A novel suicide plasmid for efficient gene mutation in *Listeria monocytogenes*

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ABSTRACT

Although several plasmids have been used in *Listeria monocytogenes* for generating mutants by allelic exchange, construction of *L. monocytogenes* mutants has been inefficient due to lack of effective selection markers for first and second recombination events. To address this problem, we have developed a new suicide plasmid, pHoss1, by using the pMAD plasmid backbone and anhydrotetracycline selection marker (*secY* antisense RNA) driven by an inducible Pxyl/tetO promoter. Expression of the *secY* antisense RNA eliminates merodiploids and selects for the loss of plasmid via a second allelic exchange, which enriches the number of mutants with deleted genes. To assess the effectiveness of pHoss1 for the generation of stable in-frame deletion mutations, the *ispG* and *ispH* genes of *L. monocytogenes* serotype 4b strain F2365 were deleted. Identification of the second allelic exchange mutants was very efficient with 80-100% of the colonies yielding desired deletion mutants. This new plasmid will be very useful for construction of marker-free deletion mutants in *L. monocytogenes*, and also we expect that it will be useful for other Gram-positive bacteria, including *Staphylococcus aureus* and *Bacillus cereus*.

Keywords: Listeria; Suicide plasmid; In-frame deletion mutants; *ispG*; *ispH*

1. Introduction

Deletion of bacterial genes by allelic exchange is a widely used method to study gene functions. The success of allelic exchange increases with the availability of selection markers that permit the effective isolation of transformants and recombinant strains resulting from single or double crossover events. Although several plasmids have been available for generating allelic exchange mutants in Gram-positive bacteria, construction of *Listeria monocytogenes* mutants has been very inefficient due to lack of a plasmid with effective selection markers for first and second allelic exchanges.

Two vectors constructed for allelic exchange in Gram-positive bacteria are pAUL-A and pLSV2 (Chakraborty et al., 1992; Wuenscher et al., 1991), which do not have a selection marker for the second allelic exchange. Thus, screening for erythromycin-sensitive deletion mutants is very labor intensive and time consuming. Previously, our lab generated two in-frame gene deletions in *L. monocytogenes* using pAUL-A (unpublished work), but the process was inefficient, and each mutant required PCR screening of hundreds of erythromycin-sensitive colonies to identify a single deletion mutant among wild-type revertant colonies.

A pMAD vector was developed and used for generating allelic replacements in several types of Gram-positive bacteria, including *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus* (Arnaud et al., 2004). This vector features a temperature sensitive origin of replication, an erythromycin selection marker, and *lacZ* gene encoding β-galactosidase (*bgaB*) for blue-white screening. However, color screening does not permit positive selection of mutants for the second allelic exchange. Our experience showed that color development is slow in *Listeria*, requiring about 10 days at room temperature, and color screening often resulted in false positives.

Recently, pKOR1 and pIMAY suicide plasmids were developed for allelic exchange in *Staphylococcus* (Bae and Schneewind, 2006; Monk et al., 2012). These two vectors have advantages of employing antisense *secY* RNA expression for positive selection of the second allelic exchange. We attempted to use these two plasmids to construct in-frame deletions in *L. monocytogenes*, but the transformation attempts were not successful.

Use of the plasmids mentioned above for constructing *L. monocytogenes* mutants was hindering in our research. Therefore, we developed a new suicide plasmid, pHoss1, that combines the pMAD backbone and the *secY* antisense cassette from pIMAY. In pHoss1, the pMAD backbone provides efficient rate of first allelic exchange in *L. monocytogenes*, and expression of the *secY* antisense RNA provides efficient selection for the second allelic exchange event and generation of a markerless deletion. To assess the usefulness of pHoss1 in *L. monocytogenes*, we constructed in-frame deletions of the *ispG* and *ispH* genes (LMOf2365_1460 and LMOf2365_1470). IspG and IspH are iron sulfate enzymes involved in isoprenoid biosynthesis via the mevalonate-independent 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. Enzymes in this pathway are encoded by six genes (*ispC, ispD, ispE, ispF, ispG*, and *ispH*) and result in production of isopentenyl pyrophosphate (IPP) or its isomer dimethylallyl pyrophosphate (DMAPP) (Hunter, 2007; Rohmer, 1999). 1-hydroxy-2-methyl-2-(E)-butenol 4-diphosphate synthase (IspG) and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (IspH) are the last two enzymes of this pathway. The IspG protein catalyzes the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) into 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (Hecht et al., 2001), whereas ispH converts 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate into IPP and DMAPP (Altincicek et al., 2002). Because the enzymes of the MEP pathway are not found in humans, this pathway has been used recently as an anti-infective drug

