ISSN (Online) 2287-3406
Journal of Life Science **2024** Vol. 34. No. 2. 79~85 DOI : https://doi.org/10.5352/JLS.2024.34.2.79

Role of *Salmonella* Typhimurium SlyA in Regulating the Expression of Virulence Factors Related to Survival in Macrophages

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Received January 4, 2024 /Revised January 15, 2024 /Accepted February 2, 2024

SlyA is known as a transcriptional regulator that regulates the expression of hemolysin (HlyE) in *E. coli*, a member of the Enterobacteriaceae family such as *Salmonella*. However, *Salmonella* has the *slyA* gene but lacks the *hlyE* gene. Then, because we were curious about the role of SlyA in *Salmonella*, we constructed and explored a mutant strain with a deletion of the *slyA* gene. *S.* Typhimurium CK295 (Δ*slyA*) was constructed using an allelic exchange approach. In a comparative analysis between the wild-type and the CK295 strain, no significant differences were observed in growth characteristics, motility, total protein analyses, and secreted protein analyses. However, the CK295 strain exhibited slightly reduced biofilm formation compared to the wild-type. Interestingly, as a result of comparing the survival ability in macrophages, the mutant strain showed a 60% decrease in survival ability compared to the wild-type. To evaluate toxicity in mice, mortality was measured after oral administration to 6-week-old BALB/c mice. As a result, the LD_{50} value of the CK295 (Δ*slyA*) was more than 100 times higher than that of wild-type *S.* Typhimurium χ3339 in BALB/c. In conclusion, SlyA is presumed to regulate the expression of genes encoding virulence factors involved in the *in vivo* survival of *Salmonella*.

Key words : *Salmonella* Typhimurium, *slyA*, transcriptional regulator, virulence factor

Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a gram-negative, facultative anaerobe, and generalist pathogen capable of causing gastroenteritis, and systemic infection [12]. *Salmonella* invades the Peyer's patches of the ileum via M cells using the type Ⅲ secretion system encoded by genes within the *Salmonella* pathogenicity island-1 (SPI-Ⅰ) [15]. Subsequently, it survives within macrophages using SPI-II-specific gene products. During the *Salmonella* pathogenesis process, various virulence determinants are essential for invasion and survival in the intracellular compartment. A live oral *Salmonella* vaccine has been developed by deleting these virulence genes for *Salmonella* pathogenesis [13].

SlyA is a member of the MarR family of transcription factors which includes the MarR, EmrR, HpaR, and HpcR proteins of *Escherichia coli*, RovA of *Yersinia*, and PecS of *Erwinia carotovora*, and SlyA-Ef of *Enterococcus faecalis* [1]. In *E. coli*, SlyA activates the expression of the cryptic hemolysin *hlyE* (*clyA*) [14], of Type1 fimbriae [6], of *pagP* involved in lipid A palmitoylation in biofilm. Interestingly, *S.* Typhimurium does not have the *hlyE* gene, but only the transcription regulator *slyA* gene.

To date, the *slyA* gene is known to exist in the chromosome of *Salmonella*, but its biological role is not precisely known. Looking at the example of *E. coli*, its function as a transcriptional regulator is expected, but research is required to find out. Therefore, we generate a *slyA* deletion mutant and compare it with the wild-type to find out the role of SlyA. In this study, various *in vitro* characteristics including growth and protein expression were compared and analyzed. In addition, the effect on the pathogenicity of *S.* Typhimurium was analyzed through survival ability in macrophages and toxicity tests in mice.

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Materials and Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1 and 2. *Salmonella enterica* ser. Typhimurium and *Escherichia coli* were grown at 37℃ in Luria-Bertani (LB) broth or LB agar [2]. LB agar containing 10% sucrose was used for *slyA* gene-based counterselection in an experiment with allele exchange. *E. coli* χ7213(Asd$^-$), a host strain for the R6K*ori* suicide plasmid, was grown in the presence of 50 μg/ml diaminopimelic acid (DAP) [8]. Antibiotics, when required, were included in the culture medium with the following concentrations: 100 μg/ml ampicillin, 15 μg/ml tetracycline.

