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Master's project in Life Sciences and Technology

Mechanism of DegU-dependent activation of flagellar gene transcription in *Listeria monocytogenes*

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1. SUMMARY

Flagellar motility is an important process for bacterial survival. It allows bacteria to move to aquire nutrients or to retreat from unfavorable environments. In the Gram-positive bacterium *Listeria monocytogenes*, flagellar motility is a temperature-dependent process, being restricted to temperatures below 37°C. This regulation is mediated by the activities of the DegU response regulator, the MogR transcriptional repressor and GmaR, the MogR anti-repressor. Transcriptional regulation of the *fliN-gmaR* promoter is central to flagellar motility regulation as p*fliN-gmaR* is transcriptionally activated by DegU and is also MogR repressed. However, the mechanism by which DegU mediates transcriptional activation of the *fliN-gmaR* promoter is not well understood. In this study, we further investigated the mechanisms controlling transcription initiating at the *fliN-gmaR* promoter by performing two comprehensive transposon mutagenesis screens to identify any additional factors involved in the activation and repression of p*fliN-gmaR*. The mutagenesis screen performed at 30°C to identify factors involved in p*fliN-gmaR* transcriptional activation led to the identification of a novel factor. We demonstrated that Lmo0866, a protein from the DEAD-box protein family, is required for transcriptional activation of p*fliN-gmaR* and therefore flagellar motility. Moreover, we identified CodY as being involved in the repression of p*fliN-gmaR* at 37°C.

2. INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium and a facultative intracellular pathogen. *L. monocytogenes* is the causative organism of the foodborne disease listeriosis, characterized by gastroenteritis, septicemia, meningitis and encephalitis, that can lead to fatal outcomes in immunocompromised individuals, pregnant women, newborns and the elderly (1).

L. monocytogenes can survive and multiply both inside and outside the host, including in soil, unpasteurized dairy products and processed food. *L. monocytogenes* has therefore evolved mechanisms that enable rapid adaptation and survival in a wide range of environments and temperatures ranging from 3°C to 43°C. Once inside the host, environmental signals sensed by *L. monocytogenes* lead to the upregulation of virulence factors and downregulation of other genes, such as flagellar genes, that are unnecessary or impede survival in the host environment.

Flagellar motility is the primary mechanism enabling bacteria to move in the extracellular environment and is widely distributed throughout bacterial species. It enables bacteria to move towards or away from environmental cues monitored by a chemotactic signal-transduction system controlling the direction of flagellar rotation. The bacterial flagellum is a complex self-assembling organelle consisting of three substructures: a membrane-bound basal body which anchors the structure in the cell envelope and contains the ion-powered rotary motor, an extracellular helical filament measuring approximately 20 nm in diameter that acts as the propeller and extends many cell lengths from the cell and a flexible hook connecting the basal body and the filament (2). Flagella from Gram-positive and Gram-negative bacteria are essentially identical, except that flagella from Gram-negative bacteria extend through the outer-membrane absent in Gram-positive bacteria. Counter-clockwise rotation of the flagella

propels the cell forward at speeds of 15-100 μm per second (3). A quick reversal to clockwise rotation induces tumbling which allows reorientation of the cell and swimming in a new direction when the motor returns to counter-clockwise rotation. Change in direction occurs approximately once a second in homogeneous environments resulting in random movement. In non-homogeneous environments the chemotactic signal-transduction system biases the overall direction of movement by decreasing the tumbling frequency.

Flagellar motility is important for bacterial survival as it enables bacteria to move to acquire nutrients or retreat from unfavorable environments (4), to colonize surfaces, and is important to establish infection. In *L. monocytogenes*, flagellar motility has been shown to increase abiotic and cellular adherence (5), is essential for biofilm formation (6) and enhances cellular invasion (7). However, in response to temperature (37°C and above) and other environmental signals, such as the host cell cytosol, flagellar motility genes are repressed (8). Indeed, flagella recognition leads to activation of host innate immune responses (9).

Flagella production is a very energy-demanding process that requires the coordinate activation of more than 40 genes and assembly of thousands of interacting proteins (10). The regulation of bacterial flagella production has been studied in different bacterial species and appears to be regulated at multiple levels in a hierarchal manner (10). Interestingly, regulation of flagellar motility in *L. monocytogenes* differs significantly from other bacterial species as conserved master regulators involved in the transcriptional hierarchal regulation of flagellar production are absent in *L. monocytogenes* (11).

The temperature-dependent regulation of flagellar motility in *L. monocytogenes* involves three key players, MogR (12), a transcriptional repressor, GmaR (13), the

MogR anti-repressor, and DegU (14), a response regulator (**Fig. 1**). At temperatures of 37°C and above, MogR binds to flagellar gene promoters to directly repress flagellar gene transcription (11). Transcription of the first operon of the flagellar motility gene cluster (*fliN-gmaR*) is activated by the DegU response regulator and is also repressed by MogR. The *fliN-gmaR* operon is of principal importance for flagellar gene regulation as it encodes the MogR anti-repressor GmaR (13). At non-permissive temperatures (37°C and above) the reciprocal activities of MogR and DegU lead to low basal production of *fliN-gmaR* transcripts, but a secondary post-transcriptional mechanism facilitates degradation of GmaR (15). As temperature decreases, GmaR undergoes a conformational change preventing its degradation (Kamp and Higgins, unpublished data). Therefore, low levels of GmaR can now bind MogR removing MogR from p*fliN-gmaR* leading to increased transcription of *gmaR*. This results in elevated levels of GmaR relieving MogR repression from all flagellar motility genes allowing production of flagella. Therefore, transcriptional and post-transcriptional regulation of the GmaR anti-repressor governs temperature-dependent control of flagellar motility in *L. monocytogenes.* However, the mechanism by which DegU mediates transcriptional activation of the *fliN-gmaR* promoter is not well understood.

DegU is an orphan two-component system response regulator as no cognate histidine kinase has been identified in the *L. monocytogenes* genome. DegU is composed of two distinct domains, a phosphorylation receiver domain and a LuxR family helix-turn-helix DNA binding domain. It shares significant homology with *Bacillus subtilis* (*Bs)* DegU as they are 63% identical and 78% similar (15). Interestingly, *Bs*DegU is also required for flagellar motility gene activation in *B. subtilis,* but activation or repression of flagellar genes is dependent on the state of phosphorylation of the *Bs*DegU receiver domain, which is phosphorylated by the cognate histidine sensor kinase *Bs*DegS (16).
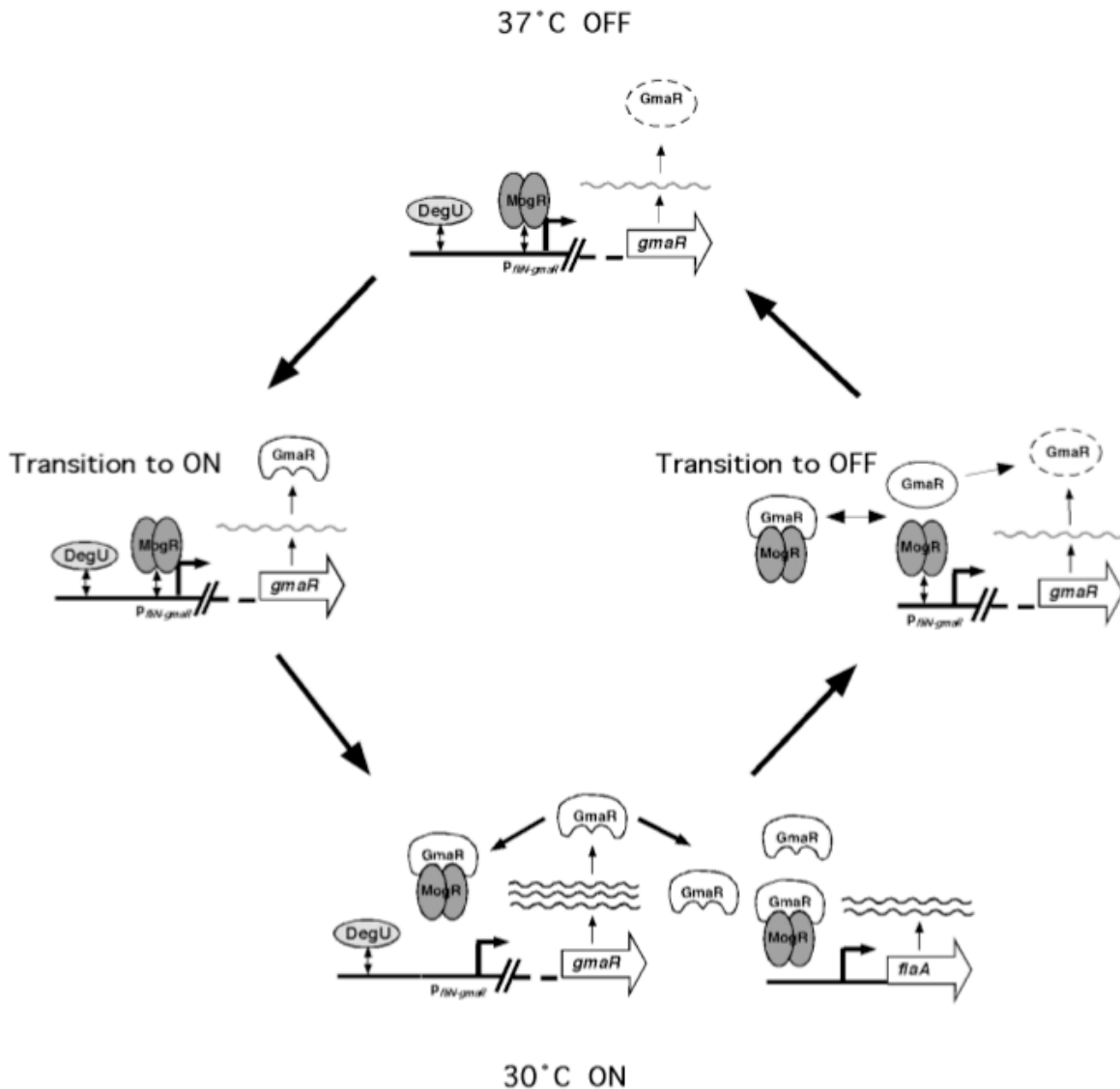
Figure 1: Temperature-dependent regulation of flagellar motility in *L. monocytogenes*

