Application of the Split GFP system to *Listeria monocytogenes* to visualize the virulence factor InlC

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I. Abstract

Listeria monocytogenes (Lm) is an opportunistic pathogen that is able to survive in a range of environments and cell types, and therefore serves as an important model system for host-pathogen studies.\(^1\) Lm can enter mammalian cells and survive within these host cells by secreting a number of virulence proteins during these steps. In the literature, there are inconsistencies in the localizations of one of these effector proteins, InlC.\(^2,3\) In order to better understand the localizations of the Lm effector protein InlC in the live cell during infections, a split GFP approach is taken to fluorescently label the protein. This system has been previously used in the Gram-negative pathogen Salmonella, and the goal of this thesis project is to create the tool to establish this method in Listeria monocytogenes. To provide more clear contrast in the fluorescence assays of the localizations of protein, an Lm strain producing a red fluorescent protein was created.

II. Introduction

1. Importance of developing live cell imaging tools

An important part of biological studies is the inherent dynamical nature of living cells. Cells are the smallest unit of life and provide an important bridge between the molecular and the multicellular worlds, thus serving as a crucial model system to guide the understanding of biology.\(^4\) In the cell, thousands of reactions including protein interactions are happening simultaneously. While many of these reactions influence each other and even govern the overall state of the cell, they do not necessarily happen synchronously and in the same order in every
cell, creating heterogeneous results even between sister cells. Often times, cells in the same state of life could have followed different paths to get there. For these reasons, the most accurate way to study how a set of interactions cause an overall effect is to investigate these interactions in their natural habitat: the cell. To understand complex intracellular interactions, both static and dynamic methods have been used. Static approaches do not provide time-resolved information, so that changes in cellular state must be extrapolated. In contrast, dynamic approaches allow these interactions to be detected in real time. Although static approaches can reveal localizations and interactions that take place at certain times, no information can be attained about how the different parts of interactions relate to each other, and how interactions evolve over time. Dynamic approaches on the other hand can reveal important information about changes in localizations of molecules of interest in real time as they come together to drive certain processes.

In dynamic studies, one of the most commonly used approaches is to fluorescently label proteins of interest in the living cell. Generally, proteins are genetically modified so that they are expressed as a fusion to a fluorescent protein, such as Green Fluorescent Protein (GFP). These proteins can then be observed using fluorescence microscopes in real time, where their localizations and dynamics can be tracked relative to other identifiable parts of the cell. Examples of cellular processes that have been studied using fluorescence techniques include the relative time of transcription and localizations before and after protein interaction of interest.

Unfortunately, introducing a foreign protein into the cell can often cause interruption of interactions and have toxic effects on the cell. Furthermore, when working with secreted proteins, fusing GFP to them can often interrupt the secretion process due to steric constraints.
Examples for systems where secreted proteins are investigated include bacterial pathogens that secrete proteins called effectors into infected host cells. Due to these limitations, it is of extreme importance to engineer tools that are compatible with the protein of interest in a particular system, such that no dynamics are inhibited or disturbed. When the appropriate tool is used on the protein of interest, immense amounts of information can be deducted from fluorescent microscopy assays.

2. *Listeria monocytogenes* Infections

The ability of the bacterial pathogen *Listeria monocytogenes* (*Lm*) to thrive in a wide range host cells and to cross tight barriers in human cells has made it a very important model system to study host pathogen interactions.\(^1\) The versatility of this organism has also made it a target for studies of cell invasion and bacterial spread, as well as intracellular survival.\(^6\) This Gram-positive food-borne pathogen is able to cross tight barriers to infect internal organs and even spread to the brain.\(^1\) Furthermore, *Lm* cells are able to adapt and survive in macrophages and other phagocytic cells. By manipulating endocytic properties of host cells, *Lm* gain entry into the cell, divide intracellularly and spread from cell to cell while escaping the host’s immune system (Fig. 1).\(^1\)

In the process of infection and spreading throughout the host, *Lm* cells secrete a series of effector proteins that help manipulate the host to enable survival and replication of the pathogen. These effector proteins, also called virulence factors, have specialized functions to facilitate survival of the bacteria in new intracellular environment within the host.\(^1,7\) Virulence factors
secreted into the host include proteins that possess structural functions and enzymatic activities, among others to enable the survival of the pathogen and promote the infection.\textsuperscript{7}

One of the best-studied \textit{Lm} virulence factors is a surface protein, ActA which mimics host proteins to polymerize the host actin. As a result, ActA utilizes actin to promote intercellular motility and allows for escape from the immune system of the host (Fig. 1). The polymerized actin (called actin tails) is the most prominent phenotype of \textit{Lm} infections and readily detectable in fluorescence microscopy studies.\textsuperscript{2} These tails appear as randomly oriented actin filaments around 5 µm long that are located at the end of the \textit{Lm} cell.\textsuperscript{8}