General DNA manipulations, transformation, and polymerase chain reaction (PCR)

DNA manipulations were carried out as described by Sambrook *et al* [10]. The plasmid transformation in *E. coli* or *Salmonella* was performed by the method either rubidium chloride-heat shock or electroporation (Biorad). PCR amplification was applied to obtain the DNA fragment for the gene identification or cloning. The PCR reaction was performed according to a commonly used method [4]. The primers used are listed in Table 3. DNA electrophoresis was performed using 0.8% agarose gel to identify or purify the DNA products. HiGene™ Gel & PCR Purification System (Biofact) was used to extract DNA from the agarose gel according to the manufacturer's manual.

Biofilm formation assay

The measurement of biofilm formation was carried out by modifying the method described by O'Toole GA [9]. After *Salmonella* culture on a 96-well plate, the culture medium was removed, and each well was washed with distilled water. 0.1% crystal violet solution was treated for 30 min. After that, it was washed again using distilled water, and the pigment dyed in biofilm was decolorized using 95% ethanol. Dissolved crystal was measured for the degree of biofilm formation by measuring the absorbance at 550 nm.

Table 1. Bacterial strains used in this study

Strains	Relevant characteristics*	References
Escherichia coli		
E. coli DH5α	Transformation host for cloning vector	Promega
E. coli χ7213	*E. coli* DH5α derivative (Δ*asd*), Km^R, DAP required	Lab collection
Salmonella		
S. Typhimurium χ3339	SL1344 *hisG,* Sm^R, wild-type, isolated from mouse	Lab collection
S. Typhimurium CK295	χ3339 derivative, Δ*slyA*	This study

*Km^R; kanamycin resistance, Sm^R; streptomycin resistance

Table 2. Plasmids used in this study

Plasmids	Relevant characteristics*	References
pDMS197	Suicide vector, R6K*ori*, *sacB*, Tet^R	
pGEM-T vector	Cloning vector for PCR product, ColE1*ori*, Ap^R	Promega
pBP1170	5'-flanking region DNA of *slyA* in pGEM-T vector, Ap^R	This study
pBP1171	3'-flanking region DNA of *slyA* in pGEM-T vector, Ap^R	This study
pBP1172	5'-flanking region and 3'-flanking region DNA of *slyA* in pGEM-T vector, Ap^R	This study
pBP1173	5'-flanking region and 3'-flanking region DNA of *slyA* in pDMS197 vector, Tet^R	This study

*Ap^R; ampicillin resistance, Tet^R; tetracycline resistance

Table 3. Primers used in this study

Primers	Nucleotide sequence (5'→3')*	Characteristics
HY700F (*Kpn* Ⅰ)	5'- ggtaccgctatctaccagggcgaaa -3'	Forward for 5'-flanking region of *slyA*
HY701R (*BamH* Ⅰ)	5'- ggatccagacatagtgogcacttgg -3'	Reverse for 5'-flanking region of *slyA*
HY702F (*BamH* Ⅰ)	5'- ggatccgtaaagcctggtttagcgtg -3'	Forward for 3'-flanking region of *slyA*
HY703R (*Sac* Ⅰ)	5'- gagctccctggcaaatatcgagaatg -3'	Reverse for 3'-flanking region of *slyA*

*Underlines indicate restriction enzyme sites for the enzymes indicated in parentheses of the primers.

Precipitation of secretory proteins using trichloroacetic acid (TCA)

The cell-free culture supernatant was made into an ice-cold state and 25% of cold trichloroacetic acid (TCA) was added to the final concentration of 6% to precipitate secretory protein for 30 min. The precipitated proteins were centrifuged and collected at 4°C at 10,000× g for 10 min. The sup-free pellet was washed twice with acetone to remove residual TCA. Then, the tube was completely dried, and the protein bound to the tube wall was re-dissolved with a 5× protein digestion buffer [50 mM Tris-HCl buffer (pH 6.8), 4%(w/v) SDS, 20%(v/v) glycerol, 10%(v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were solubilized in 5× protein digestion buffer. Protein samples digested with the digestion buffer were boiled for 5 min and then separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

RAW 264.7 cell culture

To examine the cytolysis effect of microorganisms on the cells, RAW 264.7 (KCLB No.40071) cell line was used. The cell line was used with Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco). Culture conditions were maintained using a 60 mm petri dish at 37℃, 95% humidity, and 5% CO_2.

