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Research Article

The Effect of Single Amino Acid Substitution in *SecA2* on Protein Translocation and Pathogenicity of *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is an important zoonotic pathogen that cause severe listeriosis with high mortality in immunosuppressive humans. Infection and pathogenicity of *L. monocytogenes* was mediated by several surface proteins that translocated by secretion systems. Our previous genomic study showed the secretion systems of the virulent and low-virulent strains were different in *secA2* and two hollin genes. To confirm whether the pathogenicity of the two strains was determined by the difference observed in secretion system. We deleted *secA2* and the two hollin genes to compare the pathogenic phenotypes. Our data showed that *secA2* but not the two hollin genes affected the pathogenic phenotypes. To further confirm whether the single base mutant in *secA2* affected the protein pathogenicity and translocation ability of *SecA2*, we complemented the *secA2* deletion mutant strain with $secA2_{Lm850658}$ and $secA2_{M7}$, which encode *SecA2* with Asn567 and Lys567, respectively. Our data showed that *secA2* mutant complement with $secA2_{Lm850658}$ instead of $secA2_{M7}$ significantly improved the adhesion and invasion ability to epithelial cells Caco-2 and bacterial load in mice liver and spleen at both 24 and 48 h post infection. Cell surface protein analysis indicated that only *SecA2* with Asn567 could restore the protein translocation ability. Taken together, our study demonstrated that single amino acid mutant in *SecA2* affected the protein translocation and pathogenicity of *L. monocytogenes* for the first time.

Keywords: *Listeria monocytogenes, Secretion system, SecA2, Pathogenicity*

SecA2'de Tek Amino Asit Yerdeğiştirmesinin *Listeria monocytogenes*'te Protein Translokasyonu ve Patojenite Üzerine Etkisi

Öz

Listeria monocytogenes önemli bir zoonotik patojen olup immunsupresyone insanlarda yüksek mortaliteye sahip şiddetli listeriozise neden olur. *L. monocytogenes* enfeksiyonu ve patojenitesi sekresyon sistemi tarafından salınan çeşitli yüzey proteinleri tarafından oluşturulur. Yapılan önceki genomik çalışma virülent ve düşük-virülent suşların sekresyon sistemlerinin *secA2* ve iki hollin genlerinde farklı olduğunu gösterdi. İki suşun patojenitelerindeki farkın sekresyon sistemlerinde gözlenen farktan kaynaklanıp kaynaklanmadığı araştırıldı. Çalışmada, patojenik fenotipleri karşılaştırmak amacıyla *secA2* ve iki hollin geni çıkarıldı. Elde edilen sonuçlar iki hollin geninin değil de *secA2* geninin patojenik fenotipi etkilediğini gösterdi. *secA2* geninde tek baz mutantının protein patojenitesini ve *SecA2*'nin translokasyon kabiliyetini etkileyip etkilemediğini test etmek amacıyla *secA2* gen delesyon mutant suşunu sırasıyla Asn567 ve Lys567'i kodlayan $secA2_{Lm850658}$ ve $secA2_{M7}$ ile tamamladık. Elde edilen veriler, $secA2_{Lm850658}$ ile tamamlanan *secA2* mutantının anlamlı derecede Caco-2 epitel hücrelerine adezyon ve invazyon kabiliyeti ile fare karaciğer ve dalak dokularında enfeksiyon sonrası 24 ve 48. saatlerde bakteriyel yığılmayı artırdığını gösterdi. Hücre yüzey protein analizi, sadece Asn567'li *SecA2*'nin protein translokasyon kabiliyetini koruyabildiğini gösterdi. Tüm sonuçlar değerlendirildiğinde, *L. monocytogenes SecA2*'de tek amino asit mutasyonunun protein translokasyonu ve patojeniteyi etkilediği ilk kez gösterilmiştir.