\textbf{Figure 1.} \textit{Listeria monocytogenes} infection \textit{in vitro}. Steps of infection shown schematically. Entry into the host cell, intracellular division and spread to new host are shown in the diagram. Two effector proteins ActA and InlC are shown and localizations observed in the literature are indicated. Note that InlC was proposed to localize at two distinct localizations, the cytosol and cell periphery. Figure is modified from\textsuperscript{9}. 
An important virulence factor utilized in *Lm* infections is InlC which is responsible for protrusion formation, cell spreading and modulating cell immunity (Fig. 1, red).\(^2,3\) The protein is produced once *Lm* has gained entry into the host cells and InlC secretion contributes to the infection by facilitating cell spread.\(^7,10\) However, there are discrepancies in the literature about the exact function of the InlC virulence factor in host cells. In two different static studies, InlC was visualized with immunofluorescence techniques 5 hours post infection and two different localization phenotypes were observed. (1) In one of the studies, the InlC protein was found to localize to the cytosol.\(^2\) This localization phenotype is consistent with a different study, demonstrating binding of InlC to the cytosolic human immune response protein IKKα and interference with its function.\(^11\) (2) In the other InlC immunofluorescence assay study, the protein was found to localize near the cell membrane and interact with the human adaptor protein Tuba, which is located in the apical cell junctions to promote cell spreading.\(^3\) Due to the different localizations observed and interactions detected, InlC was suggested to function as a pleotropic effector, having multiple effects in the host cell (Fig. 1).\(^2\) This thesis will focus on the development of fluorescent tools that will allow us to define the localization of InlC in live infected cells in order to resolve the controversy over its localization and provide insight into dynamics throughout the infection process.
Figure 2. (A) *Listeria* cells are transformed to contain genetic fusion of GFP11 tagged effector protein. Upon secretion of the tagged effector, spontaneous complementation with the GFP1-10 labeling components in the host cytosol results in fluorescently labeled effectors.

(B) Ribbon representation of InlC-GFP fusion. The InlC and the GFP protein are attached via a flexible linker. The linker includes an internal myc tag which can be detected with the use of anti-myc antibodies. The interaction represented with the host binding partner is an example of one of the known interactions. There might be other binding sites on the InlC protein that are not illustrated in the figure.
3. **Split GFP system**

A powerful tool in biology has been to fuse proteins of interest with the naturally derived fluorescent proteins like GFP and track them over time to gain more information on localizations of the protein of interest in relation to other markers. The common fluorescent marker protein GFP was originally identified in and purified from jellyfish *Aequorea victoria* and has 11 anti-parallel strands that fold into an overall β-barrel shape.

When working with effector proteins that get secreted from the pathogen into the host, GFP has been observed to be too bulky to be compatible with secretion and therefore cause interruption of the secretion process.\(^\text{12}\) The use of GFP may be especially problematic for *Lm* where the secretion system is not well understood, and it is reasonable to believe that FPs are likely to perturb secretion of effector proteins.\(^\text{12}\) Fortunately, this problem can be minimized by using a split GFP approach. In a split GFP system, the effector protein is only tagged with a small section of the fluorescent protein instead of the whole protein to be compatible with effector secretion systems. In a previously established split GFP system to visualize *Salmonella* effector proteins, the small GFP\(^\text{11}\) consisting of 13 amino acids was genetically fused to protein of interest.\(^\text{12,13}\) The remaining GFP\(^1\)-10 strands are introduced into the host cell by transfection. Upon infection and secretion of the effector protein, the spontaneous complementation of the two fragments result in a fluorescently tagged effector protein (Fig. 2A).\(^\text{12}\) When this system was engineered, the GFP sequence was mutated to increase solubility and complementation in both parts of the fluorescent protein.\(^\text{13}\)
When applying the split GFP system to study protein dynamics in general and effector dynamics in particular, it is important to be aware that the protein is modified by attaching a GFP label and therefore any side effects or changes in the system must be monitored carefully.

**III. Goals of this Thesis Project**

The main purpose of this thesis is to test the hypothesis that InlC changes localizations during infection: specifically, I hypothesize that InlC localizes in the cytosol in early stages of the infection, prior to its localization at the cell membrane. In order to test this, I propose to develop a split GFP system that can be used in *Lm* to track the effector protein InlC in living cells in the context of infection. Application of this system requires successful accomplishment of two independent goals outlined below.

**Goal 1: Creation of *Lm* strain expressing an InlC-GFP11 fusion**

In order to visualize the InlC effector protein during infection an InlC-GFP11 fusion has to be created and the sequence has to be cloned into a plasmid that can be expressed in the pathogen. To achieve this a plasmid containing the InlC promoter (P\_inlC), the gene encoding the InlC protein and the GFP11 fragment will need to be generated and incorporated into *inlC* knockout *Lm* cells to ensure all InlC produced in the cells are tagged with GFP11. Additionally, it will be important to verify that expression and secretion of the effector protein is similar to the wild type unmodified protein. The GFP11 tag should not interfere with the protein dynamics or cause any side effects that alter the regular dynamics. Dynamics of InlC-GFP11 can be compared to established phenotypes of wild type InlC by static methods such as immunofluorescence, as done previously. To test InlC production and secretion into the host cell, the InlC-GFP11 fusion protein will include an affinity tag which can be detected with the appropriate antibodies.
(Fig. 2 B). Finally, the InlC protein must not only be detected in the host cell, but its GFP11 counterpart must complement with the GFP1-10 protein to produce detectable fluorescence upon infection.

Goal 2: Generate an Lm strain expressing RFP to enable visualization of bacteria during infection

In order to monitor the infection simultaneously with visualization of InlC protein localization, Lm cells must also be fluorescent. To achieve the maximum contrast between the Lm cells and the tagged protein, Lm cells must be modified to constitutively express a red fluorescent protein (RFP). Currently, an RFP expressing Lm strain is not available. One reason that hampered expression of common RFPs in Lm is the significantly different codon usage of the Gram-positive Lm cells in comparison to other common bacterial strains such as E. coli. In order to generate RFP expressing Lm, the coding sequence of RFP sequences will be modified to optimize codon usage for Lm expression. In this thesis, two different RFPs, mApple with Lm codon usage (LmApple) and mCherry with Lm codon usage (LmCherry), will be tested in two different possible plasmid backgrounds.