target of various bacterial infections and malaria (Nakagawa et al., 2013; Obiol-Pardo et al., 2011). Interestingly, nonpathogenic strains of *L. innocua* and *L. monocytogenes* do not possess these two genes (Begley et al., 2008; Steele et al., 2011). Thus, we hypothesized that *ispG* and *ispH* genes could be essential for *L. monocytogenes* pathogenesis, and we tested this by developing mutants and determining their attachment properties in human Caco-2 cells, a transformed intestinal epithelial cell line.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *L. monocytogenes* strain F2365 was originally isolated from Mexican-style soft cheese implicated in a 1985 outbreak of listeriosis in California (Linnan et al., 1988). *L. monocytogenes* strains were grown routinely overnight in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI). *Escherichia coli* was grown in Luria-Bertani (LB) broth. Antibiotics (Sigma-Aldrich, St. Louis, MO) used as needed were: erythromycin (Ery, 10 µg/ml), ampicillin (Amp, 100 µg/ml), chloramphenicol (Chl, 10 µg/ml) and anhydrotetracycline (ATc, 1.5 µg/ml).

2.2. Construction of the pHoss1 suicide plasmid

A 1,371 bp fragment encoding an antisense *secY* RNA expression cassette was PCR amplified from pIMAY vector with the Anti-BseRI-F01 and Anti-BglII-R01 primers listed in Table 2. The amplified antisense *secY* RNA fragment was digested with BseRI and BglII restriction enzymes. pMAD plasmid was digested with the same two enzymes, run on 1% agarose gel, and the plasmid backbone (~7,624 bp) was gel extracted and ligated with linearized

antisense *secY* RNA fragment. The ligation reaction (1-2 µl) was transferred into *E. coli* DH5α competent cells via heat shock, and colonies with pHoss1 were selected on LB agar containing Amp. Presence of the antisense *secY* RNA expression cassette was checked first by colony PCR using the Anti-BseRI-F01 and Anti-BglII-R01 primers and then confirmed by sequencing.

2.3. Construction of L. monocytogenes in-frame deletions

In-frame deleted fragments for the LMOf2365_1460 and LMOf2365_1470 genes were produced using overlap extension PCR (Horton et al., 1990) and the primers listed in Table 2. Briefly, approximately 1 kb fragments from the upstream and downstream of each gene to be deleted were amplified separately with A and B (A/B) and C and D (C/D) primers. Then, equal volumes of these PCR products were mixed, diluted 1:20, and used as a template in the second PCR reaction using A and D primers to generate a deleted gene of about 2 kb. pHoss1 and overlap extension fragments were digested with SalI and NcoI endonucleases. After ligation and transformation into *E. coli* DH5α, plasmids p*LmΔispG* and p*LmΔispH* were obtained, and the presence of the deleted *ispG* and *ispH* fragments verified by colony PCR and confirmed further by sequencing. The resulting plasmids were transformed into the *L. monocytogenes* strain F2365 by electroporation and then incubated with shaking for 3 h at 30°C before spreading into BHI agar containing Ery (10 µg/ml).

We determined that *L. monocytogenes* is sensitive to more than 2.00 µg/ml ATc. Thus, we used 1.5 µg/ml ATc for induction of the *secY* antisense RNA. A two-step procedure was used for replacement of the wild type *ispG* and *ispH* genes. In the first step, one colony was picked from a BHI agar plate with Ery into 2 ml BHI broth and grown overnight at 30°C, then streaked on a BHI agar plate with Ery at 42°C for 2 days, which was repeated twice. In the second step,

Ery resistant colonies were grown overnight in BHI broth (no Ery) at 30°C, which was repeated twice. In the next step, cultures were inoculated in fresh BHI broth and grown at 42°C for 8 h, and mutants were selected by spreading diluted culture on a BHI agar plate containing 1.5 µg/ml ATc and incubating plates at 30°C for 3 days. Finally, 20 colonies were picked and colony PCR performed using A and D primers. Ery sensitivity was checked also. The new mutants were designed as *Lm*f2365Δ*ispG* and *Lm*f2365Δ*ispH*.