Anahtar sözcükler: *Listeria monocytogenes, Sekresyon sistemi, SecA2, Patojenite*

INTRODUCTION

Listeria monocytogenes is an important foodborne pathogens which can cause severe foodborne disease called listeriosis with an overall 20-30% mortality rate in immunosuppressive patients [1,2]. This bacterium can survive in various environments including water, soil, food, food processing plants, gastrointestinal tracts and even the

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cytosol of eukaryotic cells [3]. To successfully switch from a saprophytic to a pathogenic bacteria, *L. monocytogenes* secreted a variety of proteins involved in colonization and infection [4-6]. These surface proteins were translocated by the six well known secretion systems, including the Sec system, the Tat pathway (Twin-arginine translocation), the FEA (Flagella Export Apparatus), the FPE (Fimbrilin-Protein Exporter), the Hollins and the Wss [WXG100 (proteins with WXG motif of ~100 residues) secretion system]. Among these secretion systems, the Sec system considered to be the main force for protein secretion in *L. monocytogenes* [7]. Sec system was composed of essential SecYEG translocon, peripheral SecA ATPase and other accessory components [8,9]. Among them, SecYEG forms a protein channel through the cytoplasmic membranes, and SecA hydrolyzes ATP to provide energy for protein translocation [10]. SecA2 as one of the accessory components of the Sec system also made a contribution to the translation of several surface proteins [11-13].

SecA2 was firstly identified and demonstrated to contribute to bacterial virulence by Laurel L. Lenz [14]. Unlike SecA, which was essential for *L. monocytogenes* survival, SecA2 was not required for the bacterial viability [10], but SecA2 played important roles in pathogenesis. Soon after the discovery, seventeen SecA2-dependent secreted and surface proteins, which including two autolysins (Iap and NamA) that contributed to cell wall hydrolysis and bacterial virulence, were identified by proteomics approach [11]. More recently, Sandra et al.[12] analyzed the SecA2-dependent exo-proteome and newly found 13 proteins that were associated with cell wall metabolism, bacterial adhesion and biofilm formation. And deleted SecA2 also induces biofilm formation and impacts biofilm architecture, and promotes bacterial aggregation [13]. Moreover, SecA2 primed protective anti-*L. monocytogenes* cell immune response which might be resulted from one or several of the SecA2 secreted proteins released inside the cytosol of infected cells [15]. Taken together, SecA2 played important roles in proteins translocation and pathogenicity of *L. monocytogenes*, but the mechanisms of SecA2 in protein translocation remain unclear. Our previous study showed the secretion systems of the virulent and low-virulent strains were with a little difference [16]. We hypothesized that the difference in secretion systems between the virulent and low-virulent strains affect the virulence associated proteins translocation and finally determine their pathogenicity. To confirm this hypothesis, we deleted the components with difference between the two strains and then studied the roles. Taken together, we demonstrated the single amino acid residue mutant in SecA2 significantly affect its roles in protein translocation and pathogenicity for the first time.

MATERIAL and METHODS

Bacterial Strains, Plasmids and Culture Conditions

L. monocytogenes virulent strain Lm850658 and low-virulent strain M7 were used as the wild-type strains and cultured in brain heart infusion medium (BHI, Oxoid, UK) at 37°C. *Escherichia coli* DH5α was employed as the host strain for the shuttle plasmid pKSV7 and cultured in Luria-Bertani medium (LB, Oxoid) at 37°C. Stock solutions of ampicillin (50 mg/mL), chloramphenicol (10 mg/mL) and gentamycin (100 mg/mL) (Sangong Biotech, China) were added to media with the required concentrations.

Construction of Deletion and Complement Mutants

A homologous recombination strategy was used for the construction of *secA2* and *LMM7_0111* and *LMM7_1303* deletion in Lm850658 and M7 according to previous studies [16,17]. The homologous fragments were amplified with primer pairs listed in *Table 1* and purified with Gel Purification Kit (GK2042, Generay Biotech. Co. Ltd.). Then fragments were digested with indicated restriction enzymes (Takara, Japan) and ligated to the temperature-sensitive shuttle vector pKSV7 and transformed into DH5α. Positive clones were then confirmed by PCR and sequencing. Plasmids containing inserted fragments were subsequently extracted and electroporated into *L. monocytogenes* competent cells. Transformants were grown at a non-permissive temperature (41°C) on BHI containing chloramphenicol (10 μg/mL) to promote chromosomal integration. The recombinants were passaged in BHI without antibiotic at a permissive temperature (30°C) to enable plasmid excision and curing. The deletion mutants were identified by PCR with primers listed in *Table 1*.

For the complemental strains, *secA2* encoding sequence of Lm850658 and M7 were amplified by PCR and then purified with Gel Purification kit. After restriction digestion with appropriate enzymes, the PCR fragment was cloned into pIMK2 following the P_{Help} promoter. The recombinant plasmids were then electroporated into Lm85065-ΔsecA2 competent cells. The transformants were plated on BHI agar containing kanamycin (50 μg/mL), then positive clones were picked up and identified by PCR and designated as $C\Delta secA2_{Lm850658}$ and $C\Delta secA2_{M7}$, respectively.