IV. Methods

1. Cloning Techniques

In order to make the constructs needed to generate the InlC-GFP11 fusion and cloning of RFPs into Lm several general cloning methods were used. All DNA was using E. coli cells. All plasmids used were prepped with the Thermo-Scientific Mini-prep kit. All gel purifications were done using a Macherey-Nagel kit. Throughout the cloning process, all transformations were done
using 50 µL *E.coli* Omnimax cells. Typically, 300 ng plasmid DNA or 5 µL of the ligation reaction was transformed. An aliquot of chemically competent *E. coli* cells were incubated on ice with DNA for 10 minutes, heat shocked at 42°C for 45 seconds and recovered in 200 µL Super Optimal broth with Catabolite repression (SOC) media for an hour at 37°C shaking at 180 rpm, followed by plating on antibiotic selective plates. All restriction digests were conducted using 300 ng of plasmid DNA in 15 µL buffered solution with restriction enzymes from NEB according to the company’s recommendation. Typically, PCR reactions were done using Taq DNA polymerase using the manufacturer’s protocol.

2. **Electroporation of Listeria monocytogenes cells**

In order to incorporate the InIC-GFP11 plasmid into Lm and test different RFP variants in *Lm*, the *Lm* cells had to be transformed with the different plasmids. In order to get high efficiency transformations electrocompetent *Lm* cells were made. *Lm* EGD and EGDe cells were grown overnight in Brain Heart Infusion (BHI) media at 37°C. 2 mL of an overnight saturated culture was inoculated into 100 mL BHI media. *Lm* EGD and EGDe strains are two of the commonly used strains in *Listeria monocytogenes* studies. *Lm* cells were grown for two hours at 37°C and shaking at 180 rpm until an O.D. of 0.2 at 600 nm was reached. Penicillin-G was added to a final concentration of 0.12 µg/mL. The *Lm* cells were grown for an additional three hours until the final O.D. was between 0.6 and 0.8 at 600 nm. The *Lm* cells were harvested by centrifugation for 30 mins at 4,000 rpm. The cells were resuspended in 4 °C electroporation buffer consisting of 816 mM Sucrose, 1 mM MgCl₂ at pH 7. Cells were washed three times using 100 mL, 66 mL and 33 mL electroporation buffer in consequent steps. All resuspension steps
were performed at 4°C. The washed cells were resuspended at a concentration of approximately $1 \times 10^{11}$ cells/mL in electroporation buffer and 100 µL aliquots were kept at -80 °C.

For electroporation, 100µL cells were thawed on ice and placed in Biorad electroporation cuvettes along with 5 µL plasmid DNA at a concentration of 1µg/µL. Cells were electroporated at 2.4 kV, 200 Ohms and 25 µF. The cells were immediately transferred in 900 µL pre-warmed BHI media and incubated at 37°C for three hours, followed by plating on selective plates and incubation at 37°C overnight.

3. Mammalian Cell Culture

In this thesis all infections were conducted with the human epithelial cell line HeLa cells as the host. For effective transfection and infections, appropriate care of the HeLa cells is necessary to keep them healthy. HeLa cells were grown in 100 mm tissue culture dishes in 10 mL regular media composing of DMEM, Fetal Bovine Serum, and Penicillin-Streptomycin antibiotics. Upon reaching 90% confluency cells were passaged (also called splitting) into new dishes (typically every 2 – 3 days). HeLa cells were not used past passage 12.

Splitting HeLa Cells:

To maintain healthy HeLa cells, they need to be split every 3-5 days. All old media is aspirated out of the confluent petri-dish and the dish was rinsed with 5 mL Phosphate-buffered saline (PBS). The cells are then incubated at 37°C / 5 % CO₂ for 5 minutes in 2 mL of pre-warmed trypsin. An additional 8 mL of pre-warmed regular media was then added into the dish and the cells were resuspended by pipetting. 2 mL of resuspended culture is transferred into a new dish with 8 mL of pre-warmed regular media. The cells were equally distributed in the dish and are incubated in 37°C / 5 % CO₂ for growth.
4. Infections

In order to achieve consistency in \( Lm \) infections, it was important to follow a very systematic protocol. HeLa cells were used as the host cell for all \( Lm \) infections. One day prior to the infection, \( 0.25 \times 10^6 \) cells were seeded on a glass slides in a well of a 6-well dish. Cells were grown in standard medium (DMEM with Fetal Bovine Serum and penicillin streptomycin). On the day of the infection, HeLa cells in one well were dissociated for counting as follows: Cells were rinsed with 2 mL of PBS, dissociated from the glass using 250 µL of trypsin and quenched with 750 µL of media.

One day prior to the infection, a single colony of the desired \( Lm \) strain from a streaked plate was used to inoculate an overnight culture in BHI media supplemented with antibiotic, if appropriate. On the day of the infection, overnight \( Lm \) cultures were diluted down 5,10 and 20 fold in BHI media and grown for about three hours. The O.D. of the \( Lm \) culture at 600 nm was measured periodically. For infections, the O.D. was between 0.6-0.8. The optical density (O.D.) was converted to number of bacterial cells per mL using the conversion factor \( 10^9 \) bacteria/mL for each O.D. unit. Bacteria were then washed by centrifuging at maximum speed for 5 minutes and re-suspending in 1 mL DMEM media three times. After the last resuspension in media, the desired amount of \( Lm \) cells to infect HeLa cells at the desired multiplicity of infection (MOI, typically between 5 and 20) were diluted in 2 mL DMEM.

HeLa cells to be infected were rinsed with DMEM media three times. Then, the wash media was replaced with DMEM supplemented with \( Lm \) cells. The infected cells were incubated in \( 37^\circ C / 5\% \) CO\(_2\) for one hour. The media was then exchanged with DMEM media with FBS, supplemented with 20 ng/µL of Gentamicin to ensure only cells that had entered a host cell in the
first hour of the infection remain alive. The cells were then incubated for the remainder of the infection, for example overnight.