37°C OFF: MogR binds to flagellar gene promoters to directly repress flagellar gene transcription. The reciprocal activities of MogR and DegU at p*fliN-gmaR* lead to low basal production of *fliN-gmaR* transcripts, but GmaR is rapidly degraded and cannot interact with MogR.
Transition to ON: As temperature decreases GmaR is no longer degraded and able to interact with MogR.
30°C ON: GmaR removes MogR from p*fliN-gmaR* leading to increased transcription of *gmaR*, this results in elevated levels of GmaR relieving MogR repression from all flagellar motility genes.
Transition to OFF: At temperature increases the GmaR::MogR complex is destabilized due to a conformational change in GmaR. MogR is released and can repress transcription of all flagellar genes

DegU in *L. monocytogenes* has been shown to activate the *fliN-gmaR* promoter (15) and to bind and repress its own promoter (17). Protein levels of DegU have been shown to be temperature-independent (13). In addition, activation of the *fliN-gmaR* promoter by DegU is also temperature-independent, as quantitative RT-PCR experiments in a Δ*mogR* strain indicate that *fliN-gmaR* transcripts are produced at both 30°C and 37°C (15). It has not been determined if DegU is phosphorylated in *L. monocytogenes*, but *in vitro* experiments have shown that *Bs*DegS can phosphorylate DegU with even higher efficiency than *Bs*DegU. However, DegU containing a mutation in the phosphorylation acceptor site is still able to activate *fliN-gmaR* transcription in *L. monocytogenes*, indicating that DegU does not require phosphorylation to activate transcription of the *fliN-gmaR* promoter (15). DegU binds the *fliN-gmaR* promoter region independently of other factors and DNase I footprinting analysis has identified a unique binding region at position -154 to -184 nt upstream of the *fliN-gmaR* transcription start site (15). Furthermore, electrophoretic mobility shift analysis suggests that DegU may bind as a multimer, as a super-shifted complex at higher concentration of DegU was observed (15). Interestingly, the N-terminal portion of DegU containing the phosphorylation receiver domain was shown to be dispensable for activation of the *fliN-gmaR* promoter (15). Despite sharing significant homology with DegU, *Bs*DegU was not able to complement activation of *fliN-gmaR* in a *L. monocytogenes degU* mutant (15), underlying the differences existing between the two DegU proteins.

DegU is necessary for transcriptional activation at the *fliN-gmaR* promoter (p*fliN-gmaR*) however it is not known if it is sufficient. The DegU binding site within the *fliN-gmaR* promoter region is located far upstream of the transcriptional start site, leading to the question of whether DegU directly activates transcription of p*fliN-gmaR*. Activators typically bind close to the -35 region, but binding sites located at approximately -150 nt have also been described in bacteria (18). DegU binding may change the conformation

of promoter region DNA or DegU could induce DNA bending and directly contact RNA polymerase. Alternatively, DegU may be required for recruitment of a coactivator.

The goal of this project was to further characterize transcriptional regulation occurring at the *fliN-gmaR* promoter and to determine if DegU is sufficient for transcriptional activation of p*fliN-gmaR* or if additional bacterial factors are required. In this study, we first intended to determine if DegU was sufficient to activate transcription of p*fliN-gmaR* in *B. subtilis*, therefore using *B. subtilis* as a surrogate host bacterium. Furthermore, to fully characterize regulation of the *fliN-gmaR* promoter, we generated a mariner transposon mutant library in a p*fliN-gmaR::gusA* reporter strain and performed comprehensive library screens at 30°C and 37°C to identify any additional factors involved in regulation of the *fliN-gmaR* promoter. Here, we report the identification of a novel factor required for p*fliN-gmaR* transcriptional activation.

3. MATERIALS AND METHODS

Bacterial strains and culture conditions

Listeria monocytogenes, *Escherichia coli* and *Bacillus Subtilis* strains used in this study are listed in **Table 1**, **Table 2** and **Table 3**, respectively. Oligonucleotides used in this study are listed in **Table 4**. All *L. monocytogenes* strains are in the EGDe background and were grown in Brain Heart Infusion (BHI) broth. *E. coli* strains were grown in Luria-Bertani (LB) media for plasmid isolation. *B. subtilis* strains were grown in LB media. All bacterial stocks were stored at -80°C in LB or BHI supplemented with 33% glycerol. Antibiotics were used in the following concentrations: chloramphenicol at 20 µg/ml for selection of pPL3 derivatives in *E. coli*, 7.5 µg/ml for selection of integrated pPL3 derivatives and pAM401 vectors in *L. monocytogenes*. 30 µg/ml kanamycin for selection pIMK vectors in *E. coli* and for selection of integrated pIMK derivatives in *L. monocytogenes*. 100 µg/ml carbenicillin for selection of pDG1663 derivatives and pDR110a amyE-pSpank derivatives in *E. coli*. 100 µg/ml spectinomycin for selection of integrated pDR110a amyE-pSpank derivatives in *B. subtilis*, and 5 µg/ml erythromycin for selection of integrated mariner transposon in *L. monocytogenes* and pDG1663 derivatives in *B. subtilis*.

All plasmid constructs were confirmed by DNA sequencing. Plasmids were isolated from strains XL1-Blue and electroporated directly into *L. monocytogenes* or transformed into SM10 for conjugative transfer in *L. monocytogenes*.

Table 1 *Listeria monocytogenes* strains used in this study

Strain	Genotype and relevant features	Strain designation	Reference
DH-L478	wild-type strain EGDe	Wild-type	
DH-L1056	*gmaR* in-frame deletion in EGDe	Δ*gmaR*	(13)
DH-L1156	*mogR* in-frame deletion in EGDe	Δ*mogR*	(12)
DH-L1273	*degU* in-frame deletion in EGDe	Δ*degU*	(11)

DH-L1339	$D_{55}N$ point mutation in *degU* in EGDe	*Lm*DegU $D_{55}N$	(15)
DH-L1867	pPL3-p$_{fliN}$gusA-278 in Δ*mogR*	Δ*mogR*/p$_{fliN}$gusA-278	(15)
LM1	pIMK-p$_{fliN}$gusA-278 in EGDe	pIMK-p$_{fliN}$gusA-278	This study
LM2	pPL3-p$_{fliN}$gusA-278 in Δ*degU*	Δ*degU*/p$_{fliN}$gusA-278	This study
LM3	*Himar1* transposon generated library in LM1		This study
LM4	Transposon insertion into *lmo0866* LM1	*lmo0866*/pIMK-p$_{fliN}$gusA-278	This study
LM5	Transposon insertion into *lmo0866* in DH-L478	*lmo0866*/ wt EGDe	This study
LM6	Transposon insertion into *lmo1280* in LM1	*codY*/ pIMK-p$_{fliN}$gusA-278	This study
LM7	pAM401-*lmo0866* in LM4	*lmo0866*/pAM401-*lmo0866*	This study
LM8	pAM401 empty in LM4	*lmo0866*/pAM401empty	This study
LM9	pPL3-*lmo0866* in LM5	*lmo0866*/pPL3-*lmo0866*	This study

Table 2 *Escherichia coli* strains used in this study

Strains	Genotype and relevant features	Reference
DH-E182	XL1-Blue {F' *proAB lacI^q Δ(lacZ)*M15 Tn10} *RecA1 endA1 gyrA96 thi-1 hsdR17 supE relA1 lac*	Stratagene
DH-E474	SM10	
DH-E898	pPL3 in XL1-Blue	(12)
DH-E476	pAM401 in 1855	
DH-E1934	pIMK in DH10B	(21)
	pJZ037 in DH5 alpha	(19)
DH-E1829	pPL3-p$_{fliN}$gusA-278 in XL1-Blue	(15)
DH-E1833	pPL3-p$_{fliN}$gusA-278 in SM10	(15)
DH-E1564	pDG1663 in DH5 alpha	(32)
DH-E1565	pDR110a amyE-Pspank in DH5 alpha	(32)
EC1	pIMK-p$_{fliN}$gusA-278 in XL1-Blue	This study
EC2	pAM401-*lmo0866* in XL1-Blue	This study
EC3	pPL3-*lmo0866* in XL1-Blue	This study
EC4	pDG1663-p$_{fliN}$lacZ in XL1-Blue	This study
EC5	pDR110a-*degU* in XL1-Blue	This study

EC6	pDR110a-*degU* $D_{55}N$ in XL1-Blue	This study

Table 3 *Bacillus subtilis* strains used in this study

Strains	Genotype and relevant features	Reference
bDR124	*thrC*::*cat* in *B. subtilis* PY79	(32)
bDR993	*amyE::*pHyper-spac-*lacZ* in *B. subtilis* PY79	
BS1	*thrC::p$_{fliN}$lacZ* in bDR124	This study
BS2	*amyE::*pSpank-*degU* in BS1	This study
BS3	*amyE::*pSpank empty in BS1	This study
BS4	*amyE::*pSpank-*degU*$D_{55}N$ in BS1	This study

Table 4 Oligonucleotides used in this study

Number	Sequence	Site[a]
P1	AGATAC<u>AAGCTT</u>TGAAGGAGGAGTAGTCATTATGGCAC	HindIII
P2	AGATAC<u>GCATGC</u>TAGTCCCCCTGAGATTTCTTTAGCG	SphI
P3	AGATAC<u>GAATTC</u>GGCATACAATCACATACCTCTCTATC	EcoRI
P4	AGATAC<u>GGATCC</u>CTTTCACTCCCTTCATCAGATTAC	BamHI
P5	AGATAC<u>GGATCC</u>TGTATGGGACTAATAAAAAGCTGG	BamHI
P6	AGATAC<u>GTCGAC</u>AACTAGCACCACACTCCCGTATC	SalI
P7	GGCCACGCGTCGACTAGTACNNNNNNNNNNGTAAT	
P8	GCAATGAAACACGCCAAAGTAAAC	
P9	GGCCACGCGTCGACTAGTAC	
P10	CGCCTACGGGGAATTTGTATC	

a. The indicated restriction endonuclease site is underlined within the oligonucleotide sequence

Strain construction

A pIMK-p$_{fliN}$gusA-278 *L. monocytogenes* strain was generated and used as strain

background for the mariner transposon mutant library. To create the pIMK-p$_{fliN}$gusA-278

L. monocytogenes strain plasmid pPL3-p*fliN*gusA-278 (DH-E1829) was digested with *EagI* and *SalI* and gel purified. The p*fliN*gusA-278 insert was ligated to plasmid pIMK digested with the same restriction enzymes and transformed into XL1-Blue to create strain EC1. The resulting plasmid was introduced into wild-type EGDe (DH-L478) by electroporation. Strain LM2 was obtained by mating SM10 strain DH-E1833 containing the pPL3-p*fliN*gusA-278 plasmid with *L. monocytogenes* Δ*degU* strain (DH-L1273).

