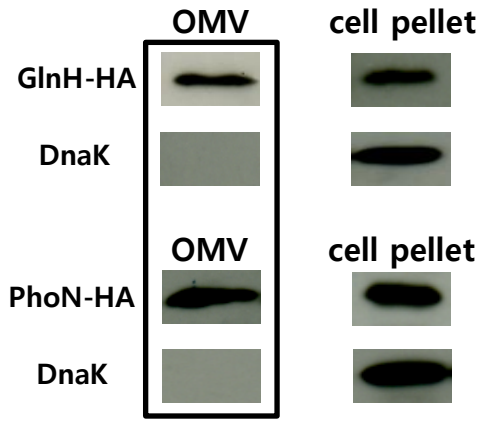

Figure 4. GlnH and PhoN were secreted via OMV *in vitro*.

Secretion of protein via OMV was confirmed by western blot. Proteins having HA tag were constructed and OMV were purified from each strain under 500 ml of acidic minimal medium after 4hours as described in materials and methods. For cell pellet sample, 5.0×10^7 of bacteria were pelleted and then both OMV and cell pellet were resuspended in 1x Laemmli sample buffer and loaded on 10% polyacrylamide gel. Anti-HA antibody and Anti-DnaK antibody were used to immunoblot the HA tagged proteins and DnaK respectively.

5. *Salmonella* strains lacking *hupA, glnH and phoN* were attenuated in survival inside macrophages.

Since secretion of HupA, GlnH and PhoN into host cell cytosol was confirmed through the results precedence, their roles in *Salmonella* survival inside host cells were examined.

For this, RAW 264.7 macrophage cells were infected with 14028s wild type strain, *phoN*, *hupA* and *glnH* mutants respectively. All mutants showed attenuated virulence after 12 hours compared to wild type and the similar numbers of bacteria at 1h 30min post-infection showed that there were no differences in phagocytosis by macrophages. (figure 5B) However, this attenuation did not reproduced in *in vitro,* mimicking intracellular condition which implicates their specific roles inside the host cells.

Attenuated replications inside macrophages were recovered to wild type level in mutants by introducing phupA and pglnH. However pphoN did not complement the absence of *phoN*, which will be discussed later. Functions and predicted locations of HupA, GlnH and PhoN are listed in Table 3.