5. Immunofluorescence

When imaging cells and particularly infections, it is important to stain parts of the cell with fluorescently tagged antibodies to provide a sense of special arrangements in the host cell compared to the Lm. Cover slides with infected cells were incubated in 1 mL of 4% paraformaldehyde solution in water for 10 minutes, rinsed in 2 mL of PBS and permeabilized by incubating in 200 µL 0.2% Triton-X100 in PBS for 5 minutes. After three more rinses in PBS slides were blocked with 200 µL 5% FBS in PBS for 20 minutes. After rinsing in PBS, slides were incubated with the primary antibody in 5% FBS in PBS. All dilutions and incubations for antibodies used are indicated in the table below (Table 1). After rinsing in PBS three times, the slides were incubated with the secondary antibody in 5% FBS in PBS. Phalloidin stain was added together with the secondary antibody. After incubation with the secondary antibody, slides were washed in PBS, rinsed in water and mounted.

<table>
<thead>
<tr>
<th>Antibody / Stain</th>
<th>Dilution and incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa 594-Phalloidin (Invitrogen)</td>
<td>1:1,000 dilution, 45 min</td>
</tr>
<tr>
<td>Anti-myc (Cell Signaling)</td>
<td>1:200 dilution, Overnight</td>
</tr>
<tr>
<td>Phalloidin-coumarin (Sigma Aldrich)</td>
<td>1:1,000 dilution, 45 min</td>
</tr>
</tbody>
</table>

Table 1. All antibodies used in this thesis are listed along with the dilutions used and the incubation time in 37 °C.
6. Imaging *Lm* Cells

In order to assess the fluorescence and the overall health of the *Lm* cells, imaging was necessary. *Lm* cultures were grown up overnight while shaking at 37°C. A 96-well plate was incubated at 37°C with 100 µL of 0.01 mg/mL poly-L-lysine overnight. 200 µL of 1:10 dilutions of overnight *Lm* cultures were added into wells, incubated at 37°C for an hour and rinsed with PBS prior to imaging.

For bright-field and fluorescence images a Nikon Ti-E widefield fluorescence microscope was used. Typically, images were collected using 60X oil objective using the filter settings indicated in Table 2.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Excitation wavelength (nm)</th>
<th>Dichroic</th>
<th>Emission wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dapi</td>
<td>370-380</td>
<td>400 LP</td>
<td>458-482</td>
</tr>
<tr>
<td>GFP</td>
<td>470-490</td>
<td>490 LP</td>
<td>500-520</td>
</tr>
<tr>
<td>RFP (mCherry/mApple)</td>
<td>550-570</td>
<td>585 LP</td>
<td>585-635</td>
</tr>
<tr>
<td>DIC</td>
<td>---------------</td>
<td>Analyzer</td>
<td>Open</td>
</tr>
</tbody>
</table>

*Table 2.* The imaging settings for all the channels used are listed among with the excitation, dichroic and the emission settlings.

V. Results

In order to apply the split GFP system to *Lm* cells, a strain of *Lm* containing a plasmid that expresses a GFP11 tagged effector protein along with constitutive expression of a red fluorescent protein had to be created. Expression of the red fluorescent protein was important as this can serve as a marker for *Lm* throughout the experiment in real time so that infection assays can be conducted for several hours. For the real time fluorescence microscopy assays, it was important to detect *Lm* cells via a red fluorescent protein and the tagged protein via GFP. Using
this approach, both means of detection were spectrally distinct for unambiguous detection of the localization of protein of interest. Previously, constitutively GFP expressing Lm strains have been generated and used in various Lm studies.\textsuperscript{10} Because these strains do not provide the contrast needed between the tagged protein and the Lm cells, the generation of a constitutively RFP expressing Lm strain was necessary to visualize the localizations of the GFP tagged effector. As no RFP expressing strains of Lm have been generated before, this thesis aimed to examine the expression of different RFPs in Lm cells.

1. Design and construction of the inlC-GFP11 fusion gene in the pMK4_LmApple plasmid

To assemble a plasmid that expresses an RFP and the inlC-GFP11 fusion simultaneously, the vector pMK4_LmApple was modified. The plasmid pMK4_LmApple was received from our collaborators from the Cossart laboratory at the Pasteur Institut in France. The plasmid composed of a pMK4 background and includes the Lm codon optimized version of the RFP mApple (referred to as LmApple). The genetic components necessary to clone the inlC-GFP11 component as a part of the split GFP system were ordered as G-blocks and ligated into the plasmid.

First, the PinlC-GFP11 cassette was cloned in the pMK4_LmApple plasmid using the NarI site upstream of the LmApple gene (Fig. 3A). This primary cassette was designed to include the promoter of the InlC effector protein (PinlC), unique digestion sites to allow cloning of the effector genetic sequence (inlC), a linker to provide flexibility between the protein and GFP11 tag, and the GFP11 sequence. In the resulting fusion protein, GFP11 will complement with GFP1-10 to form the fluorescent tag. This cassette was designed to allow applicability of
the split GFP system to other effector proteins: the promoter can be replaced easily using unique restriction sites (Fig. 3A). Similarly, the design allows for different effectors to be cloned into the plasmid using the NheI digestion site. In designing the system, LmApple and the primary cassette were intentionally cloned to be transcribed in opposite directions to eliminate any interactions that might arise from overlapping transcriptional regulatory sequences. After constructing the primary cassette, another cassette including the inlC coding sequence was cloned in using the NheI unique digestion site (Fig. 3B). This cassette included a myc epitope tag sequence in the linker region in between the GFP11 and the inlC coding region to allow for detection of the protein fusion via an anti-myc antibody. This orthogonal detection system will be useful to troubleshoot potential problems with GFP complementation.
Figure 3. Design of the split GFP system in the pMK4_LmApple plasmid backbone. (A) The pMK4_LmApple plasmid (received from the Cossart lab, unpublished) with relevant features marked in the relative positions in the plasmid including Amp resistance in *E.coli* and Cm resistance in *Lm* cells. Details of the primary cassette functionalities and transcriptional directionality are marked. (B) The plasmid map after the ligation of the primary cassette. The secondary cassette only includes the *inIC* sequence and is cloned into the plasmid using the *NheI* digestion site.
2. Assessment of *Lm* EGDe pMK4_LmApple Strain Growth