To generate the complementation constructs for the transposon insertion into *lmo0866* primer pair P5 and P6 were used with EGDe genomic DNA to amplify the 2 kb *lmo0866* gene region. It comprises the *lmo0866* open reading frame and 500 bp of intergenic sequence upstream of the *lmo0866* start codon containing the putative *lmo0866* native promoter. The 2 kb PCR product was gel purified and digested with *BamHI* and *SalI* and ligated to plasmids pAM401 and pPL3 digested with the same restriction enzymes and transformed to XL1-Blue to generate strain EC2 and EC3, respectively. The resulting plasmids were sequenced verified and electroporated into strains LM4 and LM5 to create LM7 and LM9, respectively.

*B. subtilis thrC::*p*fliN*lacZ strain was constructed by PCR amplification of the *fliN* promoter sequence from EGDe genomic DNA with primer pair P3 and P4. The resulting PCR product was gel purified and digested with *BamHI* and *EcoRI,* ligated into pDG1663 digested with the same restriction enzymes and transformed into XL1-Blue yielding strain EC4. The resulting plasmid was sequenced and transformed into bDR124 generating strain BS1. Transformants were restreaked onto chloramphenicol LB plates to select for double cross-over recombination events at the *thrC* locus. To construct pDR110a-pSpank-*degU* and pDR110a-pSpank-*degU*D_{55}N, the *degU* coding sequence was amplified with primer pair P1 and P2 from EGDe genomic DNA or from genomic DNA isolated from strain DH-L1339 containing the D_{55}N *L. monocytogenes* DegU point mutant. The resulting PCR products were digested with *HindIII* and *SphI* and ligated to

plasmid pDR110a digested with the same restriction enzymes. The ligation reactions were transformed into XL1-Blue generating strain EC5 and EC6, respectively. The resulting plasmids were sequenced and transformed into strain BS1 yielding strain BS2 and BS4. Double cross-over events were selected by screening for α-amylase deficient clones grown on 1% starch-containing plates and flooded with iodine solution.

X-gal/IPTG plate assay

Single colonies were inoculated in 3 ml of LB medium and incubated at 37 °C for 6 hours. Dilutions were plated on either 1 mM IPTG/X-gal LB plates or X-gal LB plates and incubated 16 hours at 37 °C. X-gal was used in plates at a concentration of 40 μg/ml. Color of colonies was then visually assessed.

Beta-glucuronidase assay

Single colonies of *L. monocytogenes* strains were inoculated in 3 ml of BHI medium plus appropriate antibiotics and incubated for 20 hr without shaking at the indicated temperatures. OD_{600} readings were taken to normalize β-glucuronidase activity for bacterial density. Bacteria were pelleted in 1.5-ml aliquots at 13,000 rpm for 10 min and resuspended in 150 μl ABT buffer (60 mM K_2HPO_4/40 mM KH_2PO_4/100 mM NaCl/0.1% Triton X-100, pH 7.0, filter sterilized). Reactions were set up in a 96-well plate format and done in triplicate by mixing 10 μl of GUS substrate (0.4 mg/ml 4-methyl-umbelliferyl-β-D-glucuronide in DMSO) and 50 μl of sample using ABT buffer as control. After incubation for 60 minutes at room temperature in the dark, 20 μl from each reaction were removed and diluted into 180 μl of ABT buffer in a black 96-well plate and fluorescence values were determined by using a SpectraMAX GeminiXS instrument (Molecular Devices) at excitation and emission wavelengths of 366 and 445 nm, respectively. Known concentrations of the fluorescent 4-methylumbelliferone (MU) product ranging from 25 to 4000 pmol were used to obtain a standard curve. Units were calculated as (picomoles of substrate hydrolyzed × dilution factor)/(ml culture in final

sample × OD_{600} x minute) where pmol of substrate hydrolyzed is calculated from the standard curve as (emission reading – y intercept)/(slope). A student t-test was performed for statistical analysis.

Western blot analysis
Colonies from *B. subtilis* strain BS2 were scraped from the agar plates, resuspended in 100 ml TE buffer with 5 mg/ml lysozyme and incubated at 37°C for 1 hour. An equal volume of 2X FSB was added to the supernatant and samples were boiled for 5 min at 95°C and centrifuged at 13,000 rpm for 1 min. 15 µl was loaded on a 12% SDS-PAGE gel and a Western blot was performed as previously described (11) using a polyclonal antibody for DegU.

BHI medium supplemented with appropriate antibiotics was inoculated with *L. monocytogenes* overnight culture and incubated for 6 h at the indicated temperature or until cultures reach mid-exponential phase. A culture volume equivalent of 1 ml of OD_{600}= 1.5 was pelleted and resuspended in 75 µl of TE/lysozyme (10 mM Tris-HCL pH 8.0, 1 mM EDTA, 5mg/ml lysozyme) and incubated for 1h at 37°C. 5X FSB was added to yield a 1X final concentration, samples were boiled for 5 min at 95°C and then centrifuged for 1 min at 13,000 rpm. 15 µl was loaded on a 12% SDS-PAGE gel for analysis of DegU and MogR and western blots were performed as previously described (11) using polyclonal antibodies specific for DegU or MogR.

Generation of a mariner transposon mutant library
Electrocompetent *L. monocytogenes* LM1 cells were prepared following repeated treatments with 1mM Hepes/0.5 M sucrose. Cells were grown in vegetable peptone broth instead of BHI to increase electroporation efficiency. Approximately 1 µg of pJZ037 was used to electroporate each 50-µl aliquot of electrocompetent cells. After electroporation 1ml of BHI-0.5 M sucrose was added to cells to allow recovery for 1 hr at

30°C. Cells were then plated over approximately 19 100-mm BHI 5 µg/ml erythromycin agar plates. Plates were incubated for 60 hr at 30°C the permissive temperature and then replica-plated onto BHI 5 µg/ml erythromycin plates and incubated at 42°C, the non-permissive temperature, to cure the plasmid. Colonies were counted, scraped and resuspended in BHI 5 ug/ml erythromycin 40% glycerol for storage at -80°C. To test for plasmid curing efficiency, 10-fold serial dilutions were prepared from a frozen aliquot of the library and plated onto BHI 5 µg/ml erythromycin (the resistance marker carried by the transposon) and onto BHI 7.5 µg/ml chloramphenicol plates (the resistance marker carried by the delivery vector).

Screening

Screening was performed on BHI kanamycin 30 µg/ml, erythromycin 5 µg/ml, X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) 50 µg/ml 245 mm X 245 mm plates (Corning). Dilutions from two different frozen aliquots of the library were made to reach a concentration of approximately 1500 bacteria per ml and 3 ml were plated on each plate. Plates were incubated at the indicated temperatures for 60 h and putative mutants visually looking white or less blue were picked and restreaked on BHI kanamycin 30 µg/ml, erythromycin 5 µg/ml, X-gluc 50 µg/ml to confirm phenotype. Motility phenotype was then assessed for mutants (see below) and frozen stocks of each mutant were stored at -80°C.

Identification of transposon insertion sites

Identification of transposon insertion sites was performed through semi-arbitrary PCR of a mutant colony. The first PCR from a mutant colony uses primer pair P7 and P8. Primer pair P9 and P10 is used in the second round of PCR with1 µl from the first PCR reaction used as template. The PCR products were purified and sequenced with primer P10.

Phage Transduction

L. monocytogenes bacteriophage P35 was used to transduce the mariner transposon. Bacteriophages (10^7 and 10^8 pfu) grown on the appropriate donor strains were mixed with 10^8 of mid-exponential phase recipient cells and incubated at RT for 40 min. Cells were centrifuged for 5 min at 13,000 rpm and resuspended in 1 ml of BHI medium containing 10 mM sodium citrate pH 7.5 and 0.04 µg/ml erythromycin and incubated for 2 hrs at 37°C. Cells were pelleted and resuspended in 100 µl supernatant and plated on BHI 10 mM sodium citrate 5 µg/ml erythromycin and incubated at 37 °C for 48 h.

Motility assay analysis

A single colony was inoculated with a small pipet tip into a low agar content BHI plate (0.375% agar). Plates were inoculated at 30°C for 48 h and then photographed or scanned to evaluate size of swimming area.

Transmission electron microscope

A single colony was inoculated in 2 ml BHI and grown for 14 hr at 30°C without shaking. 5µl of the culture was adsorbed for 1 minute to a carbon coated grid that had been made hydrophilic by a 30 second exposure to a glow discharge. Excess liquid was removed with a filterpaper (Whatman #1) and the samples were stained with 0.75% uranyl formate for 30 seconds. After removing the excess uranyl formate with a filterpaper the grids were examined in a TecnaiG² Spirit BioTWIN transmission electron microscope and images were recorded with an AMT 2k CCD camera.

Growth curve

A single colony was inoculated in 2 ml BHI and grown for 16 hr at 30°C without shaking. It was back-diluted 1:40 in 30 ml of BHI medium and grown shaking at the indicated temperature. OD_{600} readings from three independent cultures were taken at regular intervals until cultures reached stationary phase.

4.RESULTS

4.1 pfliN-gmaR activation by DegU in B. subtilis

To determine if activation of p*fliN-gmaR* can occur in *B. subtilis,* the *L. monocytogenes degU* gene was cloned under the control of the pSpank inducible promoter in the pDR110a plasmid, which also contains a constitutively expressed *lacI* repressor gene. The *fliN-gmaR* promoter region DNA was cloned upstream of a *lacZ* reporter gene in the pDG1663 plasmid, and both vectors were integrated into the *B. subtilis* genome. Upon induction with IPTG, DegU is produced and can bind the *fliN-gmaR* promoter region. Transcriptional activation was monitored by beta-galactosidase activity using X-gal plates. The use of *B. subtilis* to analyze activation of p*fliN-gmaR in vivo* has several advantages. It allows the use of an *in vivo* system closely related to *L. monocytogenes*. Moreover, transcriptional activation can easily be monitored in an *in vivo* environment by utilizing the *B. subtilis* transcription and translation machinery.