Adhesion and Invasion Assay

Adhesion and invasion assays in Caco-2 and HeLa cells were conducted as previous research [18]. Briefly, overnight cultures were harvested by centrifugation (5.000g for 10 min), resuspended in 10 mM PBS (pH 7.4) and adjusted to 0.25 at $OD_{600\ nm}$. The Caco-2 or HeLa cells (about 2×10^5 per well) were seeded in 12-well plates (Corning, USA), incubated at 37°C and 5% CO_2 for 18-24 h and infected with *L. monocytogenes* at MOI of 10:1. For adhesion, cells were washed with PBS and then lysed with deionized water at 1 h post infection (hpi) . For estimation of invasion, cells were washed with PBS at 1 hpi and incubated for an additional h in RMPI 1640 medium containing 10% FBS and 100 μg/mL gentamycin. The cells were lysed at indicated times and 10-fold diluted for plating on BHI agar. The agar plates

Table 1. *Primers used for construction deletion and complement mutants*

Primers	Sequences (5′-3′)
M0111-a	CGGGATCCAAATGAGTTTGAATGTTATTACAGG
M0111-b	TTTCTGTCCTTTGCTAAATTTCATCATACCTCACAATCTTTTTATGAAA
M0111-c	ATGAAATTTAGCAAAGGACAGAAA
M0111-d	GGGGTACCGTAACAAATCTCTACACCGATGGT
M0111-F	GGAATTGCAGTGATAGAGTCAGGT
M0111-R	CATCTACTGCAACATGAAAGCTAAC
M1303-a	CGGGATCCTATGCAAGTGCAAGTGCTGAAAGT
M1303-b	TTCTTTCTTCATAATACATAGTCATTTATTATTCCTCCTAATGCCAAATTA
M1303-c	ATGACTATGTATTATGAAGAAAGAAG
M1303-d	GGGGTACCATAGTAGTAAACAGGCACTTTAAAC
M1303-F	AAAGATTTAGTATTACCAATTTCA
M1303-R	TAGTAGTTGGAGTATCTGCTGTAA
secA2-a	CGCGGATCCTAGTCCCTTTACTTTGAGCGATA
secA2-b	GAAAAAATCAGACGTAGGTTGTATTTATATAACATCCTCCATACCTTCT
secA2-c	AATACAACCTACGTCTGATTTTTT
secA2-d	CGGGGTACCATGTTTGTATTCAATTTATCTTTTAT
secA2-F	ACGAATGGTTCTAAAGACATGA
secA2-R	AGCCGGGATTTCTTACTATTTA
secA2-CF	CATGCCATGGGACAGAATTATGATGATCGCAA
secA2-CR	CGGGATCCTTAACCTTGAATTAGACCATCTGG

were incubated overnight at 37°C for colony counting. Adhesion was expressed as the ratio of recovered colonies to the initial inoculated; while invasion was calculated as the ratio of recovered colonies after gentamycin treatment to initial inoculated.

Protein Samples Preparation and Detection

Five milliliter overnight cultures were incubated in 100 mL fresh BHI medium for 6 h at 37°C and harvested by centrifugation (15.000g, 10 min). Surface proteins were extracted from bacterial pellet with 2% SDS (30 mg wet weight per milliliter of 2% SDS) at 37°C for 1 h. Protein samples were analyzed by 12% SDS-PAGE gel and then probed with indicated antibodies as our previous research [19].

Virulence in Mice Model

The assay was conducted as previous research [16]. Four-weeks age female ICR mice, six per group, were acclimatized for three days in a standard class II laboratory animal facility. Overnight cultures were treated as above with adjusted $OD_{600\ nm}$ at 0.6. Mice were incubated by intraperitoneal injection with 2×10^4 CFU bacteria. At 24 and 48 hpi, mice were euthanized, liver and spleen samples were homogenized, and diluted appropriately for plating counting on BHI agar plates. For the LD_{50} assay, five mice per group were inoculated intraperitoneally with serial ten-fold dilutions of indicated bacteria in PBS. Mice in the control group were received PBS only. The LD_{50} values were calculated by using the trimmed Spearman-Karber method on the mortality data during the 10 days observation period. All animal experiments were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

Statistical Analysis

All data comparisons were analyzed using the two-tailed homoscedastic Student's T-test. In all cases, differences with $P<0.05$ and $P<0.01$ were considered as statistically significant and marked with * and ** respectively. The GraphPad Prism 5 software was used for producing the graphs.

