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Research Article

Roles of Histidine Kinase Gene *yycG* in the Pathogenicity of *Listeria monocytogenes*

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Abstract

As an opportunistic foodborne pathogen, *Listeria monocytogenes* could successfully switch self-characteristics in response to environmental conditions. In *L. monocytogenes,* sixteen two-component systems (TCSs) have been found to contribute the bacteria to sensing and adapting to various conditions. Our previous genomic study showed that mutation was observed in the histidine kinase gene *yycG* between Lm850658 and M7 among the sixteen TCSs. The YycFG TCS was found playing important roles in many other Gram-positive bacteria. While the roles of YycFG TCS in *L. monocytogenes* remain poorly known. In this study, we aimed to determine whether *yycG* play roles in pathogenicity of *L. monocytogenes*. We created a histidine kinase gene *yycG* deletion strain (Δ*yycG)* based on the wild type strain Lm850658 and a complemental strain (CΔ*yycG)*. We found the *yycG* deletion significantly impaired the growth ability and mobility, but enhanced the hemolysis ability *in vitro.* In addition, infection assays on cell and mice model showed that Δ*yycG* exhibited significantly defected in infection ability and virulence. All these phenotypes of the Δ*yycG* could be reversed largely to the levels of the wild type strain Lm850658 by gene complementation. Cell wall-associated and secreted protein analysis showed that the secreted content of Δ*yycG* was significantly increased. And western blotting revealed that Internalin protein B (InlB) and Listerolysin O (LLO) was markedly increased in the secreted fractions of Δ*yycG*, which might be responsible for decreased adhesion and invasion ability and increased hemolytic activity, respectively. Overall, we found the histidine kinase *yycG* played important roles in pathogenicity of *L. monocytogenes* for the first time. Further investigation is needed to explore how the YycFG TCS modulates the growth, mobility, cell surface proteins translocation and virulence.

Keywords: *Listeria monocytogenes, Two-component system, Histidine kinase yycG, Pathogenicity*

Listeria monocytogenes'in Patojenitesinde Histidin Kinaz Geni *yycG*'nin Rolü

Öz

Gıda kaynaklı fırsatçı bir patojen olan *Listeria monocytogenes,* çevresel koşullara tepki olarak kendi özelliklerini başarılı bir şekilde değiştirebilir. *L. monocytogenes*'te, onaltı adet iki bileşenli sistemin (TCS) bakterilerin çeşitli durumları algılamasına ve adaptasyonuna katkıda bulunduğu belirlenmiştir. Önceki genomik çalışmamız, on altı TCS'den Lm850658 ve M7 arasındaki histidin kinaz gen *yycG*'de mutasyon gözlendiğini göstermiştir. YycFG TCS'nin diğer birçok Gram pozitif bakteride önemli roller oynadığı belirlenmiştir. Oysa *L. monocytogenes*'teki YycFG TCS'nin rolü hala tam olarak bilinmemektedir. Bu çalışmada, *yycG*'nin L. monositogenes'in patojenisitesinde rol oynayıp oynamadığını belirlemeyi amaçladık. Yaban tipi Lm850658 suşunu ve tamamlayıcı bir suşu (CΔ*yycG)* temel alan bir histidin kinaz geni *yycG* silme suşu (Δ*yycG)* oluşturduk. YycG'nin silinmesinin büyüme kabiliyetini ve hareketliliğini önemli ölçüde bozduğunu, ancak in vitro hemoliz yeteneğini artırdığını bulduk. Ek olarak, hücre ve fare modelindeki enfeksiyon deneyleri, Δ*yycG*'nin enfeksiyon yeteneği ve virülansta önemli ölçüde kusurlu olduğunu gösterdi. Δ*yycG*'nin tüm bu fenotipleri, gen tamamlaması ile büyük ölçüde yaban tipi Lm850658 suşunun düzeyine tersine çevrilebilir. Hücre duvarı ilişkili ve salgılanan proteinler analizi, Δ*yycG*'nin salgılanan miktarının önemli ölçüde arttığını gösterdi. Western blot analizi, Δ*yycG*'nin salgılanan fraksiyonlarında; sırasıyla azalmış yapışma ve invazyon yeteneğinden ve artmış hemolitik aktiviteden sorumlu olabilecek Internalin protein B (InlB) ve Listerolysin O'nun (LLO) belirgin şekilde arttığını ortaya koydu. Histidin kinaz *yycG*'nin *L. monocytogenes* patojenitesinde önemli roller oynadığı ilk kez belirlendi. YycFG TCS'nin büyümeyi, hareketliliği, hücre yüzeyi proteinlerinin translokasyonunu ve virülansı nasıl değiştirdiğini belirlemek için ileri araştırmalara ihtiyaç vardır.