B. subtilis strain BS2, containing the pSpank::*degU* and p*fliN-gmaR*::*lacZ* constructs, was plated on LB agar plates containing 1 mM IPTG/X-gal or on LB plates with X-gal alone and incubated 16 hours at 37°C. *B. subtilis* colonies on the 1 mM IPTG/X-gal plates should be blue if p*fliN-gmaR* is activated by DegU, and colonies on the X-gal LB plates should remain white as no DegU is produced. A *B. subtilis* strain containing a strong IPTG inducible promoter upstream of the *lacZ* gene (pHyper-spac::*lacZ*) was used as a positive control for the assay. The results obtained are summarized in **Table 5**. The presence of DegU in strain BS2 plated on 1 mM IPTG/X-gal plates was confirmed by Western blot (**Fig. 2**). We can see a band corresponding to DegU in the well loaded with sample from BS2 strain colonies scraped from 1 mM IPTG/X-gal plates. The upper band present in both BS2 samples is a non-specific band.

Table 5: X-gal/IPTG plate assay

Strain number	Strain	1mM IPTG / X-gal	X-gal
BS1	p*fliN-gmaR::lacZ*	blue	blue
BS2	pSpank::*LmdegU* + p*fliN-gmaR::lacZ*	white	blue
BS4	pSpank::*LmdegUD$_{55}$N* + p*fliN-gmaR::lacZ*	white	blue
BS3	pDR110a empty + p*fliN-gmaR::lacZ*	blue	blue
bdr993	pHyper-spac::*lacZ*	blue	white

X-gal plate   X-gal/1mM IPTG

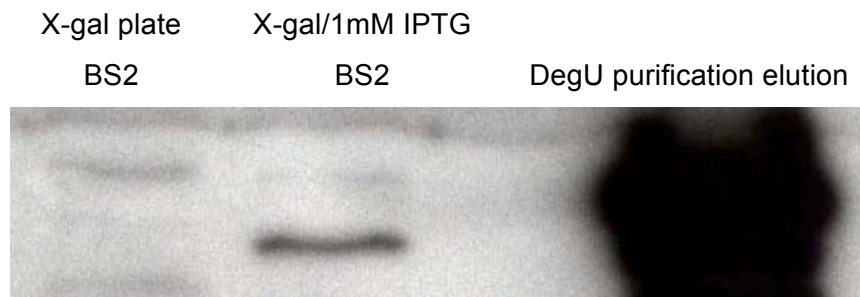BS2   BS2   DegU purification elution

Figure 2: DegU is produced in BS2 strain plated on 1 mM IPTG/X-gal plates. Western blot for DegU from BS2 strain colonies scrapped from X-gal and 1mM IPTG/ X-gal plates. DegU purification elution is a His-tagged DegU purified using a Ni-NTA (QIAGEN) column.

The results indicate that the *fliN-gmaR* promoter is active in *B. subtilis* in the absence of DegU. p*fliN-gmaR* has been shown to have basal activity in a *L. monocytogenes* Δ*degU* Δ*mogR* strain. Alternatively, *Bs*RNA polymerase may recognize another initiating sequence within the 300 bp *fliN-gmaR* promoter region DNA or further upstream. Following induction of DegU, transcription of *lacZ* was repressed (**Table 5,** BS2 strain), suggesting that DegU may bind the *fliN-gmaR* promoter region in *B. subtilis* and inhibit transcription. We know that *Bs*DegS can very efficiently phosphorylate DegU *in vitro*

(15). It is possible that phosphorylation of DegU by *Bs*DegS *in vivo* could inhibit the ability of DegU to activate p*fliN-gmaR*. Alternatively, DegU may not function with the *Bs*RNA polymerase, or other *B. subtilis* factors could associate with DegU on the *fliN-gmaR* promoter region to prevent transcriptional activation. Lastly, DegU may require additional *L. monocytogenes* factors to activate transcription. To address the possibility of DegU phosphorylation by *Bs*DegS affecting p*fliN-gmaR* activation, *L. monocytogenes degU* D55N, the *L. monocytogenes degU* gene containing a mutation in the phosphoacceptor site, therefore preventing phosphorylation by *Bs*DegS (15), was cloned behind the Spank promoter in the pDR110a plasmid. *B. subtilis* strain BS4, containing the pSpank::*degUD55N* and p*fliN-gmaR::lacZ* constructs did not show any phenotypic difference from *B. subtilis* strain BS2 (**Table 5**) indicating that possible phosphorylation of DegU by *Bs*DegS is not responsible for the transcriptional repression of p*fliN-gmaR* following IPTG induction. *B. subtilis* strain BS3, containing p*fliN-gmaR::lacZ* and the empty pDR110a vector has the same phenotype as BS1 (**Table 5**) proving that the pDR110a vector is not responsible for transcriptional repression of p*fliN-gmaR* following IPTG induction.

These results are inconclusive, but suggest that DegU binds p*fliN-gmaR* and is unable to activate transcription in *B. subtilis*. This could indicate that another factor is required for p*fliN-gmaR* activation. In light of these results, we initiated a transposon mutagenesis screen to identify *L. monocytogenes* genes involved in transcriptional activation of p*fliN-gmaR* at 30°C.

4.2 A transposon mutagenesis screen for L. monocytogenes genes involved in transcriptional activation of pfliN-gmaR at 30°C

Construction of a mariner transposon mutant library

Recently, a new *Himar1*-based transposon system was developed for use in *L. monocytogenes* (19). This new mariner transposon system provides a better alternative to the prominently used Tn917 transposon system, which has low transposition efficiency and a non-random pattern of insertion leading to insertional "hot spots" and poor genomic coverage (20). In comparison, the *Himar1*-based transposon system has been shown to be effective in multiple bacterial species and has a low site specificity, the dinucleotide TA, very common in the low G+C *L. monocytogenes* genome.

p*fliN-gmaR* fused to a *gusA* reporter gene was cloned into the pIMK vector (21). This vector was integrated into the genome of wild-type *L. monocytogenes* strain EGDe*,* resulting in strain LM1, and a *mariner* transposon library of approximately 90,000 independent insertion mutants was generated using pJZ307 (19). A curing efficiency of approximately 97% was determined based on the number of colonies that were chloramphenicol resistant (drug marker carried by delivery plasmid) compared to the number of colonies that were erythromycin resistant (resistance marker carried by the transposon).

Transposon mutagenesis screen for white mutants on X-gluc plates at 30°C

The transposon screen aims to identify additional factors required for transcriptional activation of p*fliN-gmaR* by screening the generated mariner transposon mutant library for white colonies on X-gluc plates after incubation at 30°C, indicating that p*fliN-gmaR* can no longer be activated. The p*fliN-gmaR::gusA* construct integrated into a *L. monocytogenes* Δ*degU* strain (LM2) was used as a control and produced white colonies on X-gluc plates incubated at 30°C for 48 hours verifying the ability of the

screen to identify *degU* mutants. The screen should identify mariner insertions in *degU*, *gmaR,* p*fliN-gmaR* and any additional factor(s) required for activation of p*fliN-gmaR*. A partial library screen of approximately 60,000 transposon insertions was performed on BHI plates containing erythromycin kanamycin and X-gluc incubated at 30° C for 60 h. One hundred and sixty mutant colonies either visually white or less blue were selected and restreaked on BHI plates containing erythromycin kanamycin and X-gluc to confirm the phenotype. Seven mutants that appeared bluer were also selected. One hundred and thirty seven mutants confirmed their initial X-gluc plate white/less blue phenotype. Their motility phenotype was then assessed using low agar BHI plates. X-gluc plate (white/less blue/blue/bluer) and motility (motile/less motile/non-motile) phenotypes were visually determined. Phenotypes of the wild-type (wt) p*fliN-gmaR::gusA* strain (blue/motile) and the Δ*degU* p*fliN-gmaR::gusA* strain (white/non motile) were taken as reference and mariner transposon mutants were placed in different phenotypic categories based on their X-gluc plate/motility phenotype. The number of mutants falling in each different category is reported in **Table 6**.

Table 6: **Classification of mutants based on X-gluc plate and motility phenotype**

	White	Less blue	Bluer
Motile	19	17	7
Less motile	1	7	0
Non motile	80	6	0

We expect mutants for factors involved in p*fliN-gmaR* transcriptional activation to show a white or less blue X-gluc plate phenotype corresponding to a decrease in p*fliN-gmaR* transcriptional activation, which should correlate with a decrease or loss in motility since the *fliN-gmaR* operon is required for motility. Therefore, we decided to send all the white or less blue/less motile or non-motile mutants for sequencing. Sequencing is based on purified PCR products obtained via arbitrary colony PCR which

enables us to determine the precise transposon insertion locus, to the exact base pair. The sequencing results are provided in **Table 7**. Multiple *degU* mutants, representing 10 distinct transposon insertion sites were isolated in the category white/non-motile as well as two independent insertions in p*fliN-gmaR* and 3 independent insertions in the *fliN-gmaR* operon, genes *lmo0676* and *lmo0677*. We were therefore able to retrieve all the expected controls suggesting that we were approaching screening saturation and no additional screening was necessary.