RESULTS

We previously reported the genomes of *L. monocytogenes* lineage III low virulent strain M7 and virulent strain Lm850658 [16]. To explore the mechanisms of that determine the pathogenicity of *L. monocytogenes*, we compared the pathogenic associated phenotypes of strains M7 and Lm850658. Our data showed that M7 exhibited much smaller mobility than strain Lm850658 *(Fig. 1A)*, and the invasion ability of M7 was significantly lower than that of Lm850658 to both Caco-2 cells and Hela cells *(Fig. 1B, 1C)*. As mobility and infection to host cells were mediated by the bacterial surface proteins, we analyzed the compositions of the secretion systems of strain M7 and Lm850658. Our data showed that the

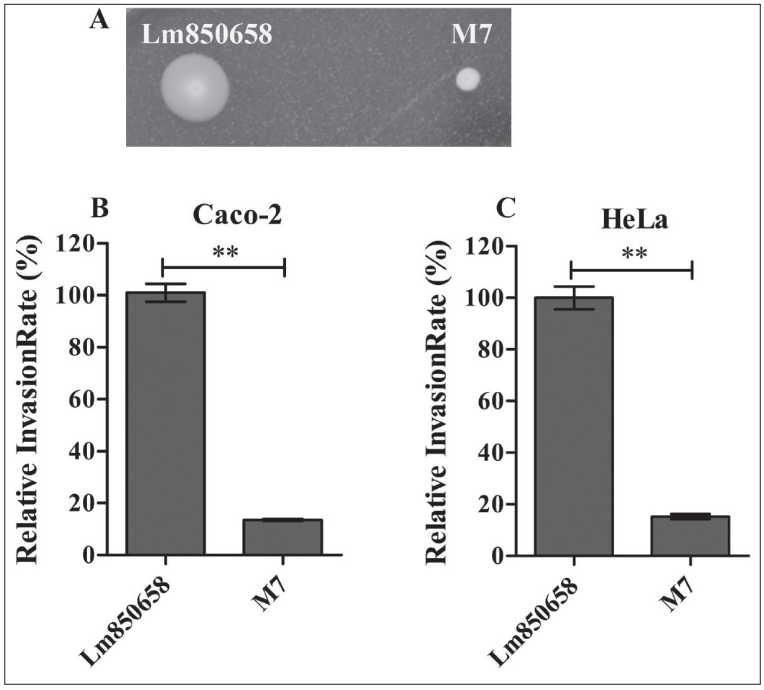


Fig 1. The virulent strain Lm850658 exhibited stronger mobility and invasion ability than that of the low-virulent strain M7. Mobility was tested on the soft agar plate (0.3% agar) under 30°C for 48 h **(A)**, and Invasion ability was conducted on epithelial cell Caco-2 **(B)** and HeLa **(C)**. Data was presented as mean±SD of three independent experiments and ** indicated the statistical significance with P<0.01