Anahtar sözcükler: *Listeria monocytogenes, İki bileşenli sistem, Histidin kinaz yycG, Patojenite*

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INTRODUCTION

Listeria monocytogenes is a non-spore forming Gram positive microorganism which contaminates food and has been frequently associated to foodborne disease. In humans, *L. monocytogenes* can cause listeriosis, a disease that ranges from febrile gastroenteritis to a more severe, invasive disease such as meningitis and septicemia [1,2]. *L. monocytogenes* infection cycle includes adhesion and invasion into host cells, escape from phagocytic or internalized vacuoles, intracellular multiplication, and cell to cell spread [1,3]. The major virulent factors mediate *L. monocytogenes* entry and survival in host cells are secreted proteins which are translocated to the bacterial surface. Among these virulent factors, internalins InlA and InlB mediate *L. monocytogenes* entry into intestinal epithelial cells or other types of cells via E-cadherin and c-Met, respectively [4]. We previous found that failed anchoring on cell surface of InlB was associated with the low-virulent phenotype of *L. monocytogenes* strain M7 [5]. Indicating that correction anchoring of the surface proteins is quite necessary for the function of these virulent factors. In Gram-positive bacteria like *L. monocytogenes*, the majority of secreted proteins are first translocated across the cytoplasmic membrane and then delivered to membrane-anchored, cell-wall anchored or extracellular milieu under suitable environments [6,7]. Anchoring of these secreted proteins were affected by complicated conditions, including ionic concentration, pH, redox potential and post-translocation modification [8-10].

Two-component regulatory signaling systems (TCSs) are used by most prokaryotic organisms to sense and adapt to various stress conditions encountered in nature, during food processing and infection [11]. A typical TCS is usually constituted of a transmembrane sensor histidine kinase and a cognate cytoplasmic response regulator [11]. In the first sequenced genome of *L. monocytogenes* EGDe, 16 TCSs have been identified [12]. These TCSs are also found in the genomes of the virulent strain Lm850658 and avirulent strain M7, which were sequenced by us. But difference was only observed in *yycG* of the YycFG TCS between Lm850658 and M7. YycFG TCSs are prevalent distribution in other Gram-positive bacteria, which share similar sequences and operon structures that contain 3-6 genes [13]. Previous studies showed that the YycFG TCS is quite important in those Gram-positive bacteria, since it plays essentially and regulatory roles in metabolism, cell division, cell wall synthesis, autolysis, biofilm formation and virulence [13-18]. While the roles of YycFG TCS in *L. monocytogenes* remain poorly known. Up to date, only Pontinen A. et al. showed that the histidine kinase YycG was involved in the growth of *L. monocytogenes* EGDe at low temperatures [19]. Whether YycFG TCS plays other roles in *L. monocytogenes* remains to be explore.

This study was aimed to determine whether *yycG* contribute to the virulence of *L. monocytogenes*. Therefore, we created a histidine kinase gene *yycG* deletion mutant using homologous recombination and a complemental strain. We found the *yycG* deletion significantly impaired the growth ability and mobility, but enhanced the hemolysis ability *in vitro*. In addition, infection assays on cell and mice model showed that the deletion of *yycG* in Lm850658 significantly defected the infection ability and virulence. Taken together, we found the histidine kinase *yycG* played important roles in pathogenicity of *L. monocytogenes* for the first time.

MATERIAL and METHODS

Bacterial Strains, Plasmids, Cells and Culture Conditions

Listeria monocytogenes virulent strain Lm850658 (serovar 4a) was used as the wild-type strain. *Escherichia coli* DH5α was employed as the host strain for shuttle vector pKSV7 and expression plasmid pIMK2. *L. monocytogenes* strains were cultured with brain heart infusion medium (BHI, Oxoid, Basingstoke, U.K.) and *E. coli* DH5α was cultured with Luria-Bertani (LB, Oxoid) medium at 37°C. Stock solutions of ampicillin (50 mg/mL), gentamycin (50 mg/mL), kanamycin (50 mg/mL) and chloramphenicol (10 mg/mL) (Sangong Biotech, Shanghai, China) were added to media at required concentrations. Macrophages RAW264.7 and epithelial cells Caco-2 (National Infrastructure of Cell Line Resource, Beijing, China) were cultured at 37°C and 5% CO_2 in complete RPMI 1640 medium (Gibco, New York, U.S.A.) containing 10% fetal bovine serum (FBS) (Gibco).

