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Effect of Gene *act*A on the Invasion Efficiency of *Listeria monocytogenes*, as Observed in Healthy and Senescent Intestinal Epithelial Cells

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Listeria monocytogenes can asymptomatically inhabit the human intestine as a commensal bacterium. However, the mechanism by which *L. monocytogenes* is able to inhabit the intestine without pathogenic symptoms remains unclear. We compared the invasion efficiency of *L. monocytogenes* strains with the 268- and 385-bp-long *act*A gene. Clinical strains SMFM-CI-3 and SMFM-CI-6 with 268-bp *act*A isolated from patients with listeriosis, and strains SMFM-SI-1 and SMFM-SI-2 with the 385-bp gene isolated from carcasses, were used for inoculum preparation. The invasion efficiency of these strains was evaluated using Caco-2 cells (intestinal epithelial cell line), prepared as normal and healthy cells with tightened tight junctions and senescent cells with loose tight junctions that were loosened by adriamycin treatment. The invasion efficiency of *L. monocytogenes* strains with the 268-bp-long *act*A gene was 1.1–2.6-times lower than that of the strains with the 385-bp-long gene in normal and healthy cells. However, the invasion efficiency of both types of strains did not differ in senescent cells. Thus, *L. monocytogenes* strains with the 268-bp-long *act*A gene can inhabit the intestine asymptomatically as a commensal bacterium, but they may invade the intestinal epithelial cells and cause listeriosis in senescent cells.

Keywords: *Listeria monocytogenes*, tight junction, Caco-2 cells, invasion, senescence

Introduction

Listeria monocytogenes is an intracellular, gram-positive, ubiquitous bacterium found in various environments such as water, soil, and plants [1]. Because *L. monocytogenes* is psychrotrophic, it can grow at refrigeration temperature, and can thus contaminate food at all steps of the food chain. Subsequently, *L. monocytogenes*-contaminated food causes listeriosis, with a mortality rate of up to 20–30% [2].

L. monocytogenes has various virulence factors such as *hly*A, *inl*A, *inl*B, *act*A, and *plc*A [3]. Because of these factors, it can survive and proliferate in phagocytes and invade host cells [4]. The onset of the pathogenic mechanism involves binding to the host cells aided by *inl*A-E cadherin, *inl*B-Met, and *act*A heparin sulfate interaction [5, 6]. As *L. monocytogenes* invades the host cell, the pathogen is trapped in the vacuole and then expresses *plc*A, *plc*B, and *act*A genes responsible for vacuole lysis [7, 8]. After the vacuole lyses, *act*A helps in the formation of the actin tail in the cytosol, which initiates the cell-to-cell spread to begin a new infection cycle [9, 10]. However, recent studies have shown that *act*A plays an important role in the invasion and aggregation of host cells [11, 12]. In addition, Appelberg and Leal [13] suggested another invasion pathway of *L. monocytogenes* not only involving internalins, but also involving ActA.

ActA is a 640-amino-acid-containing transmembrane protein and contains regions such as the N, P, and C regions, with different roles [9, 10, 14, 15]. The central P domain (amino acids 263–390) contains four proline-rich

repeats. The number of proline-rich repeats is different, and this polymorphism is characterized by a 268- or 385-bp-long *act*A gene. Some researchers have found that the *act*A size is related to the pathogenicity of *L. monocytogenes* [16, 17].

Although listeriosis is caused by *L. monocytogenes* through food, it is also caused by commensal strains of *L. monocytogenes* in the immunocompromised and elderly individuals, who have loosened tight junctions of the intestinal epithelial cells [18−20]. However, it is not clear how the commensal *L. monocytogenes* can inhabit the intestine asymptomatically, but cause listeriosis in immunocompromised individuals. In South Korea, although cases of listeriosis have been reported occasionally, *L. monocytogenes*-induced foodborne outbreaks have not yet been reported. Recently, Oh [21] obtained clinical *L. monocytogenes* isolates from 10 patients with listeriosis in S. Korea, and the patients were usually the immunocompromised and elderly. Interestingly, they found that the isolates had the 268-bp-long *act*A gene instead of the 385-bp *act*A. Thus, it can be hypothesized that *L. monocytogenes* with the 268-bp-long *act*A inhabits the healthy intestine with no pathogenic symptoms, like a commensal bacterium, but the pathogen penetrates intestinal epithelial cells, causing listeriosis, when the epithelial cells are aged or the tight junctions are loosened.