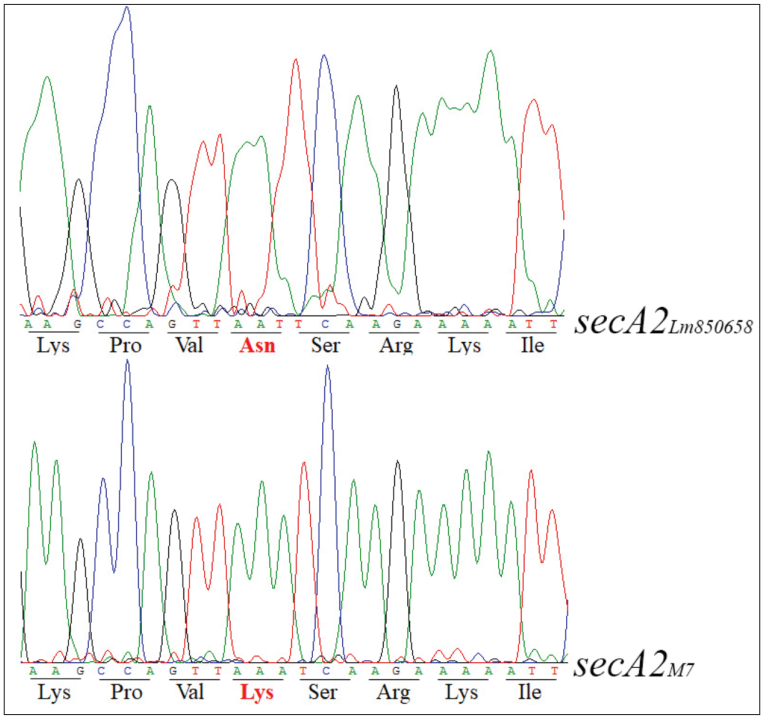


Fig 2. The *secA2*-1701T in Lm850658 was mutant to *secA2*-1701A in M7, which resulted in the Asn567 was mutant to Lys567 of SecA2

encoding genes of the six secretion systems, including the Sec system, the Tat pathway, the FEA, the FPE, the Hollins and the Wss, in strain M7 and Lm850658 were identity except for the *secA2* of Sec system and two hollin genes (*LMM7_0111* and *LMM7_1303*) of Hollin system *(Table 2)*. Sequences analysis showed the *secA2*-1701T in Lm850658 was mutant to *secA2*-1701A in M7 which resulted in SecA2 Asn567 mutant to SecA2 Lys567 *(Fig. 2)*.

To clarify whether the difference in secretion system composition affects the pathogenicity of *L. monocytogenes*, we constructed *secA2* deletion mutant in Lm850658 and *LMM7_0111* and *LMM7_1303* deletion mutant in M7, respectively. We found that deleted *secA2* in Lm850658 significantly defected the adhesion and invasion ability to Caco-2 cell and decreased virulence to mice *(Fig. 3)*. While the two hollin genes deletion made no difference on infection to epithelial cells or virulence to mice *(Fig. 3)*. To confirm the role of the mutant SecA2, the two complemental strains CΔsecA2$_{Lm850658}$ and CΔsecA2$_{M7}$ which encoding SecA2 Asn567 and SecA2 Lys567 respectively. Our data showed that the CΔsecA2$_{Lm850658}$ instead of CΔsecA2$_{M7}$,

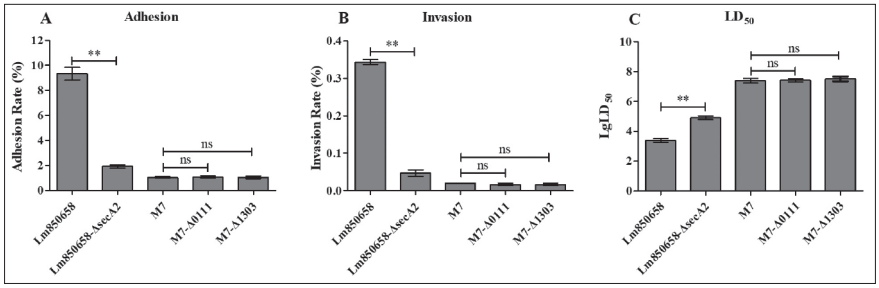
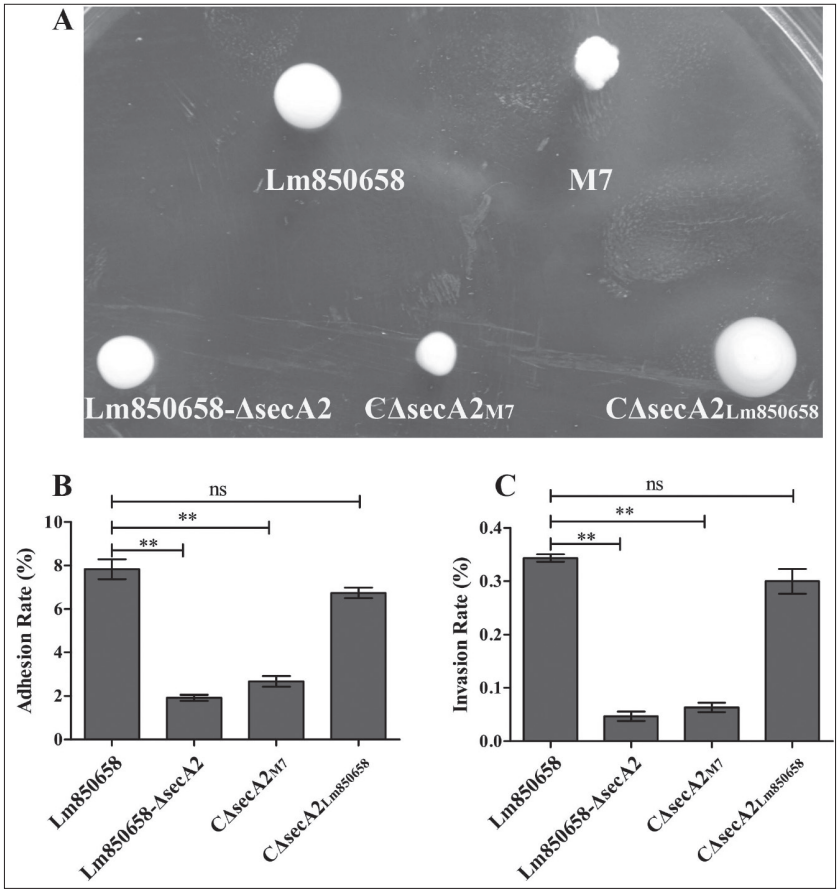