Plasmids Construction

The *yycG* gene fragment and the *yycG* homoarms were amplified from the genomic DNA of *L. monocytogenes* Lm850658 with oligonucleotide primers were synthesized by BGI-tech (Wuhan, China) *(Table 1)*. PCR fragments and plasmids were purified using the DNA Gel Extraction Kit and Unit-10 plasmid Extraction Kit (Sangong Biotech), respectively. Plasmids pIMK2 and pKSV7 were linearized with restriction enzyme *BamH*I (New England Biolabs, Massachusetts, U.S.A.) and then gel-purified. Purified PCR fragments and plasmid fragments were ligated by recombinase Exnase II with the One Step Clone Kit (Vazyme Biotech, Nanjing, China) as the manufacturer's instructions to produce recombinant plasmids (pIMK2-*yycG* and pKSV7-*yycG* homoarm). The ligation products were transformed into competent cells DH5α and positive clones were then confirmed by PCR and sequencing.

Construction of yycG Deletion and Complement Strains

A homologous recombination strategy was used for construction of *yycG* deletion mutant in *L. monocytogenes* wild-type strain Lm850658 according to the protocols [20]. The recombinant plasmids pKSV7-*yycG* homoarm were electroporated into *L. monocytogenes* competent cells. Transformants were grown at a non-permissive temperature (41°C) in BHI containing chloramphenicol (10 μg/mL) to

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

Primers	Sequences (5'-3')
yycG-A	CATGCAGGTCGACTCTAGAGATGTAACGAAACCATTCAGTAAC
yycG-B	AGCGAAATCCTGTTTTTTTCATCATAAGTCTTTCTACTCCTATTCG
yycG-C	ATGATGAAAAAAACAGGATTTCGCT
yycG-D	ATTCGAGCTCGGTACCCGGGGTCAGCTTGCTTTCATTCTTTTTA
yycG-E	ATGATGGCGATGAAGCGTTAGA
yycG-CF	GAAGGAGAGTGAAACCCATGGAAAAGATGCATAAAATGAGATTTTTTCAGTCTGTACA
yycG-CR	CGAATTCCTGCAGCCCGGGGTCATTCCCAATCATCCTCCGGT

promote chromosomal integration and recombination. The recombinants were passaged, in succession, in BHI without antibiotic at a permissive temperature (30°C) to enable plasmid to be eliminated. The *yycG* gene deletion strain Lm850658-Δ*yycG* was identified by PCR and then confirmed by sequencing. For the complement strain, recombinant plasmids pIMK2-*yycG* were electroporated into Lm850658-Δ*yycG* competent cells.

Hemolysis Assay

Listeriolysin O (LLO) was tested for hemolytic activity as described [21]. Aliquots of 950 µL 5% sheep red blood cells suspension in 10 mM phosphate buffered saline (PBS) were added 50 µL filtered overnight culture supernatants with 0.22 µm filter (Millipore, Massachusetts, U.S.A.), and 50 µL BHI with or without 10% triton-X-100 were set as negative and positive control, respectively. After incubating at 37°C for 6 h, samples were centrifugated at 1.000 g for 5 min and the optical density values of the supernatants (200 µL per well) were detected by spectrophotometer at 550 nm in 96 microwell plate.

Adhesion and Invasion Assays in Caco-2 Cells

Overnight cultures were harvested by centrifugation (5.000 g for 10 min), resuspended in 10 mM PBS (pH 7.4) and adjusted the optical density at the wavelength of 600 nm ($OD_{600\ nm}$) to 0.25. The epithelial cell line Caco-2 was grown at 37°C and 5% CO_2 for 18-24 h to confluence (about 2×10^5) in 12-well plates (Corning, New York, U.S.A.) and infected with *L. monocytogenes* at multiplicity of infection (MOI) of 10:1 for 1 h. For adhesion assay, cells were lysed after three times of washing with PBS. For invasion assay, cells were washed with PBS after 1 h infection and incubated for an additional 1 h in RPMI 1640 medium containing 10% FBS and 50 µg/mL gentamycin. At the indicated times, the cells were lysed and 10-fold diluted for plating on BHI agar. The agar plates were incubated overnight at 37°C for colony counting. Adhesion rate was expressed as the percentage of recovered colonies to those inoculated; while invasion rate was calculated as the percentage of recovered colonies after gentamycin treatment to those inoculated.