Table 7: Transposon insertions from sequenced mutants

X-gluc plate /motility phenotype	Transposon insertion	# of independent insertions	Gene name	Annotation
White/motile	*gusA*	1	*gusA*	p*fliN-gmaR* reporter gene
White/ less motile	no curing	1		Mariner delivery vector
White/non-motile	*lmo2515*	10	*degU*	Two-component DegU response regulator
	p*fliN-gmaR*	2		Promoter region of the *fliN-gmaR* operon
	lmo0676	3	*fliP*	similar to flagellar biosynthesic protein FliP
Less blue/ less motile	*lmo0889*	1	*rsbR*	highly similar to positive regulator of sigma-B activity
	lmo0896	1	*rsbX*	Indirect negative regulation of sigma B dependant gene expression (serine phosphatase)
	lmo0894	1	*rsbW*	sigma-B activity negative regulator RsbW
	lmo0539	1		similar to tagatose-1,6-diphosphate aldolase

	no curing	2		Mariner delivery vector
Less blue/ non-motile	lmo2515	2	degU	Two-component DegU response regulator
	lmo0676	1	fliP	similar to flagellar biosynthesic protein FliP
	lmo0677	1	fliQ	similar to flagellar biosynthesis protein FliQ
	lmo0866	2		similar to ATP-dependent RNA helicase
Bluer/ motile	lmo0027	1		similar to PTS system, beta-glucosides specific enzyme IIABC
	lmo0734	2		similar to LacI transcriptional regulator
	lmo0785	1		similar to transcriptional regulator NifA/NtrC

The less blue/less motile category led to the identification of 3 distinct insertions in the *sigma B* operon. SigB is an alternative sigma factor involved in stress response and virulence. SigB has been proposed to play a role in regulation of motility as Δ*sigB* mutants in *L. monocytogenes* strain 10430S have an increased motility at 30°C (22). Those results could not however be reproduced in our lab strain *L. monocytogenes* EGDe. In our screen we found that mutants with distinct transposon insertions in the *sigB* operon have a decreased motility at 30°C. It is difficult to identify which gene is responsible for this phenotype as we identified three different insertions, which may have polar effects on other genes in the operon. Due to the fact that we did not see any effect on motility in a Δ*sigB* mutant in our lab strain, the difficulty to identify which gene is directly responsible for the observed phenotype, and the fact that these mutants are still motile we decided not to further characterize these mutants. Another gene, *lmo0539*, was identified in the less blue/less motile category and encodes an enzyme similar to a tagatose-1,6-diphosphate aldolase which catalyzes the reversible condensation of dihydroxyacetone phosphate with glyceraldehyde 3-phosphate to

produce tagatose 1,6-bisphosphate. The less blue/less motile phenotype could be the result of decreased metabolism and is unlikely to be directly involved in the regulation of p*fliN-gmaR*, we therefore decided not to further characterize this mutant. The bluer/motile category retrieved mutations that seem to be involved in sugar metabolism and may be involved in X-gluc uptake. Interestingly, we also identified *degU, lmo0676 and lmo0677* mutants in the less blue/non-motile category suggesting that the phenotypic difference between white and less blue was difficult to determine. Furthermore, we identified two independent insertions in the gene *lmo0866*, which encodes a protein similar to an ATP-dependent RNA helicase. We decided to further investigate the *lmo0866::mariner* mutant, which displays a phenotype very similar to a *degU* mutant, a known transcriptional activator of p*fliN-gmaR*.

Lmo0866 is required for pfliN-gmaR activation and motility

Both transposon insertions into *lmo0866* were transduced into both p*fliN-gmaR::gusA* and wt EGDe backgrounds using bacteriophage P35 to confirm linkage between the phenotype and transposon insertion. The X-gluc plate phenotype of *lmo0866::mariner* is presented in **Figure 3**. The initial streak appears less blue than the initial streak of wt p*fliN-gmaR::gusA* strain and single colonies are white. A Δ*degU* p*fliN-gmaR::gusA* mutant has a white initial streak and white single colonies. To characterize in a more quantitative manner the level of transcriptional activation of p*fliN-gmaR* in the *lmo0866::mariner* mutant, a β-glucuronidase assay was performed (**Fig. 4**). *lmo0866::mariner* showed a β-glucuronidase activity very similar to a Δ*degU* mutant and the difference from the p*fliN-gmaR::gusA* strain is statistically significant with p-value < 0.001.

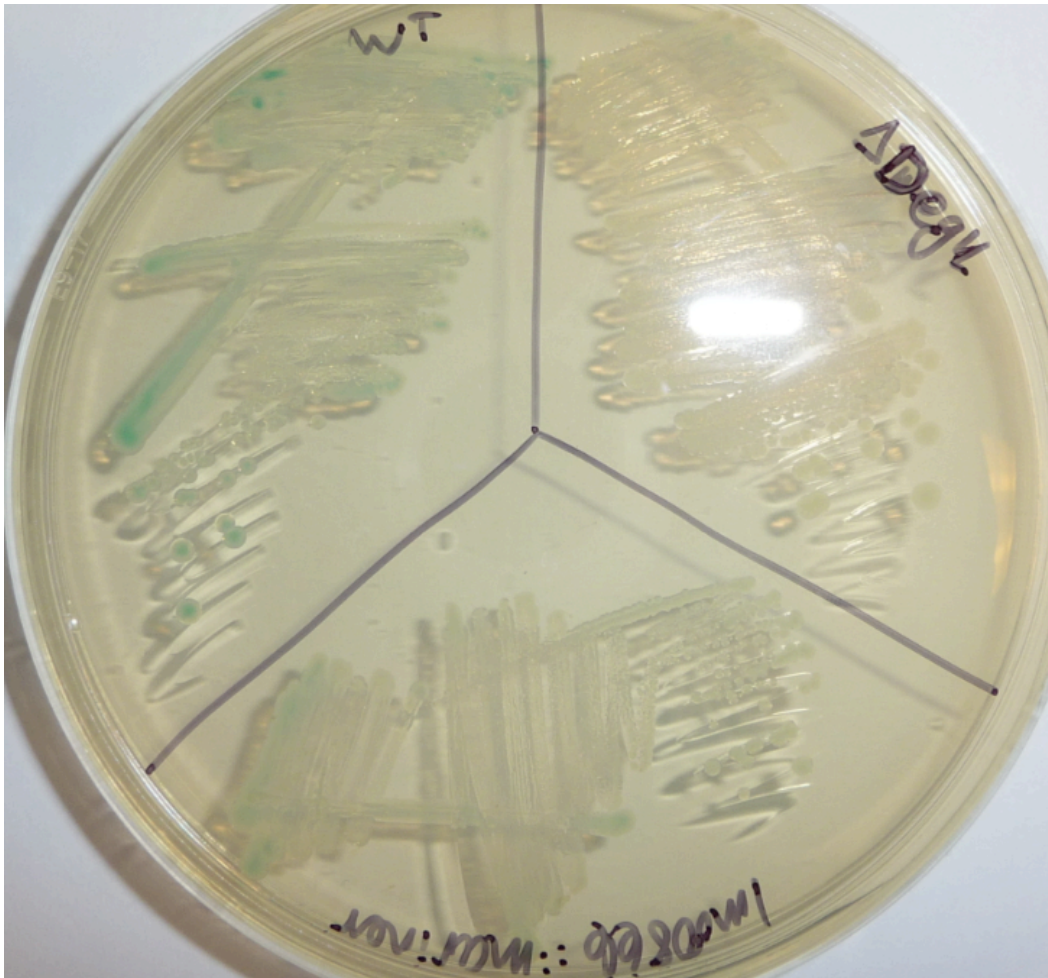**Figure 3: X-gluc plate phenotype of wt p*fliN-gmaR::gusA* strain, Δ*degU* and *lmo0866* mutant.** Wild-type p*fliN-gmaR::gusA* strain, Δ*degU* p*fliN-gmaR::gusA* and *lmo0866::mariner* were streaked on X-gluc plate and incubated 60 hr at 30°C.

The decrease in p*fliN-gmaR* transcriptional activation directly correlated with a loss of flagellar motility. Indeed, similar to a Δ*degU* mutant, *lmo0866::mariner* did not swim at 30°C in a motility plate assay (**Fig. 5**). We therefore hypothesized that *lmo0866::mariner* does not express flagella due to a decrease in transcription of the *fliN-gmaR* operon resulting in the non-motile phenotype. We further analyzed the *lmo0866* mutant strain by transmission electron microscopy. Wild-type EGDe typically expresses between 4 to 6 flagella. As expected, both the *lmo0866* and Δ*degU* mutant did not express flagella

(**Fig. 6**). Taken together, these data indicate that *lmo0866* is required for p*fliN-gmaR* transcriptional activation and flagellar motility.
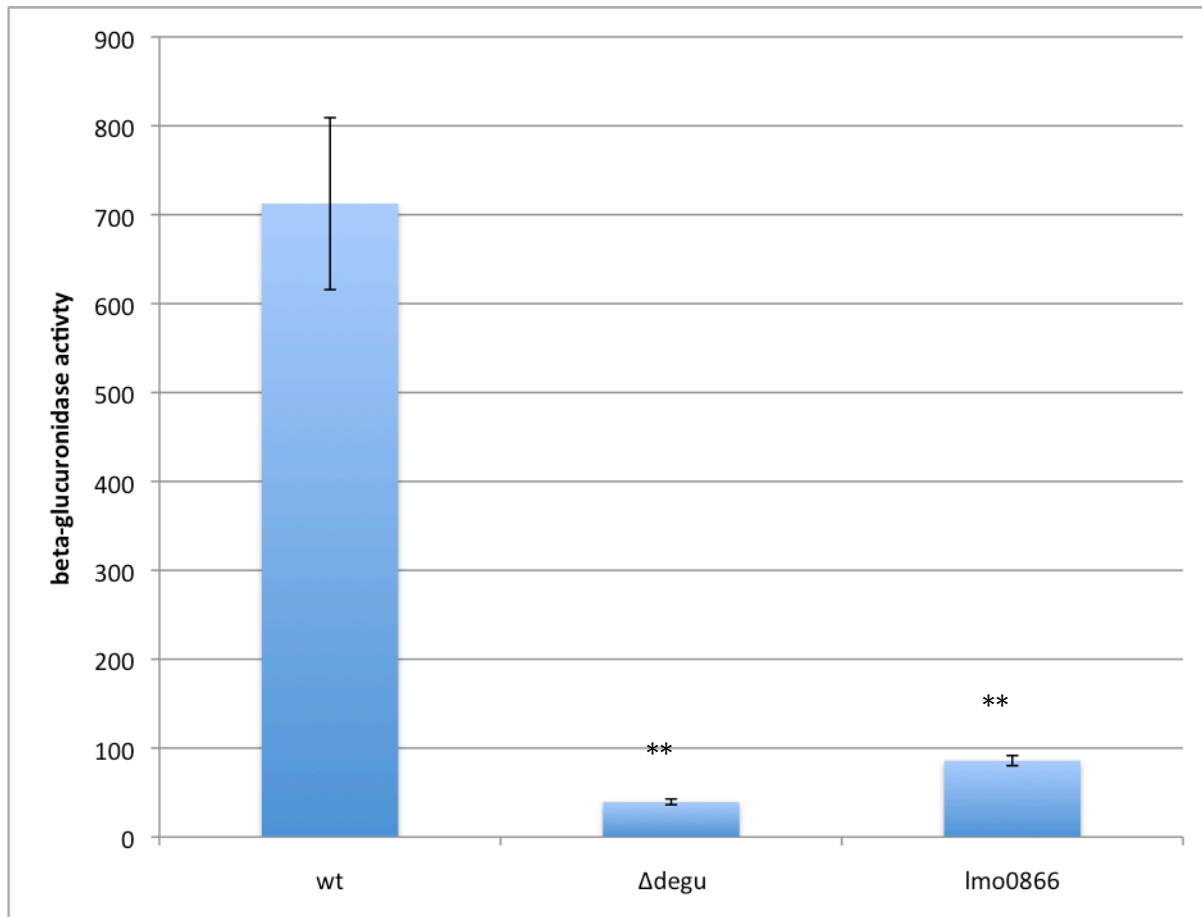