Fig 3. The secA2 deletion instead of the two hollin genes deletion defected the pathogenicity of *L. monocytogenes*. The infection ability **(A** & **B)** and LD50 **(C)** of the wild-type strains and the mutant strains was conducted on Caco-2 cells and ICR mice respectively. Results were presented as mean±SD of three independent experiments and ** indicated the statistical significance with P<0.01

Fig 4. Complement with $secA2_{Lm850658}$ instead of $secA2_{M7}$ restored the mobility and infection ability of *secA2* deletion mutant strain. Mobility was tested on the soft agar plate (0.3% agar) under 30°C for 48 hours **(A)**, adhesion **(B)** and invasion **(C)** ability was conducted on Caco-2 cells. Data was presented as mean±SD of three independent experiments and ** indicated the statistical significance with *P*<0.01



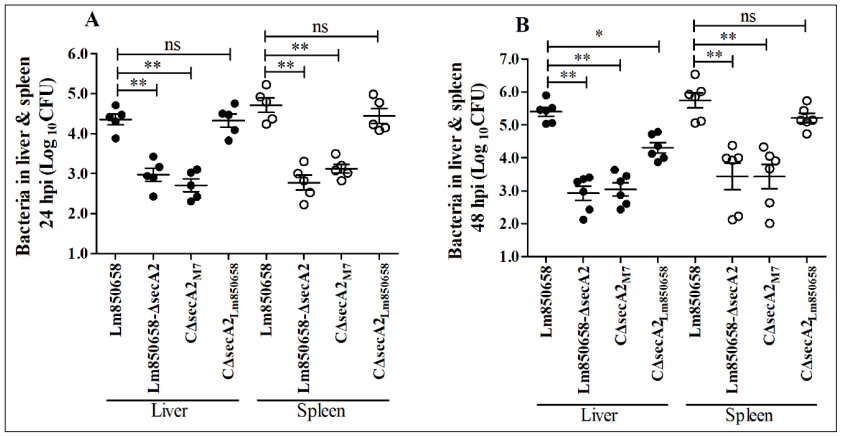


Fig 5. Complement with $secA2_{Lm850658}$ instead of $secA2_{M7}$ significantly increased bacteria load in the liver and spleen of mice at both 24 **(A)** and 48 **(B)** h post infection. Data was presented as mean±SD of five or six mice, * and ** indicated the statistical significance with P<0.05 and P<0.01 respectively

could regain the mobile ability, adhesion and invasion ability and virulence to mice as the wild type Lm850658 *(Fig. 4)*. To further determinate the role of SecA2 Asn567 on the pathogenicity of *L. monocytogenes*, we conducted virulence assay on mice model. Our data showed that the spleen and liver bacteria load of Lm850658-ΔsecA2