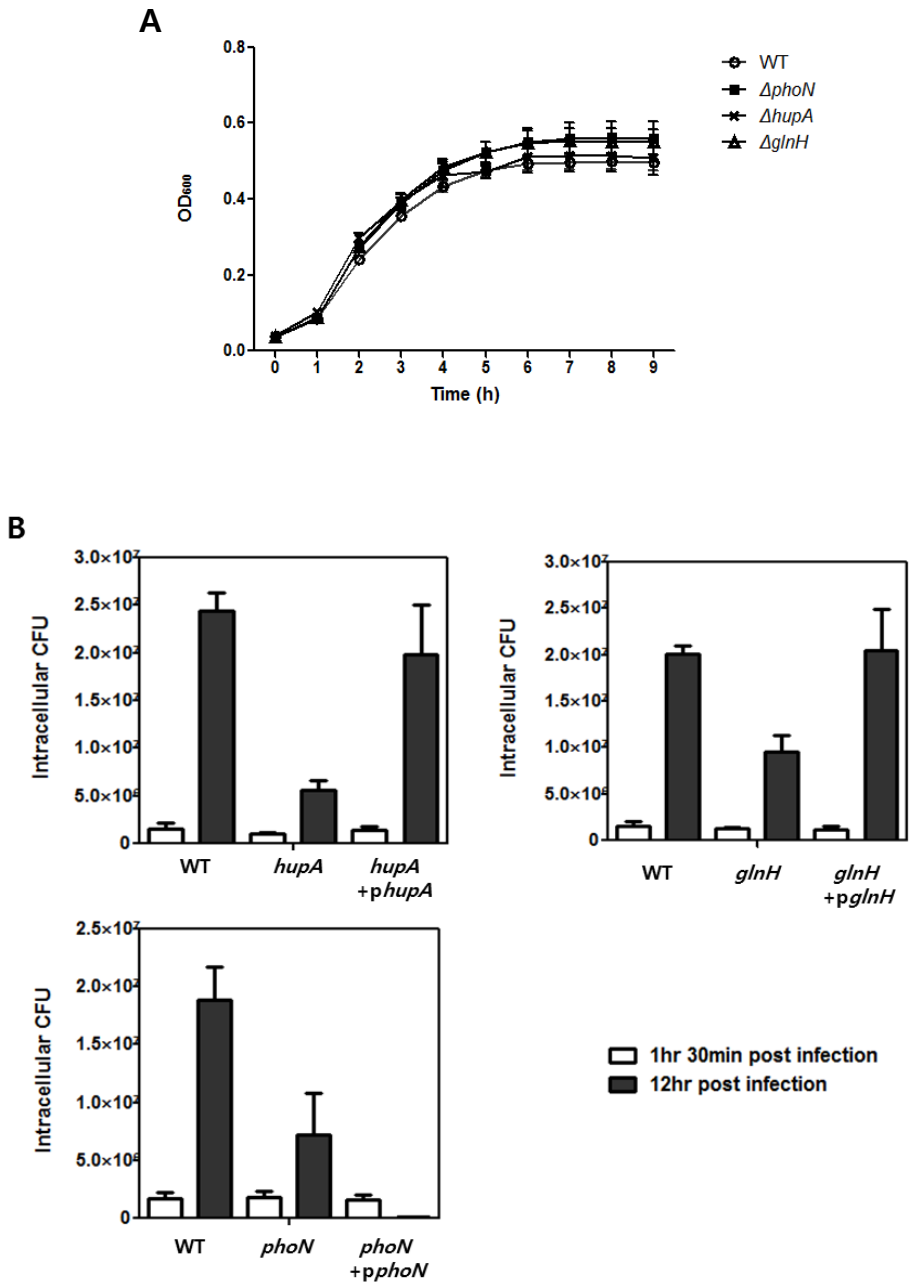
A
0.8
0.6
0.4
0.2
0.0
OD600
0 1 2 3 4 5 6 7 8 9
Time (h)
WT
ΔphoN
ΔhupA
ΔglnH
B
Intracellular CFU
3.0×10⁷
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
WT
hupA
hupA +phupA
Intracellular CFU
WT
glnH
glnH +pglnH
Intracellular CFU
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
WT
phoN
phoN +pphoN
1hr 30min post infection
12hr post infection

Figure 5. *hupA, glnH* and *phoN* mutants were attenuated in intracellular replication at 12 hour post-infection. (A) The optical density (OD_{600}) of wild-type and each mutant was measured under acidic minimal condition. (B) Intracellular survival of wild-type, mutants and mutants strains transformed with a plasmid expressing HupA, GlnH or PhoN respectively in macrophage after 12 hours. IPTG was added for GlnH expression at final concentration of 50 μM during overnight incubation and all experiments were performed in triplicate.

Table 3. List of proteins selected by cAMP assay

Protein	Function[a)]	Localization[b)]
HupA	transcriptional regulator HU subunit alpha	cytosol
GlnH	glutamine ABC transporter periplasmic protein	periplasm
PhoN	Non-specific acid phosphatase	periplasm

a) Functional description according to NCBI *Salmonella* Typhimurium 14028S database.
b) Predicted subcellular protein localization using PSORTb ver3.0.2

6. OMV-associated transporters influence *Salmonella* survival inside macrophages.

Twenty six transporters contained in OMV under acidic minimal condition were selected to verify their relations in *Salmonella* survival.

All selected proteins are transporter or their subunits and *Salmonella* lacking each transporter was subjected to infect macrophages to test the survival inside host cells.

Out of 26 strains tested, mutants of *ΔpstS, ΔdppA, ΔcysP*, *Δ*STM14_1918 and *ΔycfM* genes showed 2-folds or more decreased survival than wild-type strain and strains of *ΔrbsB, ΔpotD, ΔpagL, ΔyggE, ΔmetQ* and *ΔyehZ* showed increased survival more than 2-folds after 18 hours infection (figure 6A). After mutant screening by gentamicin protection assay, the growth of mutants selected was measured under acidic minimal media mimicking intracellular condition. As shown in figure 6B no mutant showed growth defect suggesting that the transporters play a role in *Salmonella* survival inside macrophages, which cannot be reproduced *in vitro*.