Therefore, the objective of this study was to understand how the commensal *L. monocytogenes* strains can asymptomatically inhabit the intestine and cause listeriosis when the condition of the intestinal epithelial cells is physiologically weakened.

Materials and Methods

Inoculum Preparation

L. monocytogenes strains SMFM-SI-1 (serotype: 1/2b; 385-bp *actA* gene) and SMFM-SI-2 (serotype: 1/2b; 385-bp *actA* gene) isolated in our laboratory from carcasses, and strains SMFM-CI-3 (serotype: 1/2b; 268-bp *actA* gene) and SMFM-CI-6 (serotype: 1/2b; 268-bp *actA* gene) isolated from patients in Chonbuk National University Hospital were cultured in 10 ml of tryptic soy broth (TSB; Becton Dickinson and Company, USA) with yeast extract (YE; Becton Dickinson and Company) at 30°C for 24 h, and 0.1-ml aliquots of the culture were transferred into 10 ml of TSB-YE. After incubation at 30°C for 24 h, the cells were harvested by centrifugation (1,912 ×*g*, 4°C, 15 min), washed twice with phosphate-buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4{\cdot}7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 L of distilled water), and resuspended in PBS. The bacterial cell suspensions were adjusted to an optical density of 0.05 at 600 nm (OD_{600}) for Caco-2 cell invasion assay.

Preparation of Caco-2 Cells for Transcriptome Analysis and Invasion Assay

Caco-2 cells (KCLB 30037.1) were cultured in Eagle's minimum essential medium (MEM; Gibco, New Zealand) supplemented with 20% fetal bovine serum (FBS; Gibco) plus 1% penicillin-streptomycin (Gibco). The medium was replaced every 3 days. Stabilized Caco-2 cells were seeded at a density of 5×10^4 cells/ml in 24-well plates (SPL Life Science, Korea). The intestinal epithelial cells of the immunocompromised and elderly are characterized by loose tight junctions [22]. Therefore, the intestinal epithelial cells (Caco-2 cells) were cultured for 2 days for normal cells; 10 days for healthy cells; and 2 days, followed by adriamycin (1,000 nM; ANENTION, Korea) treatment, for senescent cells with loosened tight junctions. To induce senescent intestinal epithelial cells, Caco-2 cells were treated with 250, 500, and 1,000 nM adriamycin for 4 h, which is a typical anticancer drug; it promotes senescence when treated at 500 nM or more in Caco-2 cell [23, 24], washing with Dulbecco's PBS (DPBS; Welgene, Korea), and culture in 1 ml of MEM for 3 days. The extent of senescence was evaluated using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich Co., USA), which measures the β-galactosidase produced by senescent cells [25]. Briefly, all cell supernatants were removed and washed twice with 1 ml of 1× PBS. Then, 1.5 ml of 1× fixation buffer was added to the wells and left for 6 min; the wells were washed three times with 1× PBS. The staining mixture was added to each well, followed by incubation until the cells were stained blue. All blue-stained cells were observed by microscopy (Leica Microsystems CMS GmbH, USA).

Transcriptome Analysis

Aliquots (1 ml) of Trizol (Invitrogen, USA) were added to normal, healthy, and senescent Caco-2 cells in a 24-well plate and incubated at room temperature. Two hundred microliters of chloroform (Daejung Chemicals & Metals Co. Ltd., Korea) was added to each mixture and centrifuged at 12,000 ×*g* at 4°C for 15 min. The supernatants were transferred to 1.5-ml tubes, and mixed with the same volume of isopropanol (Sigma-Aldrich Co.). These mixtures were centrifuged at 12,000 ×*g* at 4°C for 10 min, and the pellets were washed with 75% ethanol and mixed with RNase-free water (Qiagen, Germany) to extract mRNA. The total RNA concentration was measured using the Epoch Micro-Volume Spectrophotometer System (Bio Tek Instruments, USA). The QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA. The PCR mixture (25 μl) was prepared using the Rotor-Gene SYBR Green PCR Kit (Qiagen). The relative expression levels of the genes were analyzed with Rotor-Gene Q software (Qiagen) to compare the expression levels of the genes related to cell tight junctions. Duplicate per replication was performed, and an increase of more than two times was considered significant [26]. The primers used are listed in Table 1.