Upon construction of a plasmid that produces LmApple and the GFP11-tagged InlC, the plasmid was electroporated into *Lm* EGDe cells to test cell growth. *Lm* EGDe cells electroporated with the plasmid pMK4_LmApple were grown side by side with appropriate control strains: wild-type (wt) *Lm* EGDe did not have a plasmid, *Lm* EGDe cGFP expressed GFP from the chromosome and *Lm* EGDe pAT18_18 expressed GFP from the plasmid pAT18_cGFP. The plasmid pAT18_cGFP was previously confirmed to robustly express GFP.³ It was observed that the wt *Lm* cells, *Lm* that express GFP from the chromosome (EGDe cGFP), or *Lm* that express GFP from a plasmid (EGDe pAT18_cGFP) grow at a similar rates. These strains reached half of the corresponding maximum O.D.s about five hours after inoculation and within 30 minutes of each other (Fig. 4). In contrast, the *Lm* strain that expressed LmApple from the pMK4 plasmid (EGDe pMK4_LmApple) was observed to grow significantly differently. The growth did not exceed an O.D of 0.5, whereas for the other strains the maximum O.D. was about 0.9. Additionally, half of the maximum O.D. observed by the EGDe pMK4_LmApple strain was not reached until 15 hours post-inoculation, indicating a severe growth defect (Fig. 4).
Figure 4. Growth curve for four different *Lm* EGDe strains including wild-type (wt), a variant for chromosomal expression of GFP (EGDe cGFP), and *Lm* cells expressing FPs from plasmids (EGDe pAT18_cGFP and pMK4_LmApple). All cultures were supplemented with the appropriate antibiotics and grown at 37°C. Each O.D. unit corresponds to 10⁹ cells/mL. The shown values are averages of triplicate samples.

3. Assessment of RFP expression in EGDe pMK4_LmApple Strain

In addition to measuring the growth curve of the newly developed Listeria strain, I also sought to test whether the bacteria actually produced the red fluorescent protein. A fluorescent microscopy assay was conducted to qualitatively assess LmApple production and maturation in the strain EGDe pMK4_LmApple. As a control, the strain EGDe pAT18_cGFP was used, because this strain robustly expresses GFP. Cells were grown overnight and imaged live by fluorescence microscopy. Filter settings for mApple GFP, and DIC channels were used. In the mApple channel, very similar and dim red signal was detected for both the EGDe pMK4_LmApple and the EGDe pAT18_cGFP strains. In the GFP channel, no signal was
detected from the *Lm* cells expressing the pMK4_LmApple, while high counts of green signal was observed from the EGDe pAT18_cGFP strain (Fig. 5). In the DIC channel, it was observed that the GFP expressing cells appeared rod-shaped, indicative of healthy bacteria. On the other hand, pMK4_LmApple expressing cells looked elongated compared to their expected rod-shaped morphology (Fig. 5).
Figure 5. Fluorescent microscopy assay of EGDe pMK4_LmApple and EGDe pAT18_cGFP strains of Lm cells. (A) Comparison of Lm cells electroporated with pMK4_LmApple and pAT18_cGFP under mApple, GFP, and DIC channel settings. (B) Enlarged version of Lm electroporated with pMK4_LmApple and pAT18_cGFP under the DIC channel settings. All images were collected on a widefield fluorescence microscope using a 60x oil objective.
4. Constructing the \textit{inlC}-GFP11 fusion gene in pAT18\_cGFP backbone

Because \textit{Lm} EGDe pMK4\_LmApple displayed a highly irregular growth curve and red fluorescence was detected, it was hypothesized that the pMK4 backbone could harbor features that contribute to lethality, such as the antibiotic resistance marker or the promoter for expression of LmApple. In this case, the pAT18\_cGFP plasmid would provide a viable background to clone in the \textit{inlC}-GFP11 gene sequence and the robust promoter that drives GFP expression could be used to express LmApple. As stated previously, the main goal of this thesis was to assemble a plasmid that produces an RFP and the InlC-GFP11 fusion protein while displaying normal growth rates and normal physical morphology. Previously (Results sec. 2), it was noted that EGDe pMK4\_LmApple strain \textit{Lm} cells were observed to have no fluorescence and elongated cells that had a sick appearance, while EGDe pAT18\_LmApple strain robustly produced GFP and had a normal morphology. By switching the backbone of the plasmid, it was hypothesized that the LmApple gene could be expressed robustly and the cells would be healthier.