Survival in Macrophage RAW264.7

Cells were infected with resuspended bacteria as above (MOI =10:1) at 37°C and 5% CO_2 for 1 h. Extracellular bacteria were killed with additional 1 h incubation in the presence of 50 µg/mL gentamycin. Cells were lysed with ice-cold disitilled water after three times of washing with PBS and diluted appropriately for plating on BHI agar plates for colony counting to calculate the initial intracellular bacteria. To count intracellular survived bacteria, cells were cultured for another 3 h in the presence of 5 µg/mL gentamycin and then lysed with ice-cold distilled water after three times of washing and diluted appropriately for plate counting. Survival rate was calculated for each strain by dividing the final recovered colony counts with those initial intracellular counts.

Actin-tail Formation in Caco-2 and Macrophage Cells

Actin-tail formation assay was conducted as our previous research [22]. Briefly, the Caco-2 and RAW264.7 cells were seeded in 24-well plate and cultured in RPMI 1640 medium containing 10% FBS. Overnight bacterial cultures were harvested and adjusted $OD_{600\ nm}$ at 0.25. Cells were infected at MOI of 10:1 at 37°C and 5% CO_2 for 1 h. Extracellular bacteria were killed with 50 µg/mL gentamycin for 1 h and then incubated for additional 5 h. Cells was washed gently with 10 mM PBS, fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. The bacteria were probed with *L. monocytogenes* polyclonal antibodies which was produced by our laboratory for 1 h at 37°C, washed twice with PBS and stained with Alexa Fluor 488 conjugated donkey anti-rabbit antibody (Thermo Fisher Scientific, Massachusetts, U.S.A.) for 1 h at 37°C. F-actin was then stained with 6.6 µM phalloidin-Alexa Fluor 568 (Thermo Fisher Scientific). The 6-diamidine-2-phenylindole (DAPI, Thermo Fisher Scientific) was used to stain the nuclei. Actin tails recruited by the bacteria were visualized by confocal microscope (Olympus FV 1000, Tokyo, Japan) and indicated by arrows.

Virulence in Mice Model

The assay was conducted as our previous research [23]. Four-weeks age female ICR mice, twenty 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 inoculated intra-peritoneally with 2×10^5 bacteria. At 24 and 48 h post

infection (pi), mice (six per group) were euthanized, liver and spleen samples were homogenated, and diluted appropriately for plating counting on BHI agar plates. The rest mice were observed for mortalities twice a day for 7 days. Animal experiments were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

Surface Proteins Analysis

Surface proteins analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting was conducted as our previous research [23]. Bacteria were cultured in 100 mL BHI medium at 37°C for 12 h, and then harvested by centrifugation (15.000 g, 10 min). The supernatant samples were filtered through 0.22 μm filter (Millipore) and proteins were precipitated with 100% (w/v) trichloroacetic acid to a final concentration 10% (w/v) at 4°C. Cell wall associated proteins were extracted from bacterial pellet with 1% SDS (30 mg wet weight per ml of 1% SDS) at 37°C for 1 h. Protein samples were analyzed by 12% SDS-PAGE gel. Abundance of the secreted and surface-associated proteins InlA, InlB, and LLO were blotted and probed with respective polyclonal antibodies. The glyceraldehyde-phosphate dehydrogenase (GAPDH) was probed and set as loading control. All primary antibodies including anti-InlA, anti-InlB, anti-LLO and anti-GAPDH polyclonal antibodies were produced in our laboratory and the secondary antibody HRP-conjugated Goat anti-Rabbit IgG (D110058) was purchased from Sangong Biotech.

Statistical Analysis

All results were presented as mean ± SD of triplicate experiments and subjected to one-way analysis of variance (One-way ANOVA). Differences were considered as statistically significant at $P<0.05$. Notations of statistics marked as * and ** indicated the statistical significance with $P<0.05$ and $P<0.01$, respectively.

RESULTS

Our previous research showed that *yycG* of Lm850658 contains a mutant, which results in the 220th amino acid residue mutation in HAMP domain [22]. To clarify the role *yycG* in the pathogenicity of *L. monocytogenes*. We constructed a *yycG* in-frame deletion strain (Δ*yycG)* based on Lm850658 and a complemental strain (CΔ*yycG)* that express *yycG* with the integrated plasmid pIMK2. We found that the growth ability of mutant strain Δ*yycG* was significantly lower than that of the wild type strain Lm850658 in BHI broth *(Fig. 1-A)*. While the complemental strain CΔ*yycG* partially recovered the growth defect *(Fig. 1-A)*. The mobility of mutant strain Δ*yycG* was conspicuous smaller than that of the wild type and complemental strains *(Fig. 1-B)*. But the hemolytic ability of mutant strain Δ*yycG* was significantly stronger than that of the wild type and complemental strains as showed in 5% sheep red blood cells suspension *(Fig. 1-C)*. Infection assay on epithelial cells Caco-2 cells showed that the adhesion rate (1.07%±0.22%) and invasion rate (0.08%±0.01%) of mutant strain Δ*yycG* were significantly lower than those of the wild type strain and the complemental strain, respectively ($P<0.01$) *(Fig. 2-A,B)*. Survival in macrophages RAW264.7 showed that the survival rate of the wild type was 123.33%±10.12% at 4 h pi, which was significantly higher than that of the mutant strain Δ*yycG (Fig. 2-C)*. And the complemental strain exhibited the similar survival ability as the wild type strain ($P<0.01$) *(Fig. 2-C)*. Actin tail formation assay showed

