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Characterization of a Mutant *Listeria monocytogenes* Strain Expressing Green Fluorescent Protein

Ling-Li JIANG, Hou-Hui SONG[1], Xue-Yan CHEN, Chun-Lin KE, Jing-Jing XU,
Ning CHEN, and Wei-Huan FANG*

Institute of Preventive Veterinary Medicine and Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine, Zhejiang University, Hangzhou 310029, China; [1]Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China

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Abstract To construct a recombinant strain of *Listeria monocytogenes* for the expression of heterologous genes, homologous recombination was utilized for insertional mutation, targeting its listeriolysin O gene (*hly*). The gene encoding green fluorescent protein (GFP) was used as the indicator of heterologous gene expression. The gene *gfp* was inserted into *hly* downstream from its promoter and signal sequence by an overlapping extension polymerase chain reaction, and was then cloned into the shuttle plasmid pKSV7 for allelic exchange with the *L. monocytogenes* chromosome. Homologous recombination was achieved by growing the electro-transformed *L. monocytogenes* cells on chloramphenicol plates at a non-permissive temperature. Sequencing analysis indicated correct insertion of the target gene in-frame with the signal sequence. The recombinant strain expressed GFP constitutively as revealed by fluorescence microscopy. The mutant strain *L. monocytogenes hly-gfp* lost its hemolytic activity as visualized on the blood agar or when analyzed with the culture supernatant samples. Such insertional mutation resulted in a reduced virulence of about 2 logs less than its parent strain *L. monocytogenes* 10403s as shown by the 50%-lethal-dose assays in the mouse and embryonated chicken egg models. These results thus demonstrate that mutated *L. monocytogenes* could be a potential carrier for the expression of heterologous passenger genes or could act as an indicator organism in the food industry.

Key words *Listeria monocytogenes*; homologous recombination; insertional mutation; heterologous gene expression; green fluorescent protein (GFP)

Listeria monocytogenes is a facultative intracellular bacterium that can survive and replicate in professional and non-professional phagocytes. It is considered as one of the most popular vectors, when attenuated, for the expression of heterologous genes for the induction of $CD8^+$ T-cell responses against the human immunodeficiency virus (HIV), hepatitis C virus (HCV) and human papillomavirus (HPV) [1–4]. It is able to facilitate the secretion of cytokines such as IL-12 and IL-18 [5], induce persistent immune responses and favor the development of potentially protective IFN-γ-secreting Th1 $CD4^+$ T lymphocytes [6].

Because *L. monocytogenes* is pathogenic to human beings and animals, it has to be attenuated for the use as a potential vaccine vector for the delivery of passenger proteins of interest. A number of approaches for its attenuation have been examined while retaining its invasiveness to a certain extent. Major virulence factors of the bacterium include internalins (*inl*), listeriolysin O (*hly*), actA (*actA*), etc. [7]. hly is involved in the escape of *L. monocytogenes* from primary vacuoles [8], and actA in the polymerization of the host cell actins. The positive regulatory factor A (PrfA) is a regulatory transcription factor required for the expression of other genes within the gene cluster [9]. A number of studies have used the vector-based reporting system or homologous recombination-based methodology targeting gene *actA* for analysing the

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*Corresponding author: Tel/Fax, 86-571-86971242; E-mail, whfang@zju.edu.cn

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pathogenesis of *L. monocytogenes* [10–12]. However, no information is available about the role played by gene *hly* in the secretory heterologous expression of passenger genes.

This study was undertaken to examine if insertional mutation of gene *hly* in *L. monocytogenes* could affect its phenotypic characteristics and express a heterologous reporter gene *gfp* (encoding for green fluorescent protein, GFP) placed right behind its promoter and signal sequence.

Materials and Methods

Bacteria strains and plasmids

The reference strain of *L. monocytogenes* 10403s was a gift from Dr. M. Wiedmann (Cornell University, USA). *Escherichia coli* DH5α was used as the host strain for plasmids pUC18 and pKSV7 (a gift from Dr. Wiedmann). *L. monocytogenes* was grown in trypticase soya broth with yeast extract (TSB-Y) (Oxoid Ltd., Hampshire, England) for routine subculturing.

PCR primers and conditions

All the primers used in this study were synthesized by BioAsia Biotechnology Co., Ltd. (Shanghai, China) and listed in **Table 1**. All the PCRs were processed as following: 94 °C, 2 min; 30 cycles at 94 °C for 1 min, 55 °C for 45 s, 72 °C for 50 s; 72 °C, 5 min for 1 cycle.