To make the transition, the complete cassette including the \textit{inlC}-GFP11 fusion sequence was inserted in the plasmid backbone pAT18\_cGFP (Fig. 6).\textsuperscript{10} The plasmid pAT18\_cGFP already had a convenient \textit{NarI} restriction site downstream of the GFP gene. The opposite transcriptional directionality of the RFP and the effector protein was ensured in this plasmid background as well by sequencing clones after the ligation. The pAT18\_cGFP\_\textit{inlC} plasmid was then modified further to exchange the GFP sequence for two different RFP sequences, namely LmApple and LmCherry. In this case, the original promoter to express GFP (the \textit{phyper} promoter) was conserved to be used for the RFP expression (Fig. 6).\textsuperscript{10} The LmApple sequence
used in the plasmid pMK4_LmApple was PCR-amplified for cloning and a \textit{Lm} codon optimized version of the RFP mCherry was ordered as a G-block.

**Figure 6.** Plasmid constructs derived in the pAT18 background. All arrows indicate transcriptional directionality of relevant parts of the plasmids. (A) Plasmid pAT18_cGFP is Ery resistant, expresses GFP and was used to additionally express the \textit{inlC}-cassette that was previously assembled in the pMK4 background (see Fig.3). (B) Plasmid map after ligation of the complete \textit{inlC}-cassette in the pAT18_cGFP backbone. (C) The pAT18 backbone with the complete functional \textit{inlC}-cassette was further modified and the GFP-coding region was replaced with a sequence to express LmApple. (D) The pAT18 backbone with the complete functional \textit{inlC}-cassette was modified and the GFP coding region was replaced with a sequence to express LmCherry.
5. Verifying Growth of *Lm* Strains with pAT18 based Plasmids

The growth of the *Lm* strain containing the *inlC*-GFP11 fusion in the pAT18 plasmid background was assessed in comparison to the wt *Lm* (EGDe) and *Lm* strain deficient in the InlC protein (EGDe Δ*inlC*). When observing the localizations of the protein InlC in the InlC-GFP11 context, working with strains chromosomally deficient in the protein is important so that all InlC proteins produced in the cell are fluorescently tagged and can be tracked. For this reason, growth rates of stains that only produce the protein of interest via a plasmid should be comparable to wild type strains.

The *inlC* knockout strain (EGDe Δ*inlC*) was received from the Cossart lab. Growth of EGDe and EGDe Δ*inlC* transformed with various constructed plasmids was assessed by a growth curve. No significant difference in growth of EGDe wild type and the EGDe Δ*inlC* strain was observed (Fig. 7). Both strains plateaued at an O.D. around 1 at 8 hours after inoculation (Fig. 7). The strains harboring plasmids plateaued at around an O.D. of 0.9 units 10 hours after inoculation and reached halfway to maximum O.D. units around 2 hours after the strains without a plasmid (Fig. 7). This was expected, since strains with a plasmid grow under antibiotic pressure and must produce the antibiotic resistance protein. No significant difference between the EGDe and EGDe Δ*inlC* strains was observed. This was expected since the InlC promoter P*inlC* is only activated in the intracellular space of the host cell.  

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Figure 7. Growth curves for different EGDe and EGDe ΔinlC strains. Blue markers indicate strains with a plasmid; triangle markers indicate EGDe ΔinlC strain. All cultures were supplemented with the appropriate antibiotic and grown at 37°C. Each O.D. unit corresponds to 10^9 cells/mL. The shown values are averages of triplicate samples.

Following the assay, the two plasmids constructed to express LmApple and LmCherry were electroporated into EGDe ΔinlC Lm cells to verify the growth of the cells after the switch from GFP expression to the indicated RFPs. Lm strains EGDe ΔinlC pAT18_cGFP_InlC and EGDe pAT18_LmApple_InlC had very similar growth. For both strains the maximum O.D. was reached around 16 hours after inoculation (Fig. 8). All points showing growth of the EGDe ΔinlC pAT18_LmApple_inlC strain were within the standard deviation for the EGDe ΔinlC pAT18_cGFP_inlC strain. In contrast, for the first 30 hours after inoculation no growth was observed for the EGDe ΔinlC pAT18_LmCherry_inlC strains (Fig. 8). A significant change in growth for EGDe ΔinlC pAT18_LmCherry_inlC strains was observed starting at 35 hours after inoculation (Fig. 9). Two different clones of the strain showed different growth in terms of the
O.D., but started the log phase and reached the first plateau at very similar times (Fig. 9). A third clone that was also inoculated did not grow in the timeframe tested.

**Figure 8.** Growth curve for EGDe ΔInIC strains expressing the indicated plasmids for the first 30 hours of incubation in 37°C. The results displayed for Lm cells expressing pAT18_cGFP_InlC and pAT18_LmApple_InlC are averages of triplicate samples, whereas the two pAT18_LmCherry_InlC results are from single samples. Green and red error bars show the standard deviation for the pAT18_cGFP_InlC and pAT18_LmApple_InlC strains respectively. All cultures were supplemented with the appropriate antibiotic and grown at 37°C. Each O.D. unit corresponds to $10^9$ cells/mL.
Figure 9. Growth curve for two different clones of EGDe ΔinlC pAT18_LmCherry_inlC strain. A third clone tested on the same day did not grow in the time frame indicated here. All cultures were supplemented with the appropriate antibiotic and grown at 37°C. Each O.D. unit corresponds to 10^9 cells/mL. After the assay was collected, both cultures were placed on erythromycin-BHI media and growth was observed.