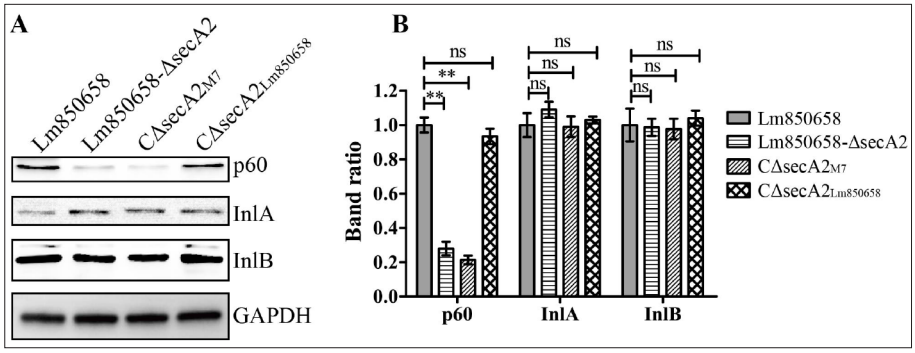


Fig 6. Complement with $secA2_{Lm850658}$ instead of $secA2_{M7}$ significantly increased translocation of p60 but not InlA or InlB. Data was presented as mean±SD of three independent experiments and ** indicated the statistical significance with P<0.01

Table 2. *Secretion systems of Lm850658 and M7*

Systems[a]	Components	EGD-e	Lm850658	M7	Length	Identity[b]
Sec	SecY	Lmo2612	LM850658_2631	LMM7_2724	431	I
	SecE	Lmo0245	LM850658_216	LMM7_0268	59	I
	SecG	Lmo2451	LM850658_2397	LMM7_2493	77	I
	SecDF	Lmo1527	LM850658_1517	LMM7_1613	754	I
	YajC	Lmo1529	LM850658_1519	LMM7_1615	108	I
	YidC	Lmo1379	LM850658_1346	LMM7_1465	275	I
	YidC	Lmo2854	LM850658_2882	LMM7_2975	287	I
	FtsY	Lmo1803	LM850658_1799	LMM7_1895	328	I
	Ffh	Lmo1801	LM850658_1797	LMM7_1893	450	I
	SecA	Lmo2510	LM850658_2456	LMM7_2552	837	I
	SecA2	Lmo0583	LM850658_560	LMM7_0613	776	N567K
	SipX	Lmo1269	LM850658_1229	LMM7_1350	188	I
	SipY	Lmo1270	LM850658_1230	LMM7_1351	189	I
	SipZ	Lmo1271	LM850658_1231	LMM7_1352	180	I
	Lsp	Lmo1844	LM850658_1841	LMM7_1937	154	I
Tat	TatA	Lmo0362				
	TatC	Lmo0361				
FPE	ComGA	Lmo1347	LM850658_1313	LMM7_1432	340	I
	ComGB	Lmo1346	LM850658_1312	LMM7_1431	343	I
	ComGC	Lmo1550	LM850658_1540	LMM7_1636	236	I
FEA	FlhA	Lmo0680	LM850658_662	LMM7_0715	691	I
	FlhB	Lmo0679	LM850658_661	LMM7_0714	348	I
	FliR	Lmo0678	LM850658_660	LMM7_0713	253	I
	FliQ	Lmo0677	LM850658_659	LMM7_0712	90	I
	FliP	Lmo0676	LM850658_658	LMM7_0711	255	I
	FliH	Lmo0715	LM850658_697	LMM7_0750	230	I
	FliI	Lmo0716	LM850658_698	LMM7_0751	433	I
Holins	TcdE	Lmo0128	LM850658_102	LMM7_0155	140	I
	φA118	Lmo2279	LM850658_2548	LMM7_2644	86	I
	Holin			LMM7_0111		
	Holin			LMM7_1303		
Wss	YukAB	Lmo0061	LM850658_56	LMM7_0056	1496	I
	EsaA	Lmo0057	LM850658_52	LMM7_0052	1071	I
	EssA	Lmo0058	LM850658_53	LMM7_0053	171	I
	YukC	Lmo0060	LM850658_55	LMM7_0055	398	I
	YukD	Lmo0059	LM850658_54	LMM7_0054	83	I
	EsaC	Lmo0062				

[a] Tat, Twin-arginine translocation, FPE, Fimbriae Protein Exporter, FEA, Flagella Export Apparatus, Wss, WXG100 secretion system; [b] I, indicated that amino acid sequences between Lm850658 and M7 were identical

infected mice were significantly reduced. Complement strain CΔsecA2$_{Lm850658}$ which expressed SecA2 Asn567 could restore these phenotypes but complement strain CΔsecA2$_{M7}$ which produced SecA2 Lys567 made no difference on the pathogenicity phenotypes *(Fig. 5)*.