Construction of the recombinant shuttle vector pKSV7-hly-gfp

The thermo-sensitive shuttle vector pKSV7 capable of replicating in *E. coli* and *L. monocytogenes* was used to construct the recombinant plasmid containing the target gene for homologous recombination. Primer pairs hly-a/hly-b and hly-c/hly-d were used to amplify two corresponding fragments for subsequent splicing by overlap extension (SOE) PCR on a Hybaid® PCR thermocycler (Hybaid Equipment Ltd., Middlesex, UK) to generate a fragment of *hly* with a 15 bp deletion [13]. The primers hly-a and hly-b were designed so that the amplified fragment contained the promoter region of *hly* and the inserted reporter gene *mgfp5* (encoding GFP) was in-frame with its signal sequence. The DNA fragment was purified and cloned into pUC18 at the enzyme sites *Pst*I and *Kpn*I introduced in the primers, yielding a recombinant plasmid pUC18-hly. Primers gfp-a and gfp-b were used to amplify a 744 bp fragment containing *mgfp5* from the recombinant plasmid pGEMT-mgfp5 (a gift from Dr. Zhong-Liang MA of the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China). The purified *mgfp5* fragment was then subcloned into pUC18-hly to yield pUC18-hly-gfp after a single digestion with *Xba*I. Correct orientation of *mgfp5* was verified by PCR identification of the hybrid DNA fragment of *hly*-ab and *mgfp5* using the primer pair hly-a/gfp-b. This was further supported by restriction digestion of the recombinant plasmid pUC18-hly-gfp with *Pst*I (introduced in primer hly-a) and *Nde*I, present both in the vector 214 bp upstream of the site *Pst*I and in *mgfp5* at 255 bp. The hybrid DNA fragment *hly-gfp* was amplified from the plasmid pUC18-hly-gfp using the primer pair hly-a/hly-d and digested with *Pst*I and *Kpn*I for ligation by T4-DNA-ligase, then subcloned into pKSV7 and digested with the same enzymes. The resulting plasmid pKSV7-hly-gfp was used for allelic exchange.

Generation of a *L. monocytogenes* mutant strain by allelic exchange with the shuttle vector pKSV7-hly-gfp

Table 1 Primer pairs used to amplify related genes and PCR product lengths

Primers *		Product length (bp)
hly-a	5′-TACTGCAGAGGTTTGTTGTGTCAGGTAGAGCG-3′ (*Pst*I)	573
hly-b	5′-AGTACATCTAGATAGGGTGGTGCCATGGATGAAATTGA-3′ (*Xba*I)	
hly-c	5′-CTATCTAGATGTACTGCCAATCGAAAAGAAACACGC-3′ (*Xba*I)	261
hly-d	5′-TGGGTACCTTACGAGAGCACCTGGATAGG-3′ (*Kpn*I)	
hly-d′	5′-GCAAATCAATGCTGAGTGTTAATGAATC-3′	744
gfp-a	5′-ATTCTAGACCATGGCCGCGGATTGA-3′ (*Xba*I)	
gfp-b	5′-CGCGCGATTCTAGAGTTTGTATAGTTCATC-3′ (*Xba*I)	

* the restriction enzyme sites were underlined.

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The recombinant shuttle vector pKSV7-hly-gfp was introduced into competent cells of *L. monocytogenes* 10403s by electroporation [14], and transformants were passaged in succession by growth at 41 °C in brain heart infusion (BHI, Oxoid Ltd., Hampshire, England) broth medium containing 10 mg/L of chloramphenicol (Sigma, St. Louis, MO, USA) to direct chromosomal integration of the plasmid DNA by homologous recombination [15]. A single colony with chromosomal integration was se rially passaged in BHI and replica-plated to obtain a mutant *L. monocytogenes hly-gfp* by allelic exchange. Chromosomal integration of the reporter gene was confirmed by PCR amplification from the genomic DNA of the chloramphenicol sensitive clones using the primer pairs hly-a/hly-d' (external to hly-d) and *mgfp5* specific primers. These were verified by sequencing the PCR products.

RT-PCR amplification of *gfp* from *L. monocytogenes hly-gfp*

The total RNA was extracted from the mutant strain *L. monocytogenes hly-gfp* using the UNIQ-10 columnTrizol total RNA extraction kit following the instruction from the supplier (Sangon Biotech Co., Ltd., Shanghai, China). RNA samples were treated with DNase I and the target mRNA was reversely transcribed using the specific primer pair to generate the *mgfp5* cDNA fragment, which was subsequently used as the template for PCR amplification. Regular PCR was performed using the *mgfp5* specific primer pair on the mutant strain chromosomal DNA template as a positive control.