2.4. Complementation of the L. monocytogenes mutants

Primer pairs Lm-ispG-comp-F01 and Lm-ispG-comp-R01, and Lm-ispH-comp-F01 and Lm-ispH-comp-R01 were used to amplify the wild type *ispG* (1,110 bp) and *ispH* (1,026 bp) genes from the *L. monocytogenes* F2365 genomic DNA, respectively. Amplified products were digested with SacI and SalI and cloned into the SacI and SalI digested pPL2 integration vector (Lauer et al., 2002). Electrocompetent *Lm*f2365Δ*ispG* and *Lm*f2365Δ*ispH* mutants were transformed with pPL2-*ispG* and pPL2-*ispH*, and positive transformants selected by plating in the presence of Chl (10 µg/ml). Complemented strains were designated *Lm*f2365Δ*ispG*::pPL2-*ispG* and *Lm*f2365Δ*ispH*::pPL2-*ispH*.

2.5. Cell culture and bacterial adhesion assays

The adhesion properties of the *L. monocytogenes* wild type and mutant strains to human Caco-2 epithelial cells were evaluated (Cowart et al., 1990). Briefly, Caco-2 cells (HTB-37) obtained from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in Dulbecco's modified Eagle medium (DMEM) (ATCC, Manassas, VA) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Caco-2 cells were grown in 75 cm^2

plastic flasks (Sigma-Aldrich) at 37°C under 5% CO_2 in a humidified incubator (Fisher Scientific, Pittsburgh, PA). The medium was changed every two days. When cells reached confluence between passage 5 to 6, as checked by phase-contrast microscopy (Olympus, IX50, Tokyo, Japan), they were trypsinized (Trypsin-EDTA 0.05%-0. 02%), diluted, and then seeded in 12- well tissue culture plates (Fisher Scientific, Atlanta, GA). On the day of the assay, fresh pre-warmed medium was added to wells. Overnight culture of *L. monocytogenes* strain F2365, *Lm*f2365Δ*ispG*, *Lm*f2365Δ*ispG*::pPL2-*ispG*, *Lm*f2365Δ*ispH*, and *Lm*f2365Δ*ispH*::pPL2-*ispH* were adjusted to OD_{600} 1.0, and approximately 4.5 X 10^7 CFU were add to each well to yield MOI of 50 to 1. After infection, the plates were centrifuged briefly for 45 s at 1,000 rpm and incubated at 37°C for 30 min. The free bacteria were eliminated by washing the cell layer five times with PBS (Sigma-Aldrich), washed Caco-2 monolayers were lysed with 0.5 ml of Triton X-100 0.5% for 10 min, and adherent bacteria were enumerated by plating on BHI agar and incubating for 48 h at 37°C.

2.6. Statistical analysis

Adhesion assays were repeated three times, and each treatment had four replicates. Visual assessment of histograms using PROC UNIVARIATE in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC) demonstrated bacterial counts were approximately normally distributed. Mixed model analysis of the generalized randomized complete block design was conducted using PROC MIXED in SAS for Windows 9.4 to determine the effect of strain on bacteria counts. The blocking effect of experiment and experiment by strain interaction were included as random effects. The Kenward-Roger method of calculating denominator degrees of freedom was used.

3. Results

3.1. Construction of the pHoss1 suicide plasmid

A novel 8,995 bp pHoss1 suicide plasmid was constructed. It contains a heat-sensitive origin of replication, four unique restriction sites (SalI, EcoRI, SmaI and NcoI), erythromycin resistance gene, and a 1,371 bp fragment encoding an antisense *secY* RNA expression cassette driven by an inducible Pxyl/tetO promoter (Fig. 1).

3.2. Construction of L. monocytogenes in-frame deletion mutants

Using the pHoss1 plasmid, we were able to construct two *L. monocytogenes* mutant strains, *Lm*f2365Δ*ispG* and *Lm*f2365Δ*ispH* (Fig. 2). Selection for the second allelic exchange was very efficient with pHoss1. Of the twenty potential *ispG* ATc-resistant mutant colonies screened by PCR, sixteen had deletion mutations, and four were wild type revertants. Of the nineteen potential *ispH* mutants screened, 100% were deletion mutants. Thus, at least 80% of the ATc-resistant colonies were deletion mutants, which confirms that antisense *secY* selection is very efficient in eliminating *L. monocytogenes* carrying the pHoss1 backbone. All positive mutants were also sensitive to Ery, confirming loss of the pHoss1 plasmid. The resulting *Lm*f2365Δ*ispG* strain contained a deletion of 1098 bp from the *ispG* gene (99% of the ORF), and the *Lm*f2365Δ*ispH* strain contained a deletion of 948 bp from the *ispH* gene (95% of the ORF).