Functions of tested transporters were predicted based on studies elsewhere and listed in table 4 and their locations are predicted using PSORTb v3.0.2 program as of the *cyaA'* fusion.

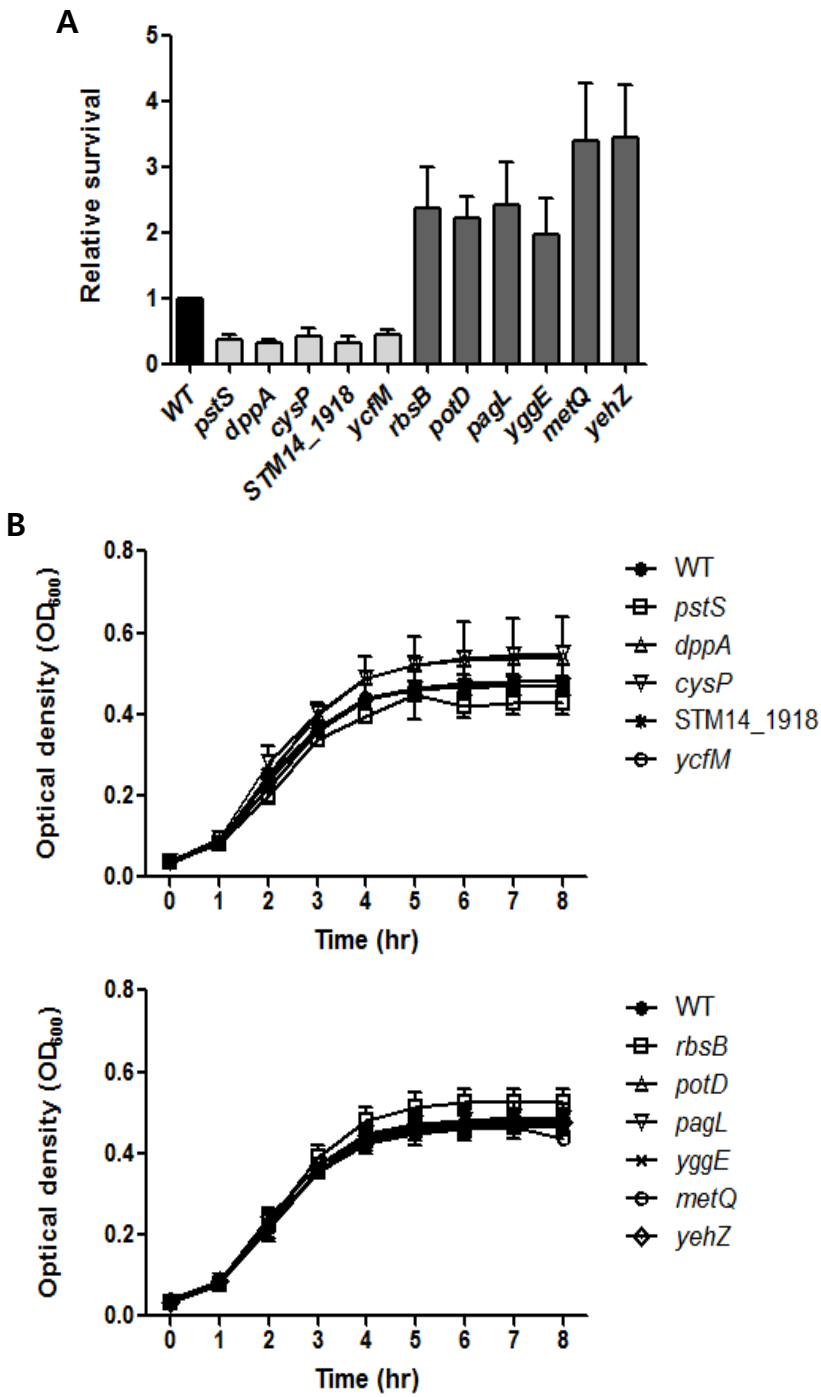
A
Relative survival
0
1
2
3
4
5
WT
pstS
dppA
cysP
STM14_1918
ycfM
rbsB
potD
pagL
yggE
metQ
yehZ
B
Optical density (OD600)
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
5
6
7
8
Time (hr)
WT
pstS
dppA
cysP
STM14_1918
ycfM
Optical density (OD600)
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
5
6
7
8
Time (hr)
WT
rbsB
potD
pagL
yggE
metQ
yehZ