Fluorescence microscopy

The mutant strain *L. monocytogenes hly-gfp* was grown for 12 h with shaking in BHI broth at 37 °C , and the bacterial cells were smeared on the slides and visualized by fluorescent microscopy using the standard FITC filter set (Olympus BH-2, Olympus Optical Co., Ltd., Tokyo, Japan). Images were taken by using a digital CCD camera.

Titration of hemolytic activity

The mutant strain *L. monocytogenes hly-gfp* and its parent strain 10403s were grown for 12 h with shaking in BHI broth at 37 °C , and then subjected to centrifugation in a microcentrifuge at 10,000 *g* for 10 min. Supernatant samples were saved for hemolytic assay. The sheep red blood cells (1% packed cells) were suspended in sterile 8.5 mg/ml NaCl. The supernatants (75 µl) were serially 2-fold diluted in a 96-well V-bottom microplate with 8.5 mg/ml NaCl and 75 µl of sheep red blood cells were added to each well. The plates were then incubated at 37 °C for 1 h. Hemolytic activity was expressed as the reciprocal of the dilution of the culture supernatant required to lyse 50% of the total erythrocytes. Experiments were carried out in duplicate and repeated twice for each strain. Both the mutant and parent strains were also subcultured on TSB-Y agar plates containing 7% sheep blood to visualize the hemolytic activity.

Invasion assay

The gentamicin-based invasion assay was performed on HeLa cell monolayers in 12-well plates (Corning Incorporated, NY, USA) according to the method described elsewhere [16]. Briefly, HeLa cell monolayers at about 80% confluency were inoculated with 300 µl of suspensions of the mutant strain *L. monocytogenes hly-gfp* and the parent strain 10403s ($1–2\times10^7$ CFU/ml) respectively to obtain a multiplicity of infection (MOI) of 10:1 and incubated for 1 h at 37 °C in 5% CO_2. The monolayers were then washed three times with phosphate-buffered saline (PBS, pH 7.2) and subjected to gentamicin treatment (100 µg/ml in Dulbecco's modified Eagle medium) for 1 h at 37 °C in 5% CO_2. The cell monolayers were washed four times again with PBS and lysed with 0.1% Triton X-100 (Sigma). The cell lysates were ten-fold diluted and plated on TSB-Y agar plates for bacterium counting. The invasion index was calculated by dividing the CFU that invaded the cells (with gentamicin) by the total bacteria CFU added to each well [$(CFU_{inv}/CFU_{total})\times1000$]. The experiment was repeated three times, each in triplicate wells for each strain.

Virulence in mice and chicken embryos

Female ICR mice of about 20–22 g (Zhejiang College of Traditional Chinese Medicine, Hangzhou, China) were housed six per cage and acclimatized for two days in a standard class II laboratory animal facility. The mutant strain *L. monocytogenes hly-gfp* and its wild parent strain were grown in BHI broth at 37 °C for 8 h and harvested by centrifugation at 10,000 *g* for 10 min at 4 °C. The cell pellets were gently resuspended in PBS (pH 7.2) and serial ten-fold dilutions were made both for cell-counting on TSB-Y agar plates and for inoculation. The mice in each group were intraperitoneally inoculated with 0.1 ml of an appropriate dilution of the two strains, and observed for 10 d. LD_{50} of the two strains was estimated using the trimmed Spearman-Karber method. In a separate experimental setting, 0.1 ml of appropriate 10-fold dilution of the mutant and parent strains in PBS was injected via the chorioallantoic membrane into 14-day-old embryonated eggs (from non-immunized layers, Hangzhou Layer's Co., Ltd.,

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Hangzhou, China) using the technique described previously [17,18]. Six eggs were used for each inoculum size in the testing of both strains. Eggs receiving PBS were included as a control. The inoculum size was confirmed by enumeration of the viable count on TSB-Y agar plates. Inoculated eggs were incubated in a horizontal position at 37.5 °C, and embryo death was monitored daily in 8 d by trans-illumination for LD_{50} calculation. In a separate experimental setting, inoculum sizes of $2\times LD_{50}$ of the mutant and wild strains were used to compare embryo mortality and mean time to death (MTD).