Intracellular survival assay

Gentamycin replication assay was applied to determine the growth of *Salmonella* inside the cell-line. Briefly, *Salmonella* is mixed with cell-line at an MOI of 1:20 and incubated in DMEM medium for 30 minutes at 37°C, 95% humidity, and 5% CO_2. After incubation, the medium in each well was removed and medium containing gentamycin (200 μg/ml) was added and incubated for 1 hr. During this process, *Salmonella* which does not enter the cell dies. After washing twice with PBS, medium containing a low concentration of gentamycin (10 μg/ml) was added and cultured. After 24 hr of incubation the wells were treated with 0.1% Triton X-100 for cell lysis and then serially diluted, and then dropped onto an agar plate for viable count [3].

Animal experiment

All animal experiments were carried out ethically and scientifically according to the Pusan National University Institutional Animal Care and Use Committee (PNU-IACUC) guidelines for the care and use of laboratory animals (approval ID number: PNU-2024-0425). Five-week-old female BALB/c mice (Samtako) were housed 1 week to acclimate them to our animal facility before inoculation . The animal room temperature was maintained at 24-26℃, with 45-55% humidity. All mice were fed NIH#31M diets (Samtako) and distilled water. Each group of mice was deprived of food and water for 4 hr before infection. The *Salmonella* cells were re-suspended in 1 ml of phosphate buffered saline (PBS, pH 7.0) [145 mM NaCl, 2.7 mM KCl, 3.6 mM KH_2PO_4, 10 mM Na_2HPO_4]. Groups of three mice were orally inoculated 100 μl per dose containing 10^4~10^6 CFU (colony forming unit) of *Salmonella* by incubation needle. Food and water were returned to the inoculated mice 4 hr after inoculation. Mortality of the inoculated mice was observed for 2 weeks.

Results and Discussion

Construction of a recombinant suicide plasmid for the deletion of *slyA* gene in a *Salmonella*

The 5'-flanking region or 3'-flanking region of *slyA* gene was amplified by PCR from *S.* Typhimurium χ3339 chromosomal DNA using a pair of primers HY700F (*Kpn*I) and HY701R (*Bam*HI) or HY702F (*Bam*HI) and HY703R (*Sac*I), respectively (Table 3). Each 1.0 kb amplified DNA fragment was cloned into the pGEM-T vector, resulting in pBP1170 or pBP1171, respectively (Table 2). To construct the recombinant DNA for the deletion of the *slyA* genes, 1.0 kb *Kpn*I-*Bam*HI 5'-flanking region DNA fragments isolated from pBP1170 was ligated into pBP1171 digested with *Kpn*I and *Bam*HI, resulting in pBP1172 (Table 2). The 2.0 kb *Kpn*I-*Sac*I DNA fragments, encompassing 5'- and 3' flanking of *slyA* isolated from the pBP1172, were inserted into the suicide vector pDMS197 [11], resulting in pBP1173 (Table 2). The pBP1173 plasmid exhibited tetracycline resistance and was approximately 8 kb in size, as expected. We also confirmed it with PCR amplification to confirm that the cloned DNA fragment matched what we wanted. Additionally, proper cloning was confirmed by analyzing the DNA fragment profile after restriction enzyme digestion.