Table 1. Oligonucleotide primers used in the quantitative real-time PCR analysis.

Gene	Primer	Sequence (5′ → 3′)	References
GADPH	GADPH-F	TCC TGC ACC ACC AAC TGC TTA G	[27]
	GADPH-R	TGC TTC ACC ACC TTC TTG ATG TC	
Claudin-1	Claudin-F	CTT GAC CCC CAT CAA TGC	[28]
	Claudin -R	CAC CTC CCA GAA GGC AGA	
Occludin	Occludin-F	TCC GTG AGG CCT TTT GAA	[28]
	Occludin -R	GGT GCA TAA TGA TTG GGT TTG	
TJP	TJP-F	CGC GGA GAG AGA CAA GAT GT	[28]
	TJP-R	AGC GTC ACT GTG TGC TGT TC	

Caco-2 Cell Invasion Assay

This experiment was performed to investigate if *L. monocytogenes* strains with the 268-bp-long *act*A gene have a lower invasion efficiency than those with the 385-bp-long *act*A in healthy intestinal epithelial cells, and if their invasion efficiency was similar in the case of immunocompromised and elderly individuals. The 0.3-ml (7.7 log CFU/ml) aliquots of the prepared *L. monocytogenes* inoculum were inoculated into 2.7 ml of MEM supplemented with 20% FBS. One-milliliter aliquots of the mixtures were then inoculated into a monolayer of Caco-2 cells (5×10^4 cells/ml) in MEM + 20% FBS and incubated in 5% CO_2 at 37°C for 2 h. The upper layer was discarded, and the Caco-2 cells were further incubated in fresh MEM + 20% FBS, along with 50 µg/ml gentamicin to remove the bacterial cells that were attached onto the Caco-2 cells, in CO_2 at 37°C for 2 h. After the incubation, the medium in the upper layer was discarded, and the Caco-2 cells were washed twice with DPBS. One milliliter of 0.5% Triton X-100 (Sigma-Aldrich Co.) was then added into each well, and the plate was placed on ice for 20 min. The resulting suspension (0.1 ml) was inoculated onto PALCAM agar to enumerate invading *L. monocytogenes*. The invasion efficiency of *L. monocytogenes* into Caco-2 cells was calculated by the following equation [29]:

Invasion efficiency (%) = [number of *L. monocytogenes* cells invading Caco-2 cells (CFU/ml)] × [initial cell count of *L. monocytogenes* (CFU/ml)]$^{-1}$ × 100 (1)

The comparison of the invasion efficiency among *L. monocytogenes* strains was expressed as the ratio of the relative increase in the invasion efficiency of the examined *L. monocytogenes* strain with respect to that of *L. monocytogenes* SMFM-CI-3.

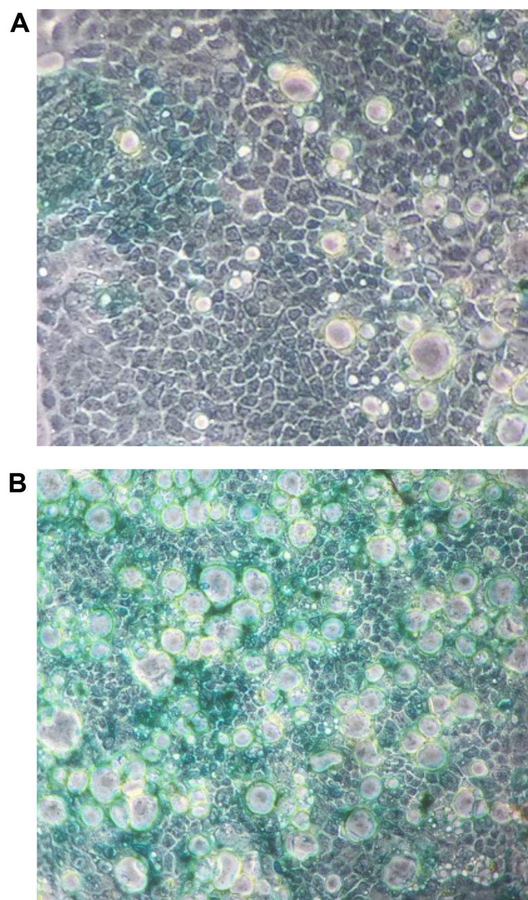


Fig. 1. Normal (**A**), and senescent Caco-2 cells by 4-h treatment with 1,000 nM adriamycin (**B**); magnification, ×10.