Results

Identification of the recombinant *L. monocytogenes* strain with *mgfp5* integration

The gene cassette containing the reporter gene and homologous regions for subsequent allelic exchange with *L. monocytogenes* chromosomal DNA was constructed first in the cloning vector pUC18 for the convenience of step-by-step verification by PCR and restriction digestion. Correct orientation of *mgfp5* was verified by PCR identification of the hybrid DNA fragment of *hly* and *mgfp5* (*hly*-ab-*gfp*) using the primer pair hly-a/gfp-b (**Table 1**). This was further supported by restriction digestion of the recombinant plasmid pUC18-hly-gfp with *Pst*I (introduced in primer hly-a) and *Nde*I present both in the vector 214 bp ahead of the site *Pst*I and in the gene *mgfp5* at 255 bp. Thus, the 828 bp fragment consisted of *hly*-ab and the upper one-third of *mgfp5* (data not shown). The pUC18-based gene cassette thus confirmed was then transferred to the shuttle vector pKSV7 via enzyme digestion and ligation, yielding pKSV7-hly-gfp (data not shown). Integration of *mgfp5* into the *L. monocytogenes* chromosome was achieved by homologous recombination under permissive conditions via allelic exchange after electroporation of the recombinant plasmid pKSV7-hly-gfp. The recombinant mutant *L monocytogenes hly-gfp* was identified via PCR using the primer pair hly-a/hly-d′ (**Table 1**). The primer hly-d′, about 73 bp behind hly-d, was used to avoid false positive amplification from residual plasmid DNA in the template. The hybrid DNA fragment *hly*-ad′-*gfp* was amplified from the recombinant strain, while the *hly*-ad′ fragment was attained from the wild parent strain, indicating that *mgfp5* had been integrated into the chromosomal DNA (data not shown). The reporter gene *mgfp5* was also transcribed in the recombinant strain, as revealed by RT-PCR amplification of a 744 bp fragment [**Fig. 1(A)**]. Furthermore, fluorescent microscopic examination revealed the expression of GFP by the recombinant strain [**Fig. 1(B)**]. Sequencing of the hybrid DNA fragment also confirmed correct insertion of *mgfp5* in the predicted site in-frame with the *hly* signal sequence (data not shown).

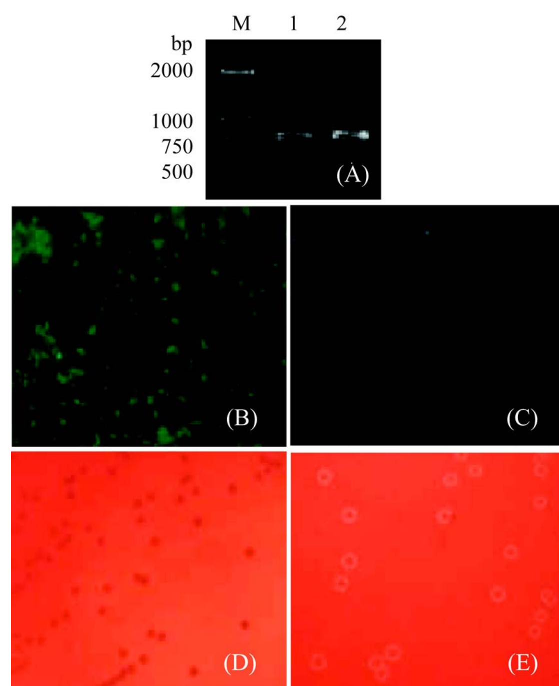

Fig. 1 Transcription and expression of *mgfp5* in the recombinant strain *L. monocytogenes hly-gfp*
(A) 1, the *mgfp5* fragment amplified by RT-PCR from total RNA of the recombinant mutant; 2, the *mgfp5* fragment amplified from the genomic DNA of the same mutant strain as a control. (B) GFP expression of the recombinant mutant *L. monocytogenes hly-gfp* revealed by the fluorescence microscopy as compared with the parent strain (C). (D) Integration of *mgfp5* into listerial *hly*-demolished listeriolysin O expression, in contrast to the clear hemolytic zones of the parent strain (E) on the TSB-Y agar with 7% sheep blood after 24 h incubation.

Loss of hemolytic activity and reduced invasiveness of *L. monocytogenes hly-gfp* mutant strain

The hemolysin gene *hly* of *L. monocytogenes* was disrupted due to the insertion of *mgfp5* as evidenced by the loss of hemolytic activity on blood agar [**Fig. 1(D)**]. The culture supernatants of the mutant strain were also devoid of hemolytic activity while the wild strain had a titer of 1:16. The mutant also showed a significantly lower invasive capability than its parent strain in the HeLa cells ($P<0.05$) (**Fig. 2**).