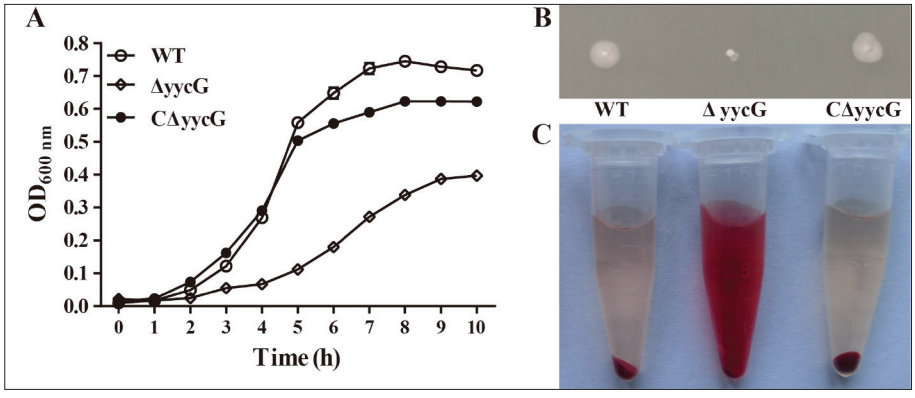
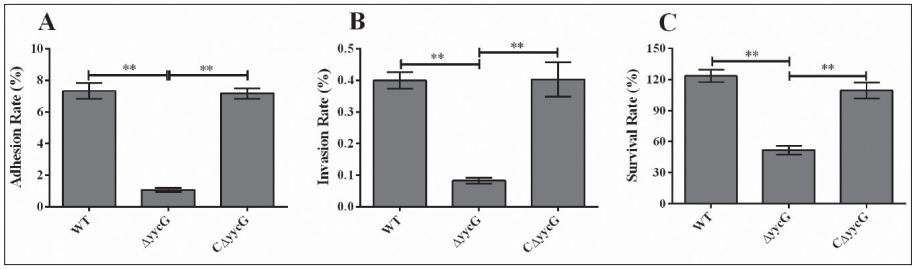


Fig 1. The *yycG* deletion strain Δ*yycG* exhibited defect in growth and mobility, but enhance in hemolysis, compared with the wild type strain Lm850658 (WT) and complemental strain CΔ*yycG*. Growth curve was made based on the OD_{600nm} values with 1 h interval in BHI broth at 37°C **(A)**. Mobility was tested on the soft agar plate (0.3% agar) under 30°C for 48 h **(B)**. Hemolysis assay was conducted in 5% sheep red blood cells suspension with the cultural supernatants at 37°C for 6 h **(C)**. The representative results were showed from at least three independent experiments

Fig 2. The *yycG* deletion significantly defected the infection ability to both epithelial cells Caco-2 and macrophages RAW264,7. Adhesion **(A)** and invasion **(B)** ability was conducted on epithelial cell Caco-2, and survival assay was conducted on macrophages RAW264.7 **(C)**. Data was presented as mean ± SD of three independent experiments and ** indicated the statistical significance with $P<0.01$