Figure 4: Analysis of the *fliN-gmaR* promoter activity determined by β-glucuronidase assay. *L. monocytogenes* strains were grown 18 to 20 hr at 30°C in BHI broth. β-glucuronidase activity represents the mean and standard deviation of three independent cultures. Miller units were calculated per 10^9 bacteria. Experiment was repeated once in triplicate and similar results were obtained.
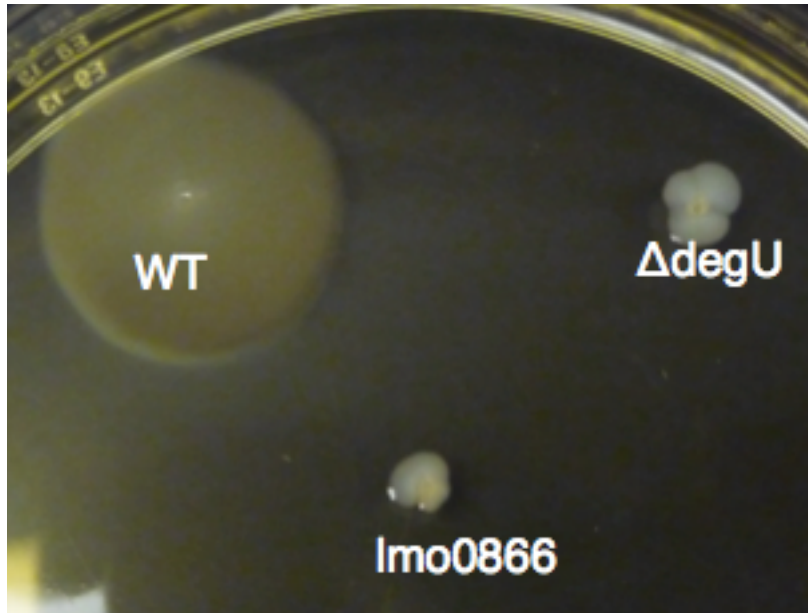
Figure 5: *lmo0866* mutant is not motile at 30°C. The swimming phenotype of wt EGDe, Δ*degU* and l*mo0866::mariner* when analyzed by motility plate assay (BHI with 0.375% agar) at 30°C.

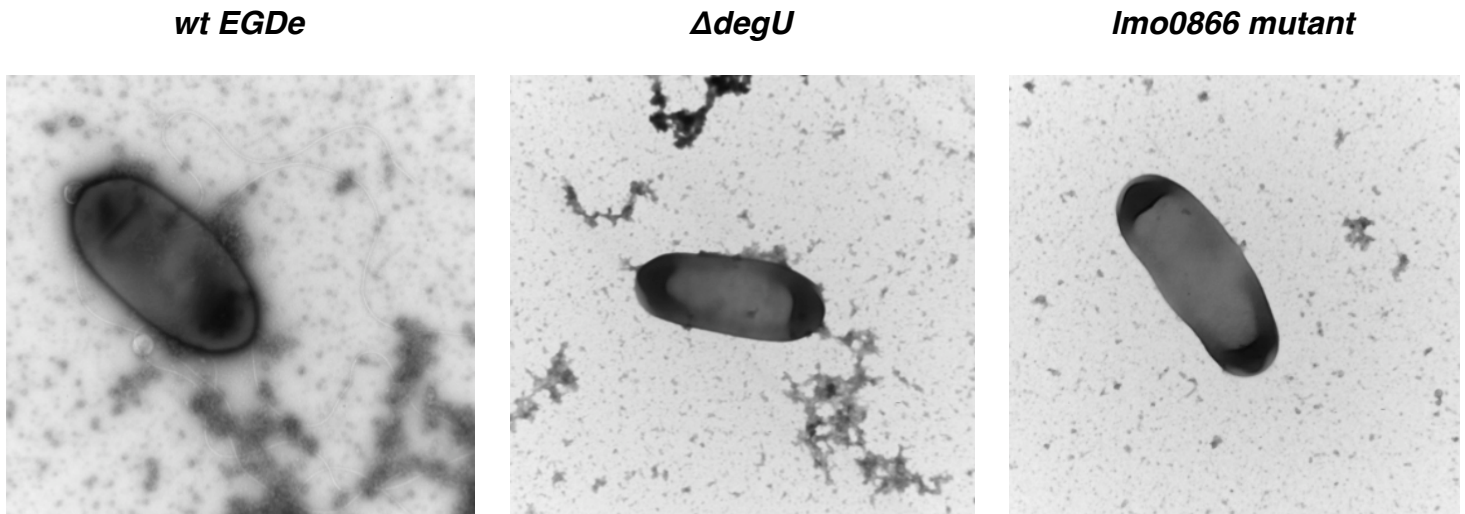| *wt EGDe*	*ΔdegU*	*lmo0866 mutant*

Figure 6: Transmission electron microscopy of *L. monocytogenes strains*. Five microliters of bacterial cultures grown for 16 hr at 30°C not shaking were used for negative staining procedure. Bacteria were imaged at 11,000 X.

Complementation of the lmo0866 transposon insertion

To verify that the observed phenotypes were specifically due to the absence of Lmo0866, the *lmo0866* mutant was complemented by the introduction of a wild-type copy of the *lmo0866* locus on the multicopy plasmid pAM401 and on the single integration vector pPL3 resulting in strains LM6 and LM8, respectively. The introduced *lmo0866* locus comprises the upstream intergenic sequence, containing the putative native *lmo0866* promoter, the *lmo0866* open reading frame and its terminator sequence. Flagellar motility was restored in both complemented strains (**Fig. 7**), however not to a wild-type level.
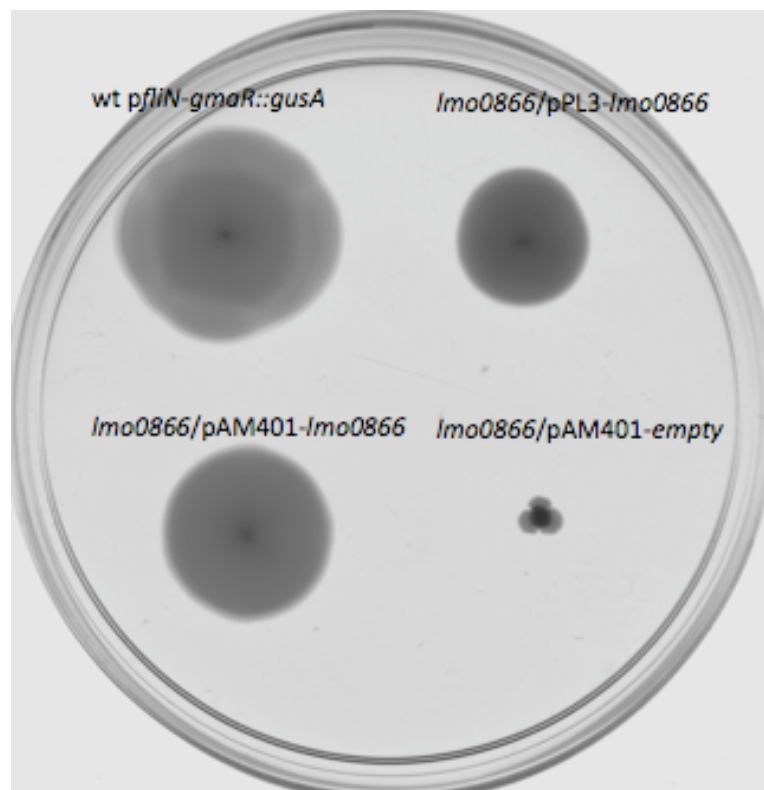

Figure 7: Motility phenotype of *lmo0866* complemented strains. The swimming phenotype of wt EGDe pPL3 p*fliN-gmaR::gusA* strain*, lmo0866*/pPL3-l*mo0866, lmo0866*/pAM401-l*mo0866* and *lmo0866*/pAM401-empty when analyzed by motility plate assay (BHI with 0.375% agar) at 30°C.

To investigate whether there was a difference in the number of flagella expressed in the complemented strains, which could account for the difference in flagellar motility, we further analyzed the complemented strains by transmission electron microscopy (**Fig. 8**).
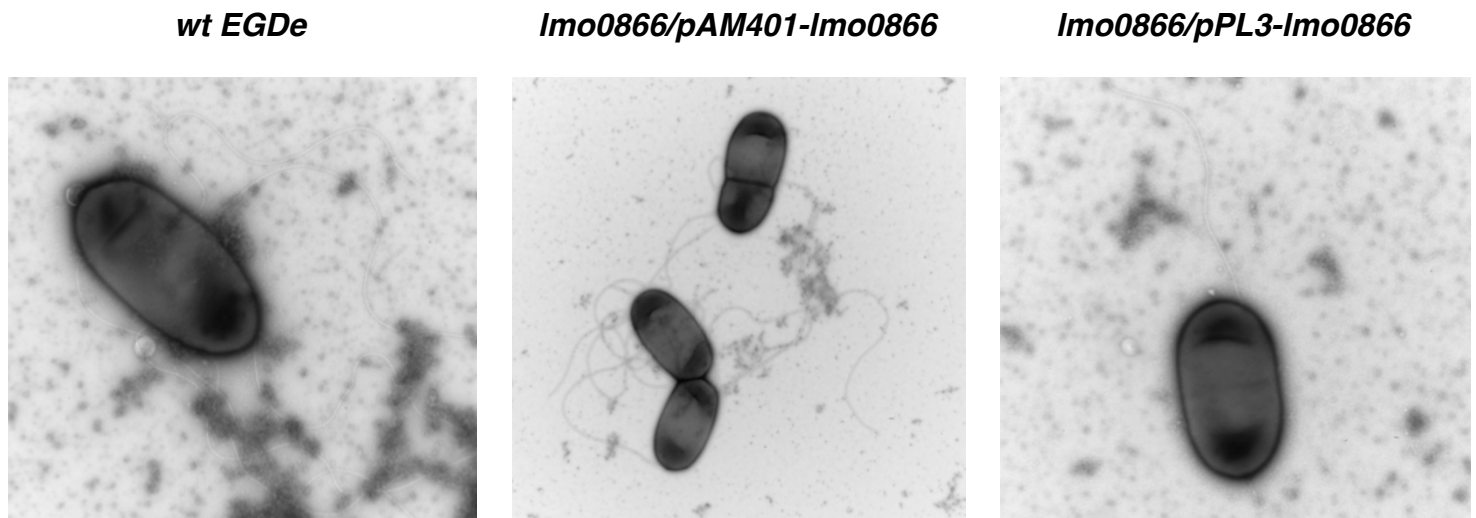
wt EGDe	lmo0866/pAM401-lmo0866	lmo0866/pPL3-lmo0866

Figure 8: Transmission electron microscopy of *lmo0866* complemented strains. Five microliters of each respective bacterial culture grown for 16 hr at 30°C were used for negative staining procedure. Wild-type EGDe and *lmo0866*/pPL3-l*mo0866* strains were imaged at 11,000X. *lmo0866*/pAM401-*lmo0866* strain was imaged at 6,800X.