Invading into host cells was an important process of *Listeria* infection, which was mediated by the internalin family members and other cell surface proteins, such as InlA, InlB, p60 and Ami [4]. But only p60 and Ami instead of InlA and InlB were reported as the substrates of SecA2 of *L. monocytogenes*. To confirm how the SecA2 mutant affects the pathogenicity of *L. monocytogenes*, we analyzed the content of these surface proteins of wild type strain Lm850658 and indicated mutant strains. Our data showed that surface p60 was significantly reduced in Lm850658-ΔsecA2 and CΔsecA2$_{M7}$ *(Fig 6)*. While the major invasion factor InlA and InlB were exhibited no significant difference in the wild-type, *secA2* mutant and complemental strains *(Fig. 6)*.

DISCUSSION

L. monocytogenes is an important foodborne pathogen because of the severity of its infections in immuno-suppressive humans, like pregnant women, old people and children [20]. *L. monocytogenes* could invade into a variety of host cell types [21], escape from the primary and secondly vacuoles, proliferate in host cytoplasm and recruit actin polymerization to spread to adjacent cells [22]. Each step of the infection process was mediated by specific virulent factors, which were secreted to the bacterial surface by secretion systems [7,23]. It was believed that the Sec secretion systems played important roles in the survival and pathogenicity of *L. monocytogenes* [7]. SecA2 as an accessory component of Sec system was found that had no threat to the bacterial life, but made a contribution to specific proteins translocation and the pathogenicity of *L. monocytogenes* [11,24]. Here we demonstrated that SecA2 Asn567 mediated the surface proteins translocation and pathogenicity of *L. monocytogenes* for the first time.

Comparative genomic analysis showed SecA2 Asn567 were conserved in most of *L. monocytogenes*, and SecA2 Lys567 was only found in strain M7 and the other two representative low-virulent strains HCC23 and L99 [25]. Although previous studies identified the substrates of SecA2 by proteomic methods and demonstrated *secA2* mutant defected the pathogenicity of *L. monoctygoenes* [11,12], little was known about the relationship between structure and function of SecA2 in *L. monocytogenes* or other bacteria. As the essential ATPase SecA and the non-essential SecA2 were conserved in many gram-positive bacteria and they contained similar domains [10], and SecA2 was shared 42% identical and/or 62% similar amino acid residues with the SecA in *L. monocytogenes* [14], SecA2 Asn567 was located in the C terminal domain (CTD). Unlike other domains, the CTD in SecA2 or SecA were various across species [10], so mutation in the CTD might result in different effects on the functions of SecA2.

In this study, we found *secA2* deletion mutant strain complement with *secA2* from the virulent strain Lm850658 and low-virulent strain M7 exhibited the same phenotypes as the original strains respectively *(Fig. 4, Fig. 5)*. These data indicated that the CTD of SecA2 might contribute to the pathogenic phenotypes of *L. monocytogenes*. These phenotypes including mobility in the environments and invading into the host cells *(Fig. 4)* but not the survival ability in macrophage and migration among the cells (data not showed). This phenomenon prompted us to analyze the cell surface proteins of the wild type and the secA2 mutant strains. Our data showed that the p60 (also named as invasion associated protein, Iap) from Lm850658-ΔsecA2 and CΔsecA2$_{M7}$ was significantly less than that of the wild-type strain Lm850658 and CΔsecA2$_{Lm850658}$ *(Fig. 6)*. But the InlA and InlB which were the major factor that contributed to the infection of *L. monocytogenes* into host cells have remained unchanged. This has consisted with previous reported that SecA2 were mediated the translocation the cell wall hydrolases p60 and Ami, and *Listeria* adhesion protein (LAP), an alcohol acetaldehyde dehydrogenase homolog (lmo1634) [11,26].

Taken together, we demonstrated that SecA2 Asn567 determinate the mobility in on agar and pathogenicity of *L. monocytogenes*. Further study might focus on how this amino acid mutant affects the structure and function of SecA2.

Statement of Author Contributions

CF and XYC and conducted experiments, analyzed data and drafted the manuscript. XYL, XWF, KLG, JC and YFG were involved in data collection and analysis. CF and YYY designed the entire experiments, contributed to data analysis and critical reading the manuscript.

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Compliance with Ethical Standards

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