Statistical Analysis

The experimental data for the quantitative analysis were analyzed with the general linear procedure of SAS ver. 9.2 (SAS Institute Inc., USA). The mean comparisons were performed by a pairwise *t*-test at α = 0.05.

Results and Discussion

The cells treated with 1,000 nM adriamycin developed a dark blue color on staining, compared with the control, indicating that the 1,000-nM-treated Caco-2 cells produced more β-galactosidase (Fig. 1), which is produced by senescent cells [25]. Senescent cells degrade X-gal at pH 6.0 and it becomes a blue color [30]. Thus, the 1,000-nM-treated cells were used to represent senescent intestinal epithelial cells.

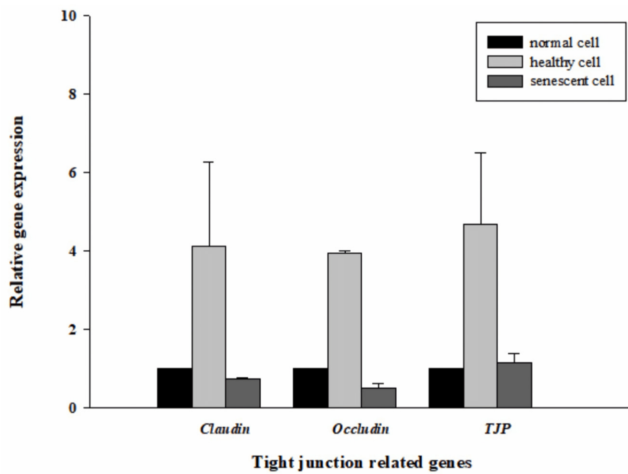
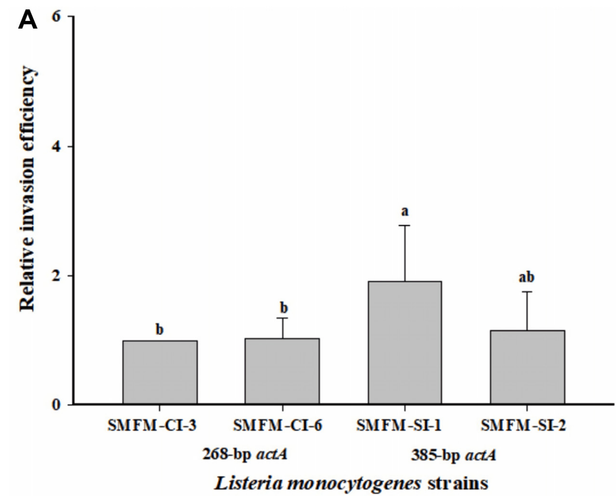
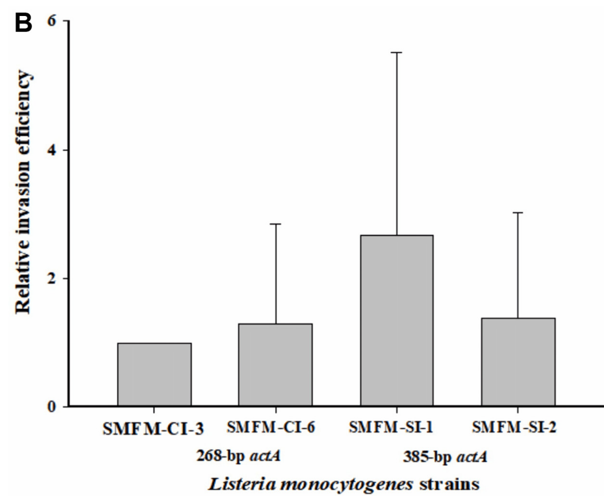


Fig. 2. Relative expression of tight junction-associated genes in normal, healthy, and senescent Caco-2 cells.