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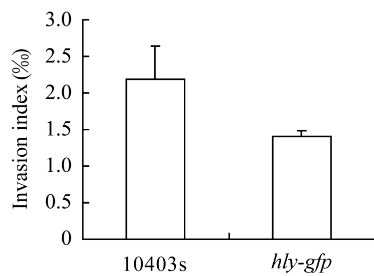

Fig. 2 Invasion assay of the mutant strain *L. monocytogenes hly-gfp*

L. monocytogenes hly-gfp was less invasive than its parent strain 10403s as tested by gentamicin-killing invasion assay in the cultured HeLa cell model ($P<0.05$). Each data point from three wells represented the average invasion index (+SD) of three experiments.

Reduced virulence of *L. monocytogenes hly-gfp* mutant strain in mice and embryonated eggs

The mutant strain had reduced virulence of about 2 logs in LD_{50} in mice as compared with that of the wild parent strain (**Table 2**). The chick embryo model test also showed a decrease in virulence of the mutant strain by over 2 logs in LD_{50} in comparison to its parent strain. At $2\times LD_{50}$, all the 12 embryos receiving the wild strain died on day 4 post-inoculation while only 83% (10/12) of the embryos inoculated with the mutant strain died by day 8, resulting in over 4 days of difference in MTD.

Table 2 Comparison of virulence between the mutant strains

Strain	LD_{50}	
	Mouse	Embryonated eggs
10403s	$10^{5.49}$	$10^{1.90}$
hly-gfp	$10^{7.44}$	$10^{4.19}$

Discussion

L. monocytogenes can invade the cytoplasm of host cell by disrupting the phagosomal membrane through the action of listeriolysin O [19]. This makes *L. monocytogenes* different from other intracellular organisms such as *Salmonella* and *Mycobacterium bovis* bacille Calmette-Guérin (BCG). The cellular peptides from *L. monocytogenes* can then be presented via major histocompatibility class I and II pathways for effective induction of both $CD4^+$ and $CD8^+$ T-cell responses [20,21]. The bacterium, when delivered orally, may stimulate mucosal immunity [22]. These characteristics might enable this particular organism, if properly attenuated, to serve as a vaccine delivery vector for genes of other pathogens [1,23,24].

As potential vectors for the delivery of passenger genes, the bacterial strains should meet a number of requirements: a safety window of 4 logs less than the parent strain in LD_{50} in the animal model, ability to express foreign genes efficiently in the cytosol of infected cells, preferential infection of professional antigen presenting cells, stability under storage conditions, and low production cost. Previous studies have examined the attenuation of *L. monocytogenes,* targeting a number of virulence-related genes such as *inl*, *hly*, *actA*, *dltA* (encoding a cytoplasmic *D*-alanine-*D*-alanyl carrier protein ligase that catalyzes the *D*-alanylation of *D*-alanyl carrier protein), etc. [25–28]. Such attenuation of insertion or deletion mutation could reduce the bacterial virulence by 2–4 logs in mouse or chicken embryo models based on the target virulence genes, as compared with the wild parent strain. We attempted the insertional mutation on *hly* by homologous recombination to see whether the reporter gene *mgfp5* transcription could be driven by the constitutive upstream *hly* promoter with its signal sequence downstream for secretory expression [29].

Restriction enzyme digestion of the PCR products and DNA sequencing indicated the correct insertion of the target gene in-frame with the signal sequence. It was also shown that *gfp* was transcribed and GFP was expressed [**Fig. 1(A,B)**]. Although the mutant strain *L. monocytogenes hly-gfp* lost its hemolytic activity [**Fig. 1 (D)**] and had reduced invasiveness in cultured cell monolayers (**Fig. 2**) and decreased virulence (**Table 2**), its virulence in the mouse and embryonated chicken egg models was reduced by only 2 logs. This makes it seem more virulent than the deletion mutants of the same gene in some other studies, which have a virulence about 3–5 logs less than the parent strain [26,30]. This implies that the mutated strain requires additional genetic manipulation to further reduce its virulence before it can be used as a potential vector for the delivery of passenger genes. However, the mutant *L. monocytogenes hly-gfp* could be utilized for studies on environmental factors affecting its expression to better understand its regulatory mechanisms or survival strategies either *in vitro* or *in vivo*, when combined with other mutagenic approaches. It could also be used as an indicator strain for monitoring the thermal or chemical inactivation efficiency in the food industry.

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