Figure 6. Intracellular survival of strains lacking outer membrane transporter in RAW264.7 cell and their growth under acidic minimal condition. (A) Mutants lacking transporters were screened by survival assay. Wild-type and each mutant were used to infect RAW264.7 macrophage-like cells at an MOI of 100. Numbers of intracellular bacteria were counted by appropriate serial dilution after 18 hours infection. Only the mutants with more than 2-fold difference from wild type are shown in figure 6A. (B) The optical density (OD_{600}) of wild-type and each mutant were measured every hour under acidic minimal medium.

Table 4. List of proteins selected by gentamicin protection assay

Protein	Function[a)]	Localization[b)]
PstS	phosphate transporter subunit	periplasm,membrane
DppA	dipeptide transport protein	periplasm,membrane
CysP	thiosulfate transporter subunit	periplasm
STM14_1918	hypothetical protein	periplasm
YcfM	putative outer membrane lipoprotein	outer membrane, inner membrane
RbsB	D-ribose transporter subunit RbsB	periplasm,membrane
PotD	spermidine/putrescine ABC transporter	periplasm
PagL	putative outer membrane protein	outer membrane
YggE	hypothetical protein	periplasm,membrane
MetQ	DL-methionine transporter substrate-binding subunit	periplasm,membrane
YehZ	putative transport protein	outer membrane

a) Functional description according to NCBI *Salmonella* Typhimurium 14028S database.
b) Predicted subcellular protein localization using PSORTb ver3.0.2

IV. DISCUSSION

Up to date, the molecular mechanisms of *Salmonella* virulence have been widely studied and T3SSs encoded by SPI-1 and SPI-2 are well known as major components for successful infection of *Salmonella* to host cells. Among two systems, T3SS encoded by SPI-2 is required for *Salmonella* to survive and replicate within macrophages by translocating series of proteins called effector factors [23]. Up to now, more than 20 effector proteins were identified to be secreted by SPI-2 T3SS.

Recently OMV got attention for delivering virulence proteins independent of T3SS [24]. Therefore, in order to get more evidences for OMV as a novel secretion mechanism, OMV cargo proteins were identified by proteomic analysis and their roles in *Salmonella* pathogenesis were examined. Differential proteomic profiles in OMV between LB and acidic minimal medium implies the presence of active sorting mechanism during vesiculation in response to environmental condition (figure 1 and 2).

cAMP secretion assay (figure 3) and immunoblot analysis (figure 4) indicate that *Salmonella* translocates HupA, GlnH and PhoN proteins into the macrophage cytosol via OMV. None of these proteins has been reported to be secreted by T3SSs as well as other secretion mechanisms present in *Salmonella*. Therefore, it is possible that they are novel virulence factors translocate to host cytosol via OMV and the possibility was tested by comparing survival of mutant strains lacking these proteins with that of wild

type in macrophages. As shown in figure 5, their attenuation in intracellular survival might be caused by mal function of cellular activity relevant to HupA, GlnH and PhoN inside bacteria not by the absent of OMV-mediated interaction of these proteins with host cellular organelles. But it still suggests that HupA, GlnH and PhoN are required for *Salmonella* survival inside macrophages and they are one of secreted proteins via OMV.

Though there have been several trial in intracellular survival, *ΔphoN* strain was not complemented by pphoN. It is possible that overexpression of PhoN in-trans might affect bacterial intracellular fitness or rather stimulate host defense mechanism.

Moreover, decreased or increased survival of mutants lacking transporters or their subunits suggests the secondary roles of transporters in *Salmonella* virulence. It was already reported that Mg^{2+} ion transporter encoded in *Salmonella* pathogenicity island 3 is related to *Salmonella* survival and its expression is regulated by PhoP/Q system which is major regulator of *Salmonella* virulence [25],[26].

Therefore, this result might implicate the existence of relations between SPI-2 and transporters tested in this study because all proteins tested here were selected from acidic minimal medium which represents SPI-2 inducing condition [27].