Construction of *S.* Typhimurium *slyA* deletion mutant

To construct a *Salmonella* mutant lacking the *slyA* gene, an allelic exchange approach was employed using pBP1173, a recombinant plasmid mentioned earlier (Fig. 1A). The pBP1173 plasmid, which can replicate only in specific strains of *E. coli*, was transformed into *E. coli* χ7213, a host capable of supporting its replication. This *E. coli* host is a DAP auxotrophic strain and efficiently facilitates screening for transconjugants in plasmid transfer by conjugation. *E. coli* χ 7213/pBP1173 strain and *Salmonella* χ3339 were simultaneously streaked onto LB agar containing DAP and incubated at 37℃ for 5 hr to facilitate contact. Subsequently, the transconjugant in which the suicide recombinant plasmid was inserted into the *Salmonella* χ3339 chromosome was screened on LB agar medium containing tetracycline without DAP. The single cross-over transconjugant was subcultured in LB broth, and the second cross-over strain was selected. Among the strains lacking the suicide plasmids, those with a deletion in *slyA* were selected. Selection of the second cross-over strain was evaluated using the *sacB*-based sucrose-sensitive counter selection system [11]. Among strains resistant to sucrose and sensitive to tetracycline antibiotics, two strains lacking s*lyA* were selected by PCR amplification, and one of them was used for further research. As demonstrated by PCR using an appropriate primer set for the selected strain, the chromosomal DNA of the mutant in the target region was smaller than that of *S.* Typhimurium χ3339, and the size of the amplified DNA fragment was as expected (Fig 1B). This confirmed the strain as a *Salmonella slyA* deletion strain, which was named it *S.* Typhimurium CK295 (Δ*slyA*).