6. RFP Expression in pAT18 Plasmid Background

After generating Lm strains containing either the pAT18_LmApple_inlC or pAT18_LmCherry_inlC plasmid, the next step was to check for expression of the RFP and morphology of the bacteria. RFP expression of these constructs were assessed qualitatively using a custom fluorescent colony screener (Fig. 10). Initially, five colonies of Lm EGDe ΔinlC transformed with pAT18_LmApple_inlC and two colonies of Lm EGDe ΔinlC transformed with pAT18_LmCherry_inlC were streaked on a BHI plate supplemented with erythromycin as the antibiotic pressure. Lm EGDe ΔinlC transformed with pAT18_LmApple_inlC were excited under the appropriate light to assess fluorescence, and red fluorescence was detected. Similarly, Lm EGDe ΔinlC transformed with pAT18_LmCherry_inlC were tested under identical
conditions but appeared to be not fluorescent (Fig. 10A). A second fluorescent assay was performed to compare LmApple producing colonies with a GFP producing control. This test was conducted to compare fluorescence and background light qualitatively (Fig. 10B,C). Under the light with the appropriate wavelength, LmApple expressing colonies 2-4 appeared to be fluorescent while the GFP expressing colony did not fluoresce (Fig. 10B). Similarly, under conditions to test green fluorescence, the GFP expressing colony appeared fluorescent, but the LmApple producing colonies did not fluoresce (Fig. 10C).

Figure 10. EGDe ΔinlC transformed with pAT18_LmApple_inlC and pAT18_LmCherry_inlC were streaked and visualized under the appropriate light. (A) Colonies visualized under the appropriate excitation emission settings (see Table 2.), colonies I-V were transformed with the pAT18_LmApple_inlC plasmid, while I and II indicated with arrows (“mCherry”) were transformed with the pAT18_LmCherry_inlC plasmid. (B,C) Strains were re-streaked to compare the fluorescence of Lm strains with pAT18_LmApple_inlC and pAT18_cGFP_inlC side by side.

To further verify expression of the RFP and analyze bacterial cell morphology Lm cells transformed with the pAT18_LmApple_inlC plasmid were compared with Lm cells transformed with the pAT18_LmCherry_inlC plasmid by widefield fluorescence microscopy. Under the mApple channel, red signal was detected from Lm EGDe ΔinlC transformed with pAT18_LmApple_inlC (Fig. 11). LmApple producing cells appear to be fluorescent and rod
shaped, while *Lm* cells containing the pAT18_LmCherry\_inlC plasmid were not fluorescent and had an elongated morphology.

<table>
<thead>
<tr>
<th>Strain / Channel</th>
<th>mCherry/mApple</th>
<th>DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGDe (\Delta)inlC pAT18_LmApple_inlC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>EGDe (\Delta)inlC pAT18_LmCherry_inlC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
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</table>

**Figure 11.** Fluorescent microscopy assay of EGDe \(\Delta\)inlC pAT18_LmApple\_inlC and EGDe \(\Delta\)inlC pAT18\_cGFP\_InlC *Lm* strains. Cells containing the plasmid pAT18_LmApple\_InlC appeared fluorescent while no red signal was detected from cells expressing the pAT18_LmCherry\_InlC plasmid. Under the DIC channel, LmApple producing cells appeared to be rod-shaped and healthy while cells expressing the pAT18_LmCherry\_InlC plasmid appeared elongated compared to natural *Lm* morphology. All images were collected on a widefield fluorescence microscope using a 60x oil objective.

In the overlay of DIC and mCherry/mApple channels for the strain EGDe \(\Delta\)inlC pAT18_LmApple\_inlC the cell morphology and fluorescence can be compared directly (Fig.}
Two different cell morphologies were prominent in the image: rod shaped bacteria indicative of healthy cells and an elongated morphology. No fluorescence was observed in elongated cells and fluorescence was observed in around 70% of the rod shaped cells. In cells going through division, three general cases of fluorescence were observed: fluorescence in both cells, no fluorescence in either cell, or fluorescence in only one cell.

Figure 12. DIC mApple overlay of EGDΔinlC pAT18_LmApple_inlC. No fluorescence is observed in elongated Lm cells. Fluorescence is observed in 70% of Lm cells with normal morphology.

In summary, the four strains created for this thesis were examined qualitatively by fluorescence and light microscopy. The differences in fluorescence and morphology were examined. Additionally, growth curves of each strain were taken. Strains displayed a range of phenotypes that are summarized below (Table 2).
<table>
<thead>
<tr>
<th>Strain</th>
<th>EGDe pMK4_LmApe</th>
<th>EGDe pATcGFP</th>
<th>EGDe ΔInlC pAT18_LmApe_inlC</th>
<th>EGDe ΔInlC PAT18_LmCherry_inlC</th>
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<tr>
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<td>-Ery resistance</td>
<td>-Ery resistance</td>
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<tr>
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<td>-Uncharacterized</td>
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<td>-phyper promoter for</td>
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<tr>
<td></td>
<td></td>
<td>-InlC-GFP11</td>
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</tr>
<tr>
<td><strong>Fluorescence</strong></td>
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<td>Fluorescence only in 70% of “healthy” cells</td>
<td>No fluorescence</td>
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<td>Normal/ Elongated</td>
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<tr>
<td><strong>Growth</strong></td>
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<td>Normal</td>
<td>Normal</td>
<td>Very slow</td>
</tr>
</tbody>
</table>

Table 2. Summary of strains and observed properties. Table summarizes the qualitative properties of strains created in this thesis. Cm= Chloramphenicol, Ery= Erythromycin

7. Infection of HeLa cells with *Lm*

Our ultimate goal is to use newly develop Lm strains expressing an RFP and InlcC-GFP11 in infections to order to track both bacteria and the effector over the course of an infection. Therefore, it must be ensured that *Lm* cells expressing the components of the split GFP system display normal infection phenotypes. To test this qualitatively, HeLa cells were infected with the strain EGDe pAT18_cGFP as a control. Any further infections with the strains expressing split GFP system will be compared to infection phenotypes displayed by the strain EGDe pAT18_cGFP.