In the *lmo0866* /pAM401-*lmo0866* complemented strain we observed the same number of flagella as in wild-type EGDe. Bacteria of the complemented strain *lmo0866*/ pPL3 *lmo0866* expressed only one flagellum. The integration of a single copy of the *lmo0866* gene at an exogeneous locus could result in lower level of Lmo0866 expression. These complementation data demonstrate that the abrogation of functional Lmo0866 is responsible for the loss of flagellar motility in the *lmo0866* mutant.

Lmo0866 is similar to an ATP-dependent RNA helicase

Lmo0866 is predicted to be a single gene, 1563 base pairs long with an intergenic region of 451 base pairs upstream of the start codon. *lmo0866* encodes a hypothetical protein similar to an ATP-dependent RNA helicase belonging to the DEAD-box protein family. It is composed of the 9 conserved motifs characteristic of DEAD-box proteins (reviewed in 23) as well as a 177 amino acid long non-conserved C-terminal domain (**Fig. 6**).
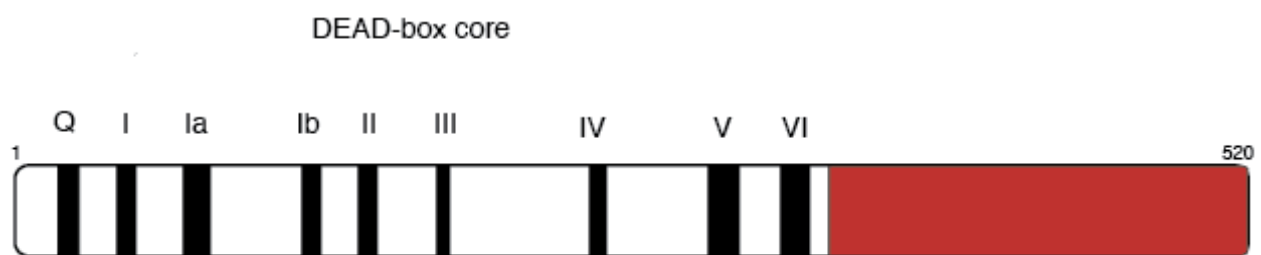

Figure 6: Schematic representation of the Lmo0866 protein. Lmo0866 is composed of a DEAD-box core containing the 9 conserved motifs, represented here as black stripes. The non-conserved C-terminal domain is shown in red.

DEAD-box proteins are a family of proteins highly conserved from bacteria through humans composed of a core of 350-400 amino acids containing 9 conserved sequence motifs. Motif II has the amino acids D-E-A-D giving the name to the family. Motifs I, II, VI and Q are required for ATP binding and hydrolysis. Motifs Ia, Ib, III, IV and V are less well characterized but may be involved in interaction with RNA. DEAD-box proteins N- or C- terminal domains are highly divergent and may provide specificity for interacting with specific RNA substrates or other factors. *In vitro* all DEAD-box proteins tested show RNA-dependent ATPase activity and many of them are ATP-dependent RNA helicases, meaning that they are able to dissociate short RNA duplexes in an ATP-dependent manner. DEAD-box proteins play important roles in many processes of RNA metabolism. In eukaryotes, they are involved in RNA degradation, pre-mRNA splicing and mRNA export as well as ribosome biogenesis and translation initiation. Interestingly, several DEAD-box proteins have been shown to be involved in

transcriptional regulation, which does not alwas require their RNA helicase activity. They are thought to act as adaptors or bridging factors between coactivators or corepressors and components of the transcription machinery. In prokaryotes, DEAD-box proteins have been involved in mRNA processing and decay, ribosome biogenesis and translation initiation. To our knowledge, no prokaryotic DEAD-box protein has been found to be involved in transcriptional regulation.

lmo0866 was identified by microarray analysis as having higher transcript levels in both log- and stationary- phase at 4°C compared to 37°C (24). Interestingly, *lmo0866::mariner* displays a growth defect when grown in BHI medium shaking at 30°C (**Fig. 9**) and 37°C (data not shown).
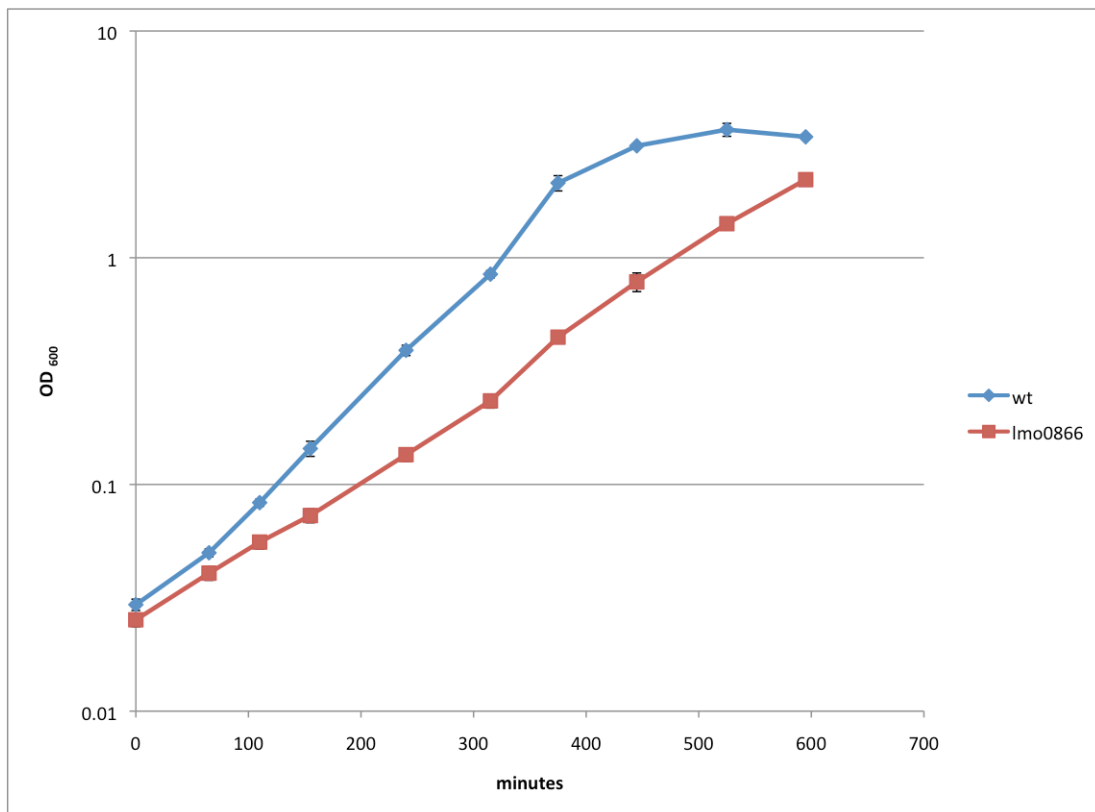

Figure 9: Growth curve of *lmo0866* mutant. *L. monocytogenes* cultures were grown at 30 °C in BHI shaking. OD_{600} readings for each strain were taken from three independent cultures at regular time intervals.

As the DEAD-box proteins shown to act as transcriptional regulators in eukaryotes are also involved in other cellular processes, the growth defect observed suggests that Lmo0866 also participates in processes other than p*fliN-gmaR* transcriptional activation. A Δ*degU* mutant did not have a growth defect under these conditions, suggesting that the growth defect is not a DegU-dependent pathway.

Lmo0866 does not affect DegU protein levels

As we identified *lmo0866* as being required for p*fliN-gmaR* transcriptional activation Lmo0866 may directly act at the *fliN-gmaR* promoter in conjunction with DegU or could alternatively be involved in DegU regulation. To test if DegU levels are affected in the *lmo0866* mutant we performed a western blot for DegU at 30°C (**Fig. 10**). Western blot analysis showed that DegU protein is still produced in the *lmo0866* mutant and protein levels appear similar or maybe slightly lower than wild-type EGDe DegU protein levels. The faint DegU band in the Δ*degU* sample is the result of some spillage from adjacent wells. We therefore hypothesize that Lmo0866 has a direct effect on transcriptional activation of the *fliN-gmaR* promoter.
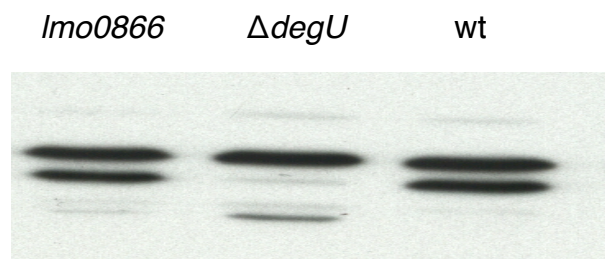

Figure 10: Western blot analysis of DegU protein levels in the *lmo0866* mutant. Cultures were grown until mid exponential phase at 30°C and a western blot with a DegU polyclonal antibody was performed.