3.3. Construction of complementation mutant strains

We introduced a copy of wild type *ispG* and *ispH* genes into the appropriate *L. monocytogenes* deletion mutants using the pPL2 integrative vector. Strains generated through

this approach are designated as *Lm*f2365Δ*ispG*::pPL2-*ispG* and *Lm*f2365Δ*ispH*::pPL2-*ispH* (Table 1).

3.4. Role of ispG and ispH in L. monocytogenes adhesion

*Lm*f2365Δ*ispG*::pPL2-*ispG* and *Lm*f2365Δ*ispH*::pPL2-*ispH* each had an approximately two-fold reduction in adhesion to Caco-2 cells compared to parent strain F2365. Complementation of the mutations restored adhesion properties. However, statistical analysis showed that there were no significant differences between the strains ($P < 0.05$), which indicates that neither *ispG* nor *ispH* genes of *L. monocytogenes* are involved in intestinal cell attachment.

4. Discussion

L. monocytogenes is an intracellular pathogen transmitted to humans and animals through the consumption of contaminated food. Clinical signs of listeriosis in healthy individuals include febrile gastroenteritis, whereas in immunocompromised individuals, listeriosis can be an invasive and systemic infection leading to sepsis, meningitis, and meningoencephalitis with a high mortality rate (25–30%) (Allerberger and Wagner, 2010). In addition, fetal infections cause spontaneous abortions, stillbirth, premature labor, and neonatal disease (Erdenlig et al., 1999). Understanding the function of genes in *Listeria* pathogenesis is essential to developing new control measures for listeriosis.

The main goal of this work was to develop an efficient suicide plasmid for production of allelic exchange mutants in Gram-positive bacteria, particularly in *Listeria monocytogenes*. Thus, we developed pHoss1, which features positive antibiotic selection markers for both the first and second allelic exchanges, resulting in an unmarked mutation on the chromosome.

pHoss1 is a modification of pMAD, which was established to facilitate construction of allelic replacement mutants in Gram-positive bacteria, including *S. aureus, L. monocytogenes,* and *B. cereus* (Arnaud et al., 2004). In our experience, when *L. monocytogenes* gene deletion constructs are cloned into pMAD, this plasmid is very efficient in transformation frequency by electroporation and insertion in the listerial chromosome (the first allelic exchange event). However, screening for the second allelic exchange event in *L. monocytogenes* with pMAD has much lower frequency. This screening depends on expression of *bgaB*, which allows bacteria to cleave the chromogenic substrate X-gal (5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside), thereby producing blue colonies. Although this technology affects neither the frequency nor selection of mutations, *bga* expression does provide a screening tool for presence or absence of plasmid. However, color development in *L. monocytogenes* required about 10 days in our laboratory, and it also resulted in many false positives.

Plasmids pIMAY and pKOR1 were used successfully for allelic replacement in *S. aureus* (Bae and Schneewind, 2006). These plasmids carry an antisense *secY* RNA under the control of an inducible Pxyl/tetO promoter, which allows positive selection for loss of the plasmid using ATc. SecY is part of the transmembrane component of the general Sec protein secretion system, which is highly conserved in Gram-positive and Gram-positive bacteria. In *Staphylococcus*, SecY protein is vital for growth and survival. Expression of *secY* antisense RNA, which inhibits *secY* expression, inhibited colony formation on agar plates (Ji et al., 2001). SecY has not been characterized in *L. monocytogenes*, but transmembrane components SecYEGDF are encoded in the genomes of *L. monocytogenes* strains EGDe (serotype 1/2a), F2365 (4b), F6854 (1/2a), and H7858 (4b) (Desvaux and Hebraud, 2006).