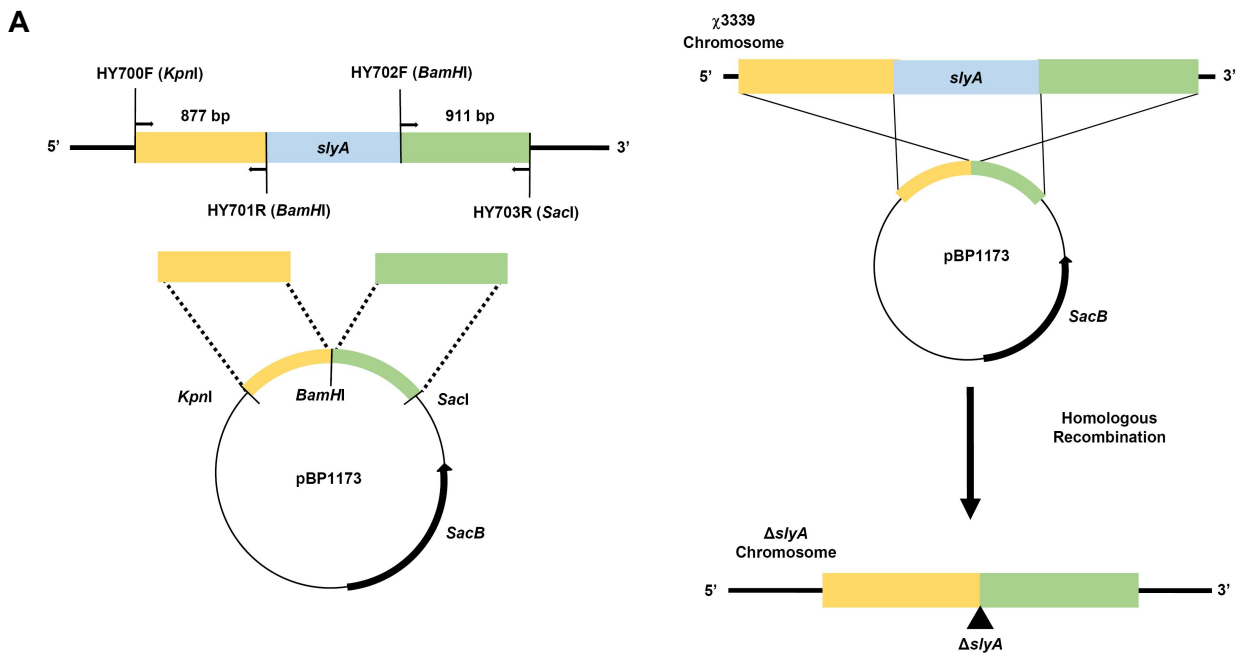

Fig. 1. Construction of *S.* Typhimurium *slyA* deletion mutant. (A) Simple schematic of the process of generating a chromosomal *slyA* deletion. Primers for PCR and DNA size when amplified were indicated. The DNA fragment of the 5' upstream and 3' downstream of the *slyA* amplified by PCR was cloned into pDMS197, resulting in a recombinant suicide plasmid pBP1173. Using this plasmid, *slyA* deletion occurs in the chromosome through allelic exchange as shown in the figure. (B) Genetic confirmation of *slyA* deletion in the *S.* Typhimurium CK295. Using the primers of HY700F (*KpnI*) and HY703R (*SacI*), the chromosomes of the wild-type and the CK295 strain were used as templates to perform PCR amplification and their sizes were compared. M: Marker, lane 1: *S.* Typhimurium χ3339 chromosome, lane 2: pBP1173, lane 3: *S.* Typhimurium CK295 (Δ*slyA*).

S. Typhimurium CK295 (Δ*slyA*) physiological characteristics

An experiment was conducted to compare the growth of wild-type *Salmonella* strain and *Salmonella* CK295, and there was no significant difference in growth in LB broth or agar medium (data not shown). *Salmonella* is known for having flagella on its surface and being motile. To investigate the effect of *slyA* on motility, motility was examined in LB medium containing 0.5% agar, which is a standard motility measurement medium, and no significant differences from the wild-type were found.

Interestingly, when the CK295 was cultured with shaking in a test tube containing LB broth, it was observed that the degree of staining of the culture medium on the surface of the test tube was weaker compared to the culture of the wild-type strain. This phenomenon was consistently observed in repeated experiments, suggesting a systematic effect. We hypothesized that it might be related to biofilm production. After culturing the wild-type strain and CK295, biofilm production was measured using the method described in materials and methods. A typical biofilm measurement method is to stain the biofilm with crystal-violet and quantify it by the degree of color development [9]. The biofilm was stained with 0.1% crystal violet solution, and the crystal violet of the stained biofilm-crystal violet complex was extracted with 95% ethanol and quantified by absorbance. Biofilm formation in *S.* Typhimurium CK295 was slightly lower than that produced by the wild-type strain, but the difference was not significant (Fig. 2). Generally, biofilms consist of various substances such as extracellular polysaccharide, surface protein, and surface glycolipid, so they cannot change dramatically if one component is not created, so even a somewhat small difference can be meaningful. Whether SlyA is involved in regulating the production of certain components of biofilms remains to be solved in the future.

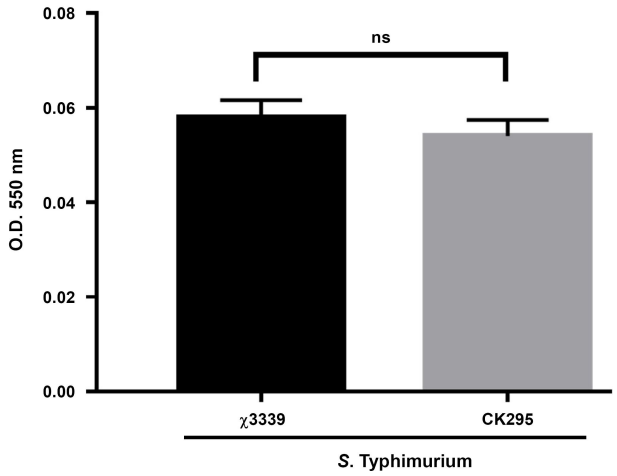

Fig. 2. Comparison of biofilm production between *S.* Typhimurium χ3339 and CK295. After removing the *Salmonella* cells and medium cultured in a 96-well plate, the biofilm attached to the plate surface was stained with crystal violet. Then, the dye was dissolved in ethanol and the color was measured at 550 nm with a spectrometer. The ns stands for no significance.