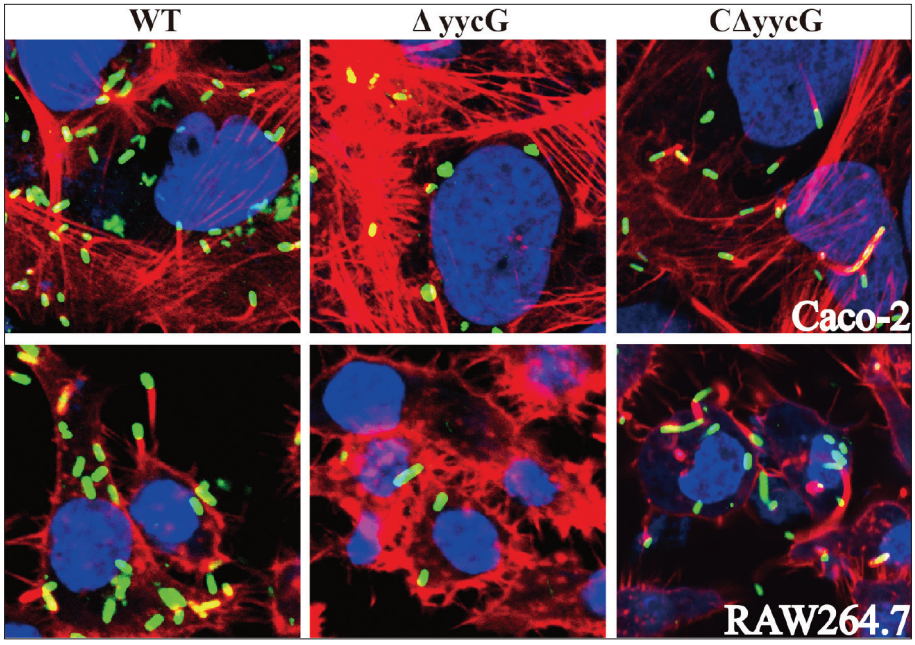


Fig 3. The *yycG* deletion significantly defected the actin tail formation ability to both epithelial cells Caco-2 (up) and macrophages RAW264,7 (down)

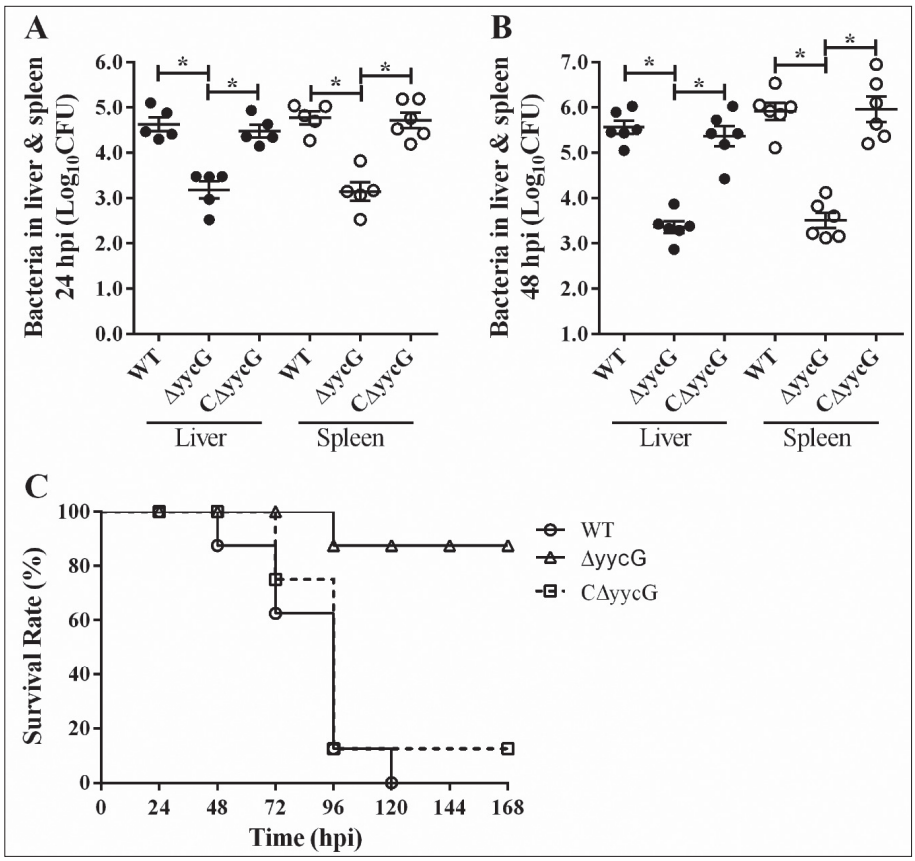


Fig 4. The *yycG* deletion significantly impaired the pathogenicity of *L. monocytogenes* on mice model. The *yycG* deletion significantly decreased bacteria load in the liver and spleen of infected mice at both 24 **(A)** and 48 **(B)** hr post infection (pi), and increased the mortality rate of infected mice **(C)**. Data was presented as mean ± SD, * indicated the statistical significance with $P<0.05$

that deletion of *yycG* almost absolutely abolished the cell-to-cell spread ability of Lm850658 in both epithelial cells Caco-2 and macrophages RAW264.7, and this phenotype was partially recovered in the complemental strain *(Fig. 3)*.