However, despite repeated electroporation attempts, when *L. monocytogenes* gene deletion constructs were cloned into pIMAY and pKOR1, we were unsuccessful in achieving insertion of these plasmids in the listerial chromosome (the first allelic exchange event). This result could be due to low transformation efficiency of these plasmids in *L. monocytogenes* or inefficient selection for plasmid insertion. Thus, we constructed pHoss1 by replacing the *bgaB* fragment in pMAD with an antisense *secY* RNA expression cassette under the control of an inducible Pxyl/tetO promoter. As we expected based on our experience with pMAD, we found that electroporation of pHoss1 carrying *ispG* and *ispH* deletions resulted in efficient plasmid integration into the listerial chromosome by allelic exchange. The Ery-resistant colonies showed the expected merodiploid genotype with both wild type and mutated alleles being amplifiable by PCR. We also found that selection for loss of the pHoss1 plasmid by a second allelic exchange event based on *secY* antisense expression was very efficient in *L. monocytogenes*, yielding about 80% frequency of deletion mutants from the ATc-resistant colonies. Our result confirmed that expression of *secY* is essential for *L. monocytogenes*, and 1.5 µg/ml ATc induced expression of antisense *secY* RNA, inhibiting growth of *L. monocytogenes* containing the pHoss1 plasmid. By comparison, in *Staphylococcus aureus*, ATc at a concentration of 1 µg/ml induced the expression of antisense *secY* RNA and suppressed the growth of bacteria containing the pKOR1 plasmid (Bae and Schneewind, 2006).

To assess the usefulness of pHoss1 in *L. monocytogenes*, we constructed in-frame deletions of the *ispG* and *ispH* genes, which are iron sulfate enzymes involved in isoprenoid biosynthesis via the mevalonate-independent 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. Because the enzymes of the MEP pathway are not found in humans, this pathway has been used recently as an anti-infective drug target of various bacterial infections and malaria

(Nakagawa et al., 2013; Obiol-Pardo et al., 2011). Additionally, both IspG and IspH have been associated with intracellular survival and induction of cellular immune responses in other bacterial pathogens (Heuston et al., 2012). Interestingly, nonpathogenic strains of *L. innocua* and *L. monocytogenes* do not possess these two genes (Begley et al., 2008; Steele et al., 2011). Thus, we hypothesized that *ispG* and *ispH* genes could be essential for *L. monocytogenes* pathogenesis, and we tested this by developing mutants and determining their attachment properties in human Caco-2 cells, a transformed intestinal epithelial cell line.

In the current study, we detected a decrease in adhesion of *L. monocytogenes* when the *ispH* and *ispG* genes were deleted; however, this difference was not statistically significant. In bacteria, there are two independent pathways to synthesize isoprenoids: the classical mevalonate pathway, or the alternative, non-mevalonate (MEP) pathway. The distribution of these two pathways is highly complex. *L. monocytogenes* is the only pathogen known to contain two complete pathways for isoprenoid biosynthesis (Begley et al., 2004). The genome sequence of *L. monocytogenes* strain F2365 confirms that it contains the intact genes of both pathways. Thus, it is possible that failure to detect a significant effect of IspG/H on intestinal cell adhesion is due to the listerial ability to synthesize isoprenoids via the alternative pathway. Previously, murine studies revealed that during intraperitoneal infection, *L. monocytogenes gcpE* and *lytB* (*ispG* and *ispH*) mutants were impaired in virulence relative to the parent strain. Also, a double *L. monocytogenes* pathway mutant lacking both the classical and alternative pathways was not recovered from the livers and spleens of mice three days post infection, suggesting that the in vivo mevalonate levels were not sufficient to allow growth of a strain deficient in both pathways (Begley et al., 2008; Begley et al., 2004). Thus, although our findings could not confirm a role

289 for isoprenoid biosynthesis in intestinal cell adhesion, these pathways are important during other
290 phase(s) in listerial pathogenesis.

291 In summary, we report for the first time a plasmid capable of efficient construction of
292 deletion mutations in *L. monocytogenes*. Deletion mutations have several advantages over
293 mutations that incorporate an inserted antibiotic resistance gene. First, insertion of an antibiotic
294 resistance gene may have a polar effect on the expression of downstream genes in an operon,
295 which would complicate the characterization of each gene's phenotype. Furthermore, integration
296 of an antibiotic resistance cassette onto the chromosome excludes this marker for further genetic
297 manipulation, making it more difficult to construct strains carrying more than one mutation.
298 Chromosomal insertion of antibiotic resistance genes also has ethical considerations, especially if
299 the bacterial strain will be released in the environment or used as a vaccine. In addition, antisense
300 *secY* RNA efficiently selects for plasmid loss in *Staphylococcus* (Bae and Schneewind, 2006).
301 Thus, it is expected that pHoss1 could be used successfully for construction of gene deletions in
302 *S. aureus, B. cereus*, and other Gram-positive species.
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370 **Table 1**