To determine whether SlyA plays a role as a regulator in protein expression of *Salmonella*, total protein expression was analyzed by SDS-PAGE. Upon comparing the Coomassie-stained gels, no protein bands showing notable differences were observed in the protein expression of wild-type strains and CK295 strains (data not shown). Secreted proteins were also analyzed to investigate how SlyA affects protein secretion in *Salmonella* bacteria. As described in materials and methods, to examine the secreted proteins of *S.* Typhimurium χ3339 and CK295, the medium supernatant from which the bacteria were removed from the culture medium was treated with TCA to precipitate the proteins, which were then analyzed by SDS-PAGE. As shown in Fig. 3, no significant dif-

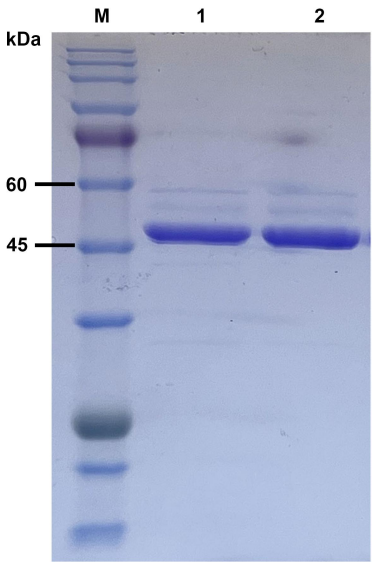

Fig. 3. SDS-PAGE analysis of proteins secreted by *S.* Typhimurium χ3339 and CK295. After culturing *Salmonella* in LB broth, the cell-free supernatant from which the bacteria were removed by centrifugation was treated with 10% TCA to precipitate proteins secreted in the culture medium. Residual TCA was removed with acetone, and the protein was dissolved in buffer and analyzed by SDS-PAGE. Proteins were visualized by Coomassie staining. M: marker, lane 1: *S.* Typhimurium χ3339 secretion, lane 2: *S.* Typhimurium CK295 (Δ*slyA*) secretion.

Table 4. Virulence of *S.* Typhimurium strains in BALB/c mice*

Strains	Inoculation dose (CFU)	Survivors/total
S. Typhimurium χ3339 (SL1344 *hisG,* Sm^R, wild-type)	3.92×10^4	1/3
S. Typhimurium CK295 (χ3339 derivative, Δ*slyA)*	3.96×10^4	3/3
	3.96×10^6	3/3

*Six-week-old BALB/c female mice were infected by oral route with a single dose of *Salmonella* strains. Morality observation was recorded daily for an additional 14 days post-infection. Both inoculating doses were measured in CFU.

ferences were found in the secreted protein pattern between *Salmonella* χ3339 and CK295 (Δ*slyA*).

Intracellular survival assay

As intracellular pathogens, *Salmonella* are capable of replicating within macrophages. In this process, they employ complex mechanisms to overcome the killing mechanism of macrophages and survive. If SlyA regulates the expression of genes required for this process, SlyA-deficient strains will likely exhibit reduced survival ability in macrophages. To investigate this hypothesis, an experiment was conducted as described in the materials and methods to assess the replication ability in macrophages [3]. *Salmonella* CK295 (Δ*slyA*) showed that its survival ability in macrophages was reduced by more than 60% compared to χ3339 (Fig. 4). These results suggest that SlyA is involved in the expression of genes necessary for survival in macrophages. This result can be seen as supporting the results of previous studies that showed the possibility of SlyA regulating genes encoded in SPI-I [7]. This is because SPI-II is a system required for the intracellular survival of *Salmonella* [5].