To evaluate the role of *yycG in vivo*, the intraperitoneal infection model showed that the bacterial load in liver and spleen samples of the mutant strain Δ*yycG* infected mice were significantly less than those of the wild type and complemental strains infected mice at 24 and 48 h pi ($P<0.05$) *(Fig. 4-A,B)*. All mice infected with the mutant strain Δ*yycG* remained alive before 96 hr pi, and only one (12.5%, 1/8) mouse died during the observation period, while the mice infected with wild type strain died within one week *(Fig. 4-C)*. Moreover, 87.5% (7/8) of mice infected

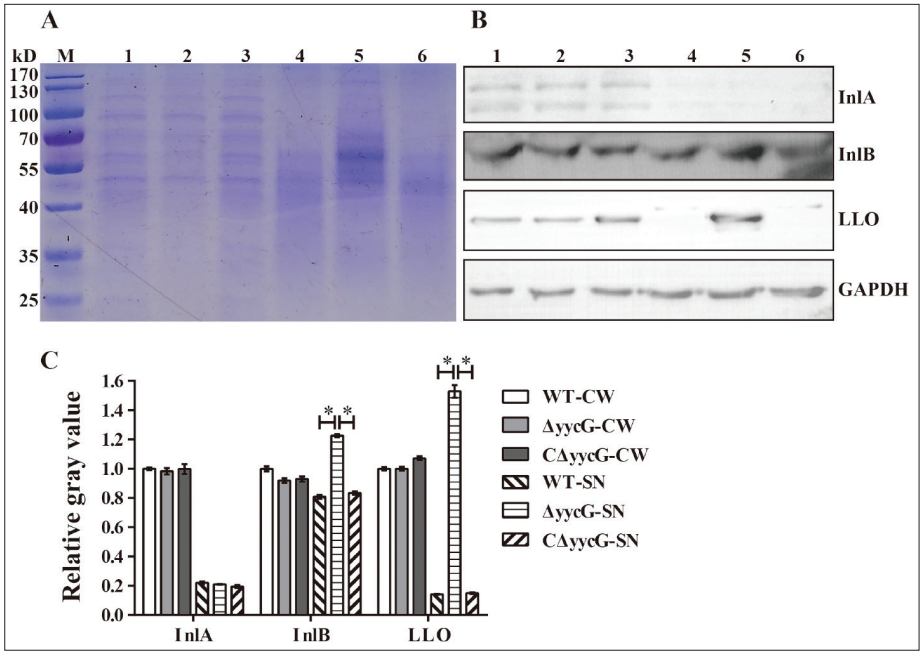


Fig 5. The *yycG* deletion significantly increased secretion of InlB and LLO. Cell surface extracted fractions (lane1, WT; lane 2, Δ*yycG*; lane 3, CΔ*yycG*) and secreted fractions (lane4, WT; lane 5, Δ*yycG*; lane 6, CΔ*yycG*) were analyzed by SDS-PAGE **(A)** and western blot **(B)**. The relative contents of InlA, InlB and LLO in different strains were analyzed by calculated the density of the specific bands **(C)**. * indicated the statistical significance with P<0.05

with the complemental strain died during the seven days observation period *(Fig 4-C)*. These results suggest that *yycG* deletion significantly weaken the pathogenicity of *L. monocytogenes*.

As pathogenicity of *L. monocytogenes* were mainly determined by the cell surface virulent factors. To explore the mechanisms that *yycG* affected the virulence of *L. monocytogenes*, we analyzed the bacterial surface associated virulent factors of wild type and *yycG* deletion strain. Our data showed that the content of some proteins extracted from the cell surface of the mutant strain Δ*yycG* were obvious less than those of the wild type and *yycG* complemental strain *(Fig. 5-A)*. While opposite pattern was observed in cultural supernatant samples of these strains *(Fig. 5-A)*. Western blotting analysis showed that content of InlB and LLO of mutant strain Δ*yycG* were significant more than those of wild type and *yycG* complemental strain in secreted fraction (P<0.05), while no significant difference was observed in the cell surface extraction *(Fig. 5-B,C)*. And the distribution of InlA remained unchanged among the three strains in cell surface extraction and secreted fraction *(Fig. 5-B,C)*.