371 Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Description	Source/Reference
E. coli		
DH5α	Competent cells	Invitrogen
TOP10	Competent cells	Invitrogen
L. monocytogenes		
F2365	Wild-type serotype 4b strain	(Nelson et al., 2004)
*Lm*f2365Δ*ispG* mutant	F2365Δ*ispG* mutant strain	This study
*Lm*f2365Δ*ispH* mutant	F2365Δ*ispH* mutant strain	This study
*Lm*f2365Δ*ispG*::pPL2-*ispG*	F2365Δ*ispG*::pPL2-*ispG* complemented strain	This study
*Lm*f2365Δ*ispH*::pPL2-*ispH*	F2365Δ*ispH*::pPL2-*ispH* complemented strain	This study
Plasmids		
pMAD	9,666 bp, ori, Ery^r, Amp^r	(Arnaud et al., 2004)
pIMAY	5,743 bp, *secY* antisence, Chl^r	(Monk et al., 2012)
pPL2	6,123 bp, PSA *attPP*, Chl^r	(Lauer et al., 2002)
pHoss1	8,995 bp, pMAD, ::*secY* antisense, Δ*bgaB*	This study
*pLm*Δ*ispG*	pHoss1, ::Δ*ispG*	This study
*pLm*Δ*ispH*	pHoss1, ::Δ*ispH*	This study
pPL2-*ispG*	pPL2, ::*ispG*	This study
pPL2-*ispH*	pPL2, ::*ispH*	This study

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Table 2

Primers used to generate and verify in-frame deletion.

Primers		Sequence (5'→3')[b]	RE[a]
Lm-ispG-F01	A	AA**GTCGAC**TAGCCTACCATGCTCCTGAAA	SalI
Lm-ispG-R847	B	CATAGAGACCGCTCCTTTAG	
Lm-ispG-F801	C	**CTAAAGGAGCGGTCTCTATG**AGATAATCGTATCGGGGTTT	
Lm-ispG-R01	D	AA**CCATGG**ATGGTAGGAAGTGATGCGAGT	NcoI
Lm-ispH-F01	A	AA**GTCGAC**CGCTAAATAAGGCTGTGAACC	SalI
Lm-ispH-R900	B	TCCGTAGCAATAACCACGAG	
Lm-ispH-F900	C	**CTCGTGGTTATTGCTACGGA**GCTAAAAACCGAGCAACTCCT	
Lm-ispH-R01	D	AA**CCATGG**TCCGTTTCTATATCGGCCAAC	NcoI
Anti_secY F01		AA**GAGGAG**GATCTAATGATTCAAACCCTTGTG	BseRI
Anti_secY R01		AA**AGATCT**TGAAGTTACCATCACGGAAAAAGG	BglII
Lm-ispH-comp-F01		AAA**GAGCTC**TGAGGATGTTTTCGAATGGA	SacI
Lm-ispH-comp-R01		AA**GTCGAC**ACAAAGGAGTTGCTCGGTTTTT	SalI
Lm-ispG-comp-F01		AAA**GAGCTC**GGAGCGGTCTCTTTGAATG	SacI
Lm-ispG-comp-R01		AA**GTCGAC**GGCTTTCCAAATCTGTTTTCTTT	SalI

[a]RE stands for restriction enzyme added to the 5' end of the primer sequence.

[b]Bold letters at the 5' end of the primer sequence represent RE site added. AA or AAA nucleotides were added to the end of each primer containing a RE site. Underlined bases in primer C indicate reverse complemented primer B sequences.

Figure legends

Fig. 1. Construction of the suicide plasmid pHoss1. pHoss1 is derived from pMAD by replacing the β-galactosidase gene (*bgaB*) with tetracycline-inducible antisense *secY* gene (anti-*secY*) amplified from pIMAY. *ermC*, Ery resistance gene; *bla*, beta-lactamase; ori, pBR322 origin of replication.

Fig. 2. PCR verification of Δ*ispG* and Δ*ispH* deletion using A and D primers. Size of 1 Kb Plus DNA Ladder (Life Technologies) bands are indicated on the left. WT indicates PCR fragment amplified from wild type *L. monocytogenes* F2365. Numbers at the top are ATc-resistant listerial colonies picked randomly for PCR screening. 16/20 (80%) of *ispG* and 19/19 (100%) of *ispH* colonies showed the gene deletion fragment only. In *ispG*, four colonies showed wild type revertants or merodiploids (lanes 1, 4, 10, and 12).