4.3 A transposon mutagenesis screen for L. monocytogenes genes involved in the repression of pfliN-gmaR at 37°C

The screen aims to identify additional factors required for transcriptional repression of p*fliN-gmaR* by screening the p*fliN-gmaR::gusA* transposon mutant library for blue colonies on X-gluc plates after incubation at 37°C, indicating that p*fliN-gmaR* is derepressed. The p*fliN-gmaR::gusA* construct integrated into a *L. monocytogenes* Δ*mogR* strain was used as a control and produced blue colonies on X-gluc plates incubated at 37°C for 48 hours (data not shown), verifying the ability of the screen to identify *mogR* mutants. The screen should identify mariner insertions in *mogR* and any additional factor(s) involved in the transcriptional repression of p*fliN-gmaR*. A partial library screen of approximately 50,000 transposon insertions was performed on BHI plates containing erythromycin kanamycin and X-gluc incubated at 37° C for 60 h. Ninety-seven blue colonies were selected and restreaked on BHI plates containing erythromycin kanamycin and X-gluc to confirm the phenotype. Ninety-six mutants confirmed their initial blue X-gluc plate phenotype. We performed a β-glucuronidase assay to better quantify the transcriptional activation level of p*fliN-gmaR* to select interesting mutants to send for sequencing. From the 96 mutants, 41 mutants showed a β-glucuronidase activity very similar to the wild-type p*fliN-gmaR::gusA* strain, 9 showed an intermediate phenotype and 46 showed an activity similar to a Δ*mogR* p*fliN-gmaR::gusA* strain. We decided to send the intermediate and high β-glucuronidase activity mutants for sequencing. We only identified insertions in *mogR* in the high β-glucuronidase activity mutants with 6 independent transposon insertions in *mogR*. The intermediate β-glucuronidase activity category led to the identification of four independent transposon insertions in the *codY* gene and one insertion in *mogR*, inserted 12 bp upstream of the stop codon. It is possible that this mutant is expressing a shorter MogR protein that still retains some activity.

As we were able to isolate multiple *mogR* mutants, the expected control, representing 7 independent insertions it suggested that we were approaching saturation and we did not perform any additional screening.

Interestingly, *codY* was identified in a previous screen performed in our lab looking at the regulation of p*flaA*, the promoter of the flagellin gene, at 37°C. Furthermore, a microarray of Δ*codY* EGDe mutant and EGDe strain showed that genes involved in flagellar biosynthesis were derepressed in the Δ*codY* mutant (24). We therefore decided to further investigate the role of CodY in the transcriptional repression of p*fliN-gmaR* at 37°C.

MogR protein levels are decreased in a codY mutant

To determine if MogR levels are affected in a *codY* mutant at 37°C we performed a Western blot for MogR (**Fig.11 A**). Western blot analysis suggested that MogR protein levels are reduced in a *codY* mutant. To try to quantify the decrease in MogR protein levels we performed a western blot with serial dilutions of the samples (**Fig.11 B**). The reduction in MogR protein levels can be estimated to an approximate two-fold decrease. We propose that CodY is involved in the regulation of MogR protein levels at 37°C by an unknown mechanism.

A.

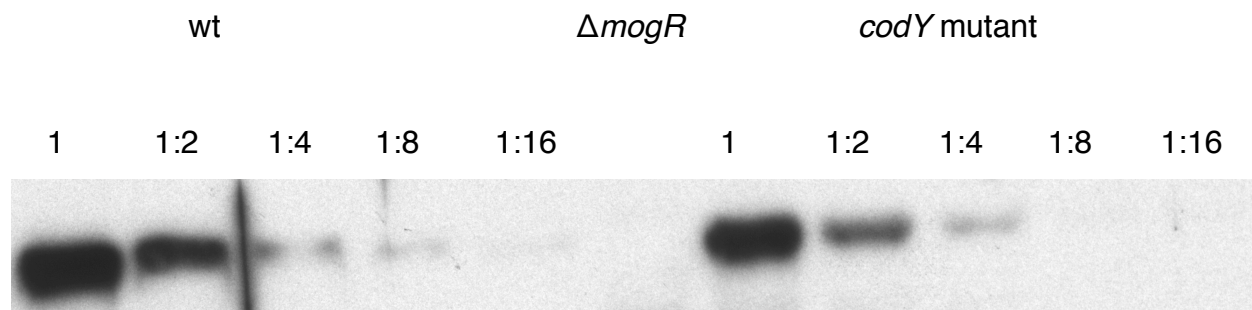| wt | Δ*mogR* | *codY* mutant |

36
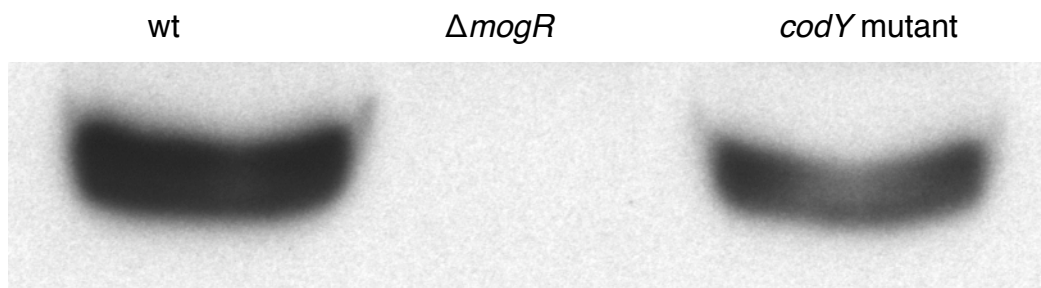
B.

Fig 11: Western blot analysis of MogR protein levels in *codY* mutant. A. *L. monocytogenes* cultures were grown until mid exponential phase at 37°C shaking and a western blot with a MogR polyclonal antibody was performed. **B**. Serial dilutions of the samples used in **A**. were used to perform a western blot using a MogR polyclonal antibody.

5. DISCUSSION

Transcriptional regulation of the *fliN-gmaR* promoter, the promoter of the first operon of the flagellar gene cluster, is central to the temperature-dependent regulation of flagellar motility in *L. monocytogenes* and is mediated by the activities of the DegU response regulator, the MogR transcriptional repressor, and GmaR, the MogR anti-repressor. In this study, we further investigated the mechanisms controlling transcription initiating at the *fliN-gmaR* promoter by performing two comprehensive transposon mutagenesis screens to identify factors involved in the activation and repression of p*fliN-gmaR*. The mutagenesis screen performed at 30°C to identify factors involved in p*fliN-gmaR* transcriptional activation led to the identification of a novel regulatory factor in *L. monocytogenes*. We demonstrated that Lmo0866, a protein from the DEAD-box protein family, is required for transcriptional activation of p*fliN-gmaR* and therefore flagellar motility. Moreover, we identified *codY* as being involved in the repression of p*fliN-gmaR* at 37°C.

Comprehensive transposon mutagenesis screens for factors involved in the

transcriptional regulation of pfliN-gmaR

To further investigate the regulation of transcription initiating at the *fliN-gmaR* promoter, we generated a mariner transposon library in a p*fliN-gmaR::gusA* reporter strain and performed two screens at 30°C and 37°C to, respectively, identify additional factors involved in the activation and repression of p*fliN-gmaR*. Comprehensive transposon mutagenesis in *L. monocytogenes* was made possible by the development of a new *Himar1*-based transposon system (19). This transposon system provides increased genomic coverage and is far superior to the well-established Tn917 transposon system. Indeed, due to the poor genomic coverage of Tn917, a previous

screen performed in our lab using the Tn917 transposon was unable to identify *degU* when screening for activating factors of p*flaA,* the promoter for the flagellin gene. We were able to isolate 75 *degU* mutants representing 10 distinct transposon insertions, in our mariner transposon screen for additional factors involved in the transcriptional activation of p*fliN-gmaR*. We also isolated transposon insertions in p*fliN-gmaR* and the *fliN-gmaR* operon. In the mutagenesis screen to identify factors involved in repression of p*fliN-gmaR* at 37°C, we isolated 46 *mogR* mutants representing 7 independent insertions. We therefore conclude that the newly generated transposon mutant library allowed for comprehensive screening for factors controlling regulation of transcription occurring at p*fliN-gmaR*.

Identification of a novel factor involved in transcription of pfliN-gmaR and flagellar motility

We identified two independent transposon insertions in *lmo0866* in the 30°C screen to identify factors involved in the activation of p*fliN-gmaR*. Both mutants were less blue on X-gluc plates (**Fig. 3**) and non-motile (**Fig. 4**). Furthermore, complementation with the cloned *lmo0866* locus was able to restore flagellar motility (**Fig. 7**) confirming that Lmo0866 is required for transcription of p*fliN-gmaR* and therefore flagellar motility. As we preferentially screened for white colonies rather than less blue colonies on X-gluc plates, it is possible that we could have identified additional independent *lmo0866* mutants by screening more extensively for less blue colonies.

Western blot analysis showed that DegU is still produced in the *lmo0866* mutant (**Fig. 10**). DegU protein levels were similar to those in wild-type bacteria suggesting that Lmo0866 does not regulate DegU protein levels, but may act directly at the *fliN-gmaR* promoter in conjunction with DegU. Overexpression of DegU in the *lmo0866* mutant and

testing for suppression of the non-motile phenotype may provide insights into the function of Lmo0866.

Lmo0866 is highly similar to an ATP-dependent RNA helicase from the DEAD-box protein family

DEAD-box proteins are highly conserved from bacteria through humans and play important roles in many cellular processes including mRNA export from the nucleus to the cytoplasm, ribosome biogenesis and RNA degradation. The *L. monocytogenes* genome encodes four ATP-dependent RNA helicases from the DExD-box protein family (where x can be any amino acid), two of them being DEAD-box proteins. Interestingly, Lmo0866 shares significant homology with the *B. subtilis* cold-shock protein A, CshA. They are 59 % identical and 74% similar (**Fig. 13**). *cshA* transcript levels are upregulated in response to cold-shock (26) and CshA was proposed to function in conjunction with cold-shock proteins to help rescue misfolded mRNA molecules to maintain proper initiation of translation at low temperatures in *B. subtilis* (27). Similarly, *lmo0866* transcript levels were found by microarray analysis to be upregulated at 4°C compared to 37°C (24). It is therefore also possible that Lmo0866 is involved in cold adaptation in *L. monocytogenes*.

Recently, CshA has been identified as the major RNA helicase of the RNA degradosome complex in *B. subtilis* (28). CshA was shown to be able to interact with several proteins of the degradosome complex, including RNase Y and the polynucleotide phosphorylase. Interestingly, the non-conserved C-terminal domain of CshA was shown to be required for dimerization of CshA and for interactions with proteins of the RNA degradosome. Based on the significant homology with CshA and