DISCUSSION

Listeria monocytogenes is an environmental saprotroph that becomes a pathogen following ingestion by mammalian hosts [2]. Yet, not all *L. monocytogenes* strains have the same pathogenicity because transition from environmental organism to pathogen requires significant changes in expression and translocation of virulent factors. These changes happened upon bacteria sensing subtle changes of the environments. TCSs play important roles in adapting to arduous environmental stress factors. YycFG as one of the most highly conserved TCSs in Gram-positive bacteria, exhibits diverse functions in different bacteria. The *yycG* has been shown to response to low temperature, but not to heat, acid, alkali, osmotic, ethanol and oxidative stresses [19,24]. In this study, we found *yycG* deletion significant impaired the growth ability and mobility, but enhanced the hemolysis ability *in vitro (Fig. 1)*, indicating multiple roles for the histidine kinase YycG in *L. monocytogenes*.

Further study showed that *yycG* in *L. monocytogenes* Lm850658 also plays roles in adhesion and invasion to epithelial cells and survival in macrophage RAW264.7 *(Fig. 2)*. Combination with actin tail formation assay and animal assay, we found that *yycG* deletion significantly affect the pathogenicity of Lm850658 *(Fig. 3, Fig. 4)*. Our findings expanding the roles of *yycG* in strain EGDe [12,19]. This might be explained by individual difference between Lm850658 and EGDe, as strain-specific gene function could be observed in other *L. monocytogenes* strains [25]. That is to say YycFG TCS might play different roles in various strains. Although Lm850658 and EGDe were belong to the same species, but they were clustered into different phylogenetic lineage that exhibited different characteristics. In Lm850658, *yycG* deletion results in severe defects in growth, survival in host cells and pathogenicity, and *yycFG* double mutant was failed to obtain. These evidences support that YycFG TCS is essential for *L. monocytogenes*. Since the essential genes knocking out will result in severe defects or lethal effects. Moreover, the essential *yycG* of *Bacillus subtilis* could be deleted only in the background of containing an inducible plasmid that expressed *yycG* [18]. In *Bacillus subtilis*, YycG senses cell division and co-ordinates cell wall remodeling by controlling the transcription of genes for autolysins and their inhibitor [18]. As a regulator of cell wall metabolism, dysfunction of YycFG TCS results in growth defect, cell wall architecture disorder and even cell death via multiple YycFG regulated genes [26].

The abnormal distribution of InlB and LLO in *yycG* deletion strain might be result from disturbing the cell wall remodeling. Since the anchoring of InlB onto cell surface need lipoteichoic acid (LTA) and suitable cationic conditions [27]. And the secretion and maturation of LLO was also associated with the environments between cell membrane and cell wall [28]. Furthermore, increase of the dissociated InlB and LLO from the secreted fraction do not seem to be benefit for the pathogenicity of *L. monocytogenes*, as demonstrated by *in vitro* and *in vivo* infection assays *(Fig. 2, 3* and *4)*. Previous research showed that exogenous InlB could also activate the c-Met signaling [29,30], which might compete with the cell surface InlB for bacteria invasion. In this way, secreted InlB dissociated from the bacterial surface might inhibit the adhesion and invasion of *L. monocytogenes* to host cells.

Listerolysin O is a versatile virulent factor involving in several infection progresses of *L. monocytogenes* [28]. Extracellular LLO is sufficient to direct bacterial internalization into HepG2 cells via activating tyrosine kinases and their downstream signaling [31]. Once entry into the cytosol, LLO could induce mitochondrial fragmentation, endoplasmic reticulum stress, unfolded protein response, protein degradation, inflammasome activation and cell-to-cell spread [28]. In the phagosome, LLO could perforate the membrane of phagosomes in graded, manage reactive oxygen species, establish chronic infection and induce autophagy [28]. But most of the functions of LLO appear to be by-products of either pore formation or entry of *L. monocytogenes* into the cytosol [28,32]. Since LLO is a multiple function factor whose expression, translocation, activation and even degradation was tightly regulated [28,33]. It is not difficult to find that un-controlled LLO is not benefit for the subtle intracellular lifecycle of *L. monocytogenes* as observed in previous reports [34,35] and in this study.

Taken together, we demonstrated that *yycG* played important roles in growth and pathogenicity of *L. monocytogenes* Lm850658. Further study might focus on the mechanisms of the *yycG* involved in the growth and pathogenicity of Lm850658 and the roles of the whole YycFG TCS in *L. monocytogenes*.

Statement of Author Contributions

XWF, WH and CF designed the project, conducted most of the experiments and drafted the manuscript. XWF, WH, YZ, CW, QPL, HW, XYL, YFG and CF were involved in study design and animal experiments. CF and YYY supervised the study and critically read the manuscript.

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest: All authors declare no conflict of interest.

Research involving Human Participants and/or Animals: This article does not contain any studies with human participants. Mice used for virulent assays were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

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