Fig. 3. Adhesion of wild type *L. monocytogenes* strain F2365, mutant strains, and complemented mutant strains to human intestinal cell line Caco-2. Numbers on the Y axis indicate bacterial numbers (CFU/ml). Statistical analysis did not show any significant differences ($P < 0.05$).

401 **Fig. 1.**

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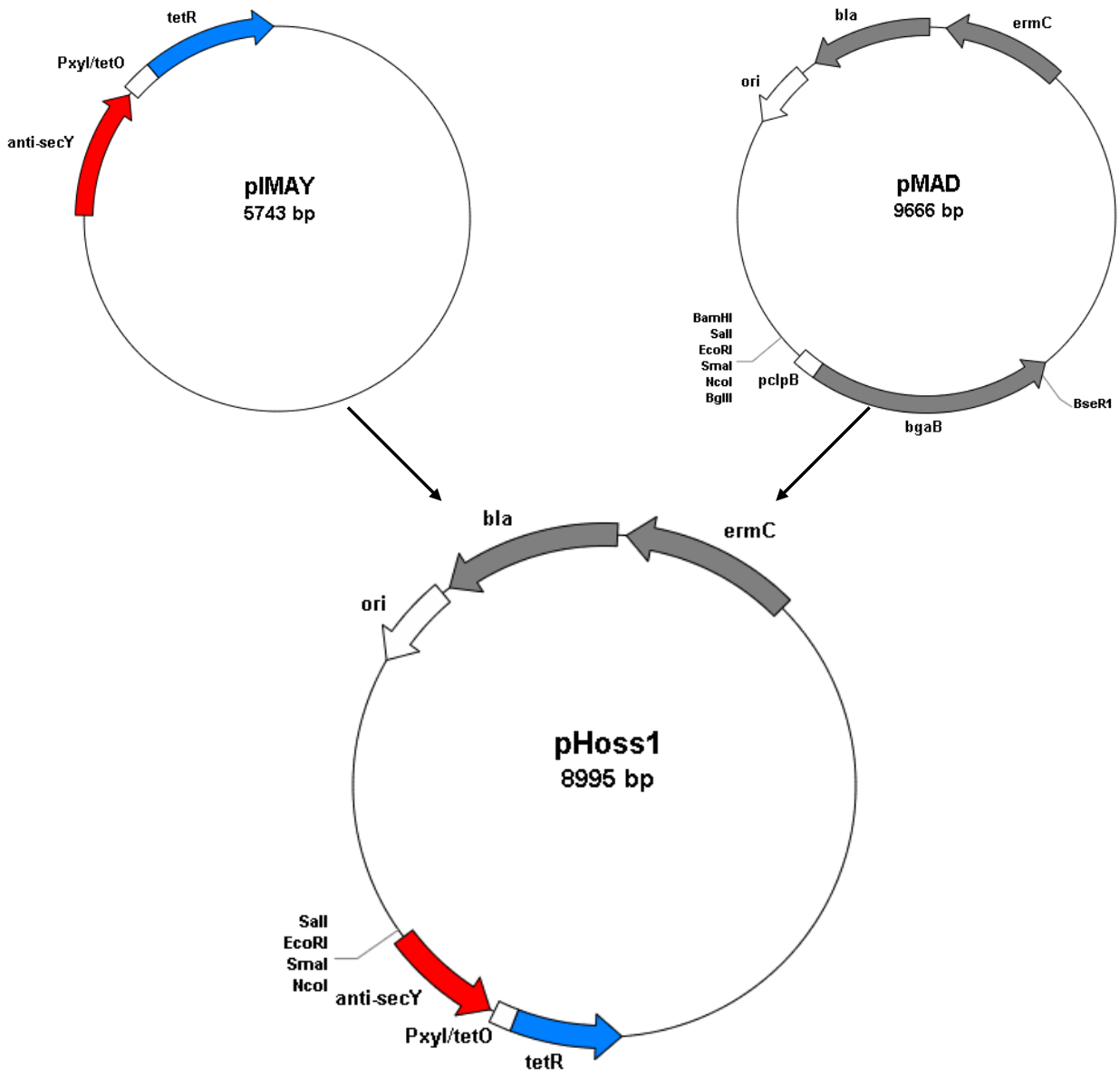