To visualize infections of HeLa cells by *Lm*, GFP producing *Lm* cells were used (Fig. 13). HeLa cells were infected at a multiplicity of infection (MOI) of 20 and the infection was carried out overnight. Cells were then fixed, permeabilized and stained with Alexa 594-Phalloidin to label actin. Fluorescently labelled actin was observed to co-localize with *Lm*, as
seen previously for actin tails.\textsuperscript{2} The \textit{Lm} cells were observed to be heavily localized in the intracellular space of HeLa cells in certain locations (Fig. 13). \textit{Lm} cells appeared to be concentrated in regions of the dish, suggesting spreading from the initial infected cell to nearby cells.

![Image](image_url)

**Figure 13.** Fluorescent microscopy of fixed and stained infection with \textit{Lm} stain EGDe pAT18_cGFP under RFP and GFP channels. Alexa 594-Phalloidin was used to label actin.

GFP producing \textit{Lm} cells are tracked with red streaks of actin (Fig. 14). In the periphery of one particular HeLa cell, \textit{Lm} cells were observed to protrude into the extracellular space. These \textit{Lm} cells were localized with these “streaks” of actin connecting them to the HeLa cell. Actin localization with the GFP producing \textit{Lm} cells were at a single end of the rod shaped bacteria.
VI. Conclusion

In this project an LmApple producing *Lm* strain was successfully created. Initial tests of a gene coding for an *Lm*-codon optimized version of LmApple was tested in the pMK4 plasmid backbone and resulted in a lethal phenotype. Upon cloning the gene into the pAT18 plasmid backbone, the combination of switching the plasmid backbone, promoter, and the antibiotic resistance pressure yielded healthy LmApple producing *Lm* EGDe cells, as judged by several metrics (growth curves, fluorescence assays, see Table 2). Switching from the pMK4 based backbone to the pAT18 based backbone showed change in both the fluorescence and the morphology of the cells. This indicates that the *Lm* cells are very sensitive to properties of the plasmid introduced. This thesis also revealed that the nature of the fluorescent protein itself also has an effect on cell morphology and growth, as LmCherry which was also expressed in the pAT18 plasmid background, showed growth defect and no fluorescence. This indicated that in

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**Figure 14.** Fluorescence microscopy of fixed and stained infection with *Lm* strain EGDe pAT18_cGFP under RFP and GFP channels. Alexa 594-Phalloidin was used to label actin.
addition to the backbone *Lm* cells are also very sensitive to the proteins produced (in this case mCherry vs. mApple, both with *Lm* codon usage).

It is possible that the elongated morphology is caused by un-matured RFP, where the protein is produced, but does not fold and/or mature properly. The defective protein might be interfering with other cellular processes and cause “sickness” of *Lm*. This hypothesis is also consistent with the observation that cells producing correctly matured fluorescent LmApple protein appear healthy.

Interestingly, *Lm* transformed with the pAT18_LmApple_inlC displayed heterogeneous fluorescence; while all cells should be genetically identical, not all cells were red fluorescent. The reason for the heterogeneity in the phenotype is unknown and must be analyzed with further protein and microscopy assays.

A split GFP system has been genetically established for *Lm* cells in this project. Although the system has not been tested, the *Lm* strains created contain all genetic components of the system. Live infection fluorescence assays must be conducted with host cells transiently transfected with DNA encoding for GFP1-10 to test for complementation of the GFP to confirm that the system works properly.

If the GFP complementation assay is inconclusive, the production and secretion of InlC in the context of the GFP11 fusion construct can be tested by immunofluorescence via the myc-tag.
VII. Future Directions

To confirm GFP complementation upon InlC secretion, the strain *Lm* EGDe pAT18_LmApple_inlC will be used to infect the HeLa host after transfection with a plasmid encoding for GFP1-10. As an initial test, protocols that were previously developed for similar experiments in *Salmonella* will be used.\(^\text{12}\) InlC is expected to be secreted into the host cell approximately 4 h post infection.\(^\text{2}\) Therefore, this time frame will be used for initial imaging tests. Fluorescent microscopy assays will be conducted to both confirm infection phenotypes in fixed cells (such as actin tails) and the complementation of the GFP protein. Then, long term live cell fluorescent assays will be used to determine the localizations of the effector InlC in live infections over time.

Another goal will be to create an *Lm* strain expressing RFPs while retaining normal morphology and a homogeneous strong fluorescence signal in each cell. It is known that red fluorescent proteins often induce cytotoxicity, and current efforts in the literature aim to improve red fluorescent proteins.\(^\text{15}\) Thus a new *Lm* strain producing different RFPs like tdTomato or mRuby will be created and tested for fluorescence, growth and morphology. These RFPs will be codon optimized for *Lm*. Although, codon usage was not directly tested as a parameter in this thesis, discussions with members of the Cossart lab and other works in the literature indicate that codon optimization is especially important for expression of fluorescent proteins in Gram positive pathogens with low GC content, like *Lm*.\(^\text{16}\)

As a long term goal, the split GFP system can be applied to study other *Lm* effector proteins. Due to the versatility of the system and the versatile plasmid developed in this project, all components including the promoter and the effector protein can be replaced relatively easily.
by standard cloning procedures. Together, this system opens the opportunity to investigate
dynamics of a broad range of secreted effector proteins, and investigate localization phenotypes
of effectors with previously unknown functions.
VIII. Acknowledgements

I would sincerely like to thank Dr. Palmer for giving me the opportunity to work in her lab. She has encouraged and reassured me throughout this research and has allowed me to perform my best. My experience in her lab has truly been the most rewarding and memorable of my undergraduate time in University of Colorado, Boulder.

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IX. References