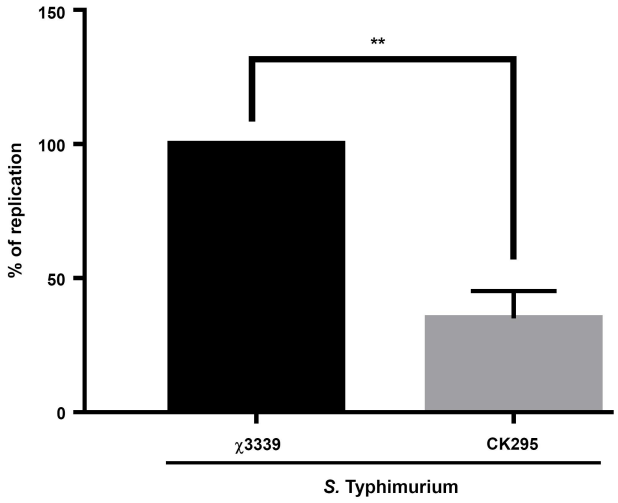

Fig. 4. *Salmonella* survival ability in macrophages. RAW 264.7 macrophage cells were infected by contacting them with *Salmonella* cells at an MOI of 1:20 for 30 minutes. *Salmonella* cells that did not enter the cell were killed by treatment with Gentamycin (200 μg/ml). After culturing the infected macrophages for 24 hr, they were lysed, and the *Salmonella* cells were counted after culture on LB agar medium. The survival ability of the CK259 strain was expressed as a relative percentile to *S.* Typhimurium χ3339 (n=3; $p<0.01$(**)).

Virulence of the *S.* Typhimurium deletion mutants *in vivo*

It was previously mentioned that the mutant strain lacking *slyA* has a significantly reduced survival ability in macrophages, which means that it can be expected that there will be a difference in pathogenicity compared to the wild-type in the pathogenicity test in the mouse model. To monitor survival dynamics, mice were infected with various doses of *S.* Typhimurium χ3339 wild-type or CK295 (Δ*slyA*) (10^6 to 10^4 CFU doses). For wild-type infected mice administered a dose of 3.92×10^4 CFU, 2 of 3 mice died. In contrast, all mice infected with CK295 (Δ*slyA*) at similar doses survived. Furthermore, when mice were infected with CK295 (Δ*slyA*) at a dose of 10^6 CFU, all mice in the group survived (Table 4). These results suggest that the *slyA* gene is essential for *Salmonella* infection. This result is consistent with previous studies showing that SlyA is crucial for the full virulence of *Salmonella*.

Overall, we believe that SlyA serves an essential regulator for the pathogenicity of *Salmonella*, particularly in regulating genes involved in survival within macrophages. Therefore, it is considered necessary to analyze the regulation of expression of SPI-II genes in future studies. Unfortunately, no significant differences could be observed in various phenotypes at the *in vitro* stage compared to the wild-type.

Acknowledgement

This work was supported by a 2-Year Research Grant of Pusan National University.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest

with the contents of this article.

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초록 : 대식세포 내 생존과 관련된 독성인자 발현 조절에서의 *Salmonella* Typhimurium SlyA의 역할

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SlyA는 *Salmonella*와 같은 장내세균과(Enterobacteriaceae)에 속하는 *E. coli*에서 용혈소(HlyE)의 발현을 조절하는 전사 조절인자로 알려져 있다. 그러나 *Salmonella*에는 *slyA* 유전자가 있지만 *hlyE* 유전자는 없다. *Salmonella*에서 SlyA의 역할을 탐구하기 위해 *slyA* 유전자가 결실된 돌연변이주를 사용하였다. *S.* Typhimurium CK295 (Δ*slyA*)는 allelic exchange 방법으로 제작되었다. 야생형 균주와 CK295 균주의 비교시험에서 생육특성, 운동성, 총 단백질 분석, 분비 단백질 분석 등에서 특별한 차이가 발견되지 않았다. CK295 균주는 야생형에 비해 생물막을 약간 적게 생성하는 패턴을 보였다. 흥미롭게도, 대식세포에서의 생존능력을 비교한 결과, CK295 균주는 야생형에 비해 생존능력이 60% 감소하는 것으로 나타났다. 마우스의 독성을 테스트하기 위해 6주령 BALB/c 마우스에 경구 투여한 후 사망률을 측정하였다. 그 결과, BALB/c에서 CK295 (Δ*slyA*)의 LD_{50} 값이 야생형 *S.* Typhimurium χ3339의 값보다 100배 이상 높게 나타났다. 종합적으로, SlyA는 살모넬라균의 *in vivo* 생존에 관여하는 독성 인자를 코딩하는 유전자의 발현을 조절하는 것으로 추정된다.