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406 **Fig. 2.**

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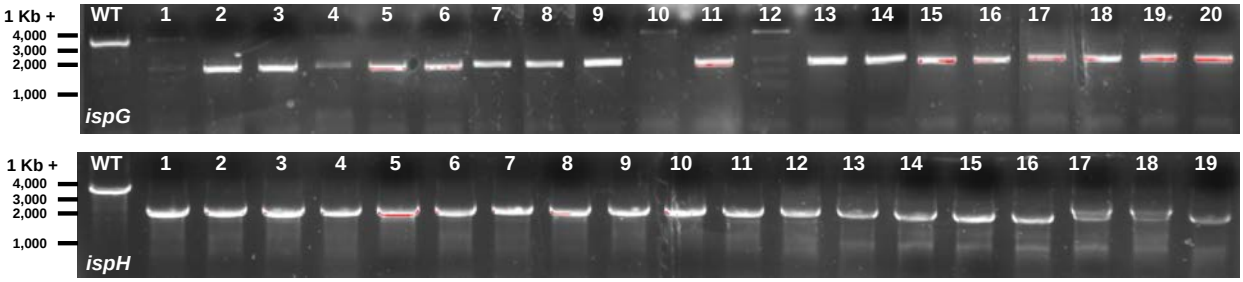

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411 **Fig. 3.**

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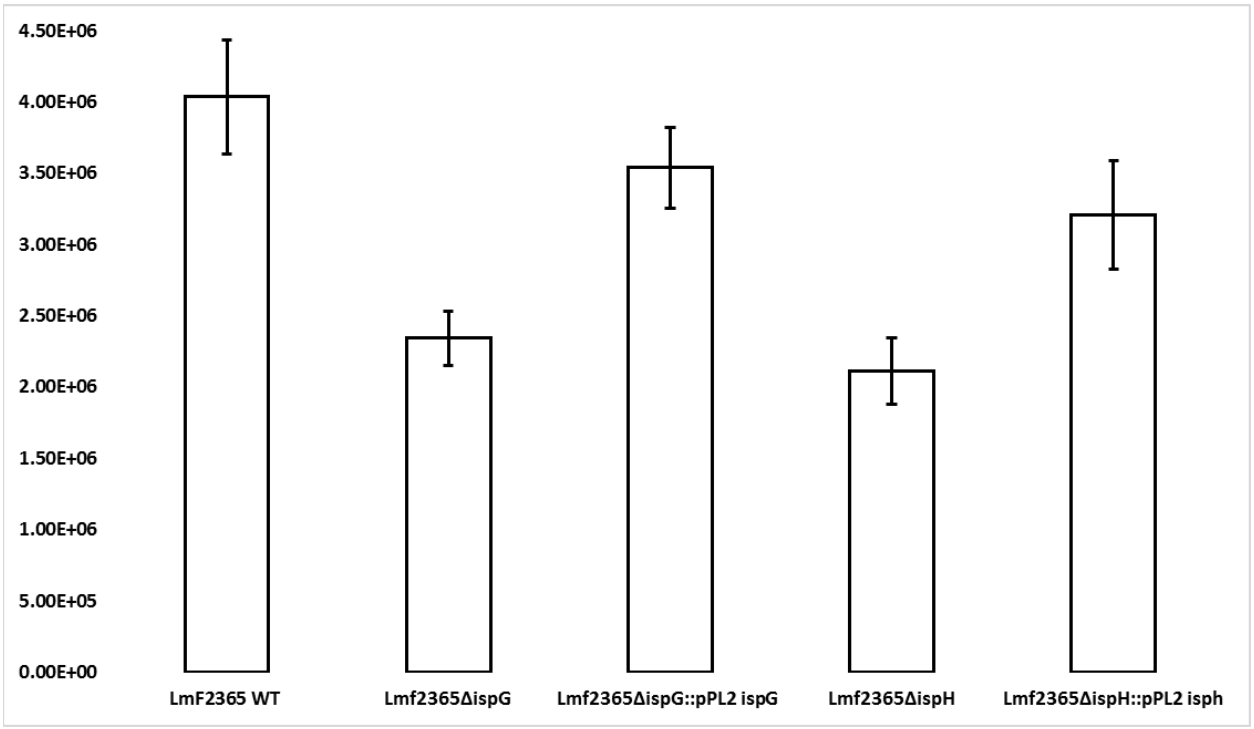

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