To determine whether the Caco-2 cells are healthy or senescent, the transcriptomes of the tight junction-associated genes Claudin-1, Occludin, and tight junction protein (*TJP*) were analyzed, because these genes encode transmembrane proteins that form the structure of tight junctions and help regulate permeability [31-33]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene in the analysis. Because of the expression levels of the tight junction-associated genes, the relative gene expression levels of claudin-1, occludin, and *TJP* were higher in the healthy cells than in the normal and senescent cells (Fig. 2). The relative expression level of *TJP* in the healthy cells was increased by more than four times of that in the normal cells (Fig. 2). Senescent cells showed lower relative gene expression levels of claudin-1 and occludin, compared with the other cells (Fig. 2). Thus, senescent cells possess loosened tight junctions.

The *L. monocytogenes* strains with the 385-bp-long *act*A gene had an invasive efficiency 1.1−1.9-times higher than that of the strains with the 268-bp-long gene in normal cells (Fig. 3A), and 2.6-times higher than that of the strains with the 268-bp-long gene in healthy cells (Fig. 3B). This indicates that the polymorphism of *L. monocytogenes act*A is related to intestinal epithelial cell invasion, and that the invasion efficiency is influenced by the expression of the tight junction protein. The difference between 385-bp *actA* and 268-bp *actA* comes from the proline-rich region. There are many reports suggesting that this region is related to pathogenicity [16, 17], and *L. monocytogenes* strains with 385-bp *actA* have higher virulence than those with 268-bp *actA*.





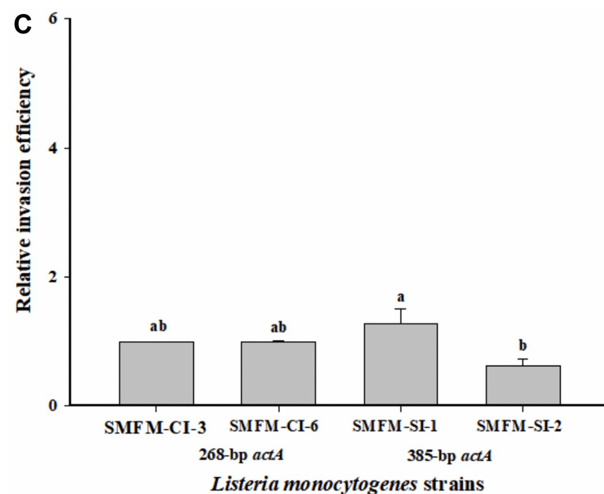


Fig. 3. Comparison of different sizes of *act*A for relative invasion efficiency in normal (**A**), healthy (**B**), and senescent Caco-2 cells (**C**).

Bars with different letters are significantly different ($p < 0.05$).

Although ActA protein is known to play a role in cell- to-cell spread [10], recent studies [11−13] showed that ActA is also related to aggregation and host cell invasion, which corroborate our findings. In senescent cells, the relative invasion efficiency of *L. monocytogenes* strains with the 268-bp-long *act*A was similar to that of the strains with the 385-bp-long gene (Fig. 3C). The invasion efficiency of *L. monocytogenes* strains with the 268-bp-long *act*A in senescent cells might be influenced by the loosened tight junctions of senescent cells, as shown in Fig. 2, as well as their loose morphology and larger flattened cytoplasm, which are characteristic of senescent cells [34]. In addition, He and Sharpless [35] suggested that cell aging causes host toxicity and affects recovery in the event of pathogenicity. This result indicates a higher chance that *L. monocytogenes* strains with the 268-bp-long *act*A will invade senescent intestinal epithelial cells, compared with normal and healthy cells.

In summary, *L. monocytogenes* strains with a 268-bp-long *act*A gene may inhabit the intestine as a commensal bacterium without pathogenic symptoms in healthy people who have tightened tight junctions of intestinal epithelial cells, but the strains may invade the intestinal epithelial cells of immunocompromised and elderly individuals who have loosened tight junctions and cell morphology, and cause listeriosis. However, further epidemiological studies need to be performed to confirm this conclusion.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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