Although many studies have been figure out the strategy of *Salmonella* survival inside the host since it had discovered, still the specific molecular

mechanisms are not fully understood.

Therefore, the possibility that HupA, GlnH and PhoN can play roles as secreted factors in macrophages can provide new information to understand the interactions between *Salmonella* and host cells. Moreover, the effects of transporters to *Salmonella* virulence though their specific mechanisms still unknown, also will be a key to appreciate diverse roles of transporters in *Salmonella*.

Although the specific mechanism how these proteins selected by OMV cargo and whether functions of each protein in *Salmonella* virulence are fully associated to OMV or not remain to be established, this study discovered three translocated proteins and suggested additional roles of transporters that are secreted via outer membrane vesicles in response to environmental conditions.

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국문 초록

살모넬라는 전 세계적으로 넓은 범위의 동물뿐만 아니라 사람에게도 살모넬라증 이라 불리는 질병을 유발하는 주요 식중독 원인 균 중 하나이다. 외막소포체는 살모넬라를 비롯한 많은 그람 음성균 에서 분비되며 박테리아 생존, 영양분 획득, 항균제제에 대한 저항 그리고 바이오 필름 형성 등 다양한 역할을 하는 것으로 알려져 있으나 소포체 형성 및 분비 기작에 대해서는 많이 알려진 바가 없다.

이에, 본 연구에서는 살모넬라의 독성과 생리적 기능에 있어 외막소포체의 기능을 이해하고자 proteomics 프로파일링을 수행하였다. 이를 위해 서로 다른 두 가지 배양 조건에서 외막소포체를 분리, 정제하고 그 구성 단백질 성분을 LC-MS/MS 방법을 이용하여 확인 하였다. LC-MS/MS 분석 결과에 따르면 탄소와 지방 대사에 관련한 효소, 리보솜 단백질, 수송 단백질 심지어는 SPI-1 과 2 단백질 또한 두 조건 모두에서 분비 되었다. 흥미로운 점은 리보솜 단백질은 LB배지 조건에서, 수송 단백질은 산성 제한배지 조건 에서 분비된 외막소포체 각각에서 보다 다량으로 분비 되었으며 이러한 배지에 따른 외막소포체 단백질 구성의 차이는 다양한 환경 변화에 따른 특이적인 외막소포체 형성 매커니즘이 존재함을 제시한다.

나아가, 산성 제한배지 조건에서만 특별하게 분비되는 다양한 단백질 중, *hupA, glnH* 그리고 *phoN* 유전자에 의해 암호화된 단백질들은 각각 대식세포에 감염되어 대식세포의 세포질로 분비되는 단백질임을 확인 하였다. Immunoblotting을 통해 세 개의 단백질 중 GlnH 그리고 PhoN 단백질은 숙주 내 환경과 유사한 조건에서 외막소포체에 존재함을 다시 확인 하였다. 또한, 이들 각각의 기능이 소실된 변이균주들은 대식세포에 감염 시 병원성이 떨어지는 것으로 나타났다. 이 단백질들이 외막소포체와는 별개로 세균의 생존에 관여하는 가능성을

배제 할 수는 없으나 이 결과는 외막소포체를 통해 분비 된 HupA, GlnH 그리고 PhoN 단백질이 살모넬라의 숙주 내 생존에 있어 중요한 역할을 할 것임을 제시한다.

또한, 외막소포체를 통해 분비 된 수송 단백질이 대식 세포 내 살모넬라 성장에 관여한다는 것을 알아냈다. PstS, DppA, CysP, STM14_1918 또는 YcfM 단백질이 결여 된 균주에서 생존 능력의 감소가 나타난 반면 RbsB, PotD, PagL, TggE, MetQ 또는 YehZ가 결여된 균주에서는 생존 능력이 증가했다.

살모넬라 생존과 스트레스 저항에 있어서 이러한 수송체들의 구체적인 역할은 추후 연구를 통한 확인이 필요할 것이나, 이 결과는 살모넬라 생존과 관련한 세포 외막 수송 단백질의 부차적인 기능의 가능성을 시사한다.

주요어: 외막소포체, 살모넬라, Proteomics, 병원성

학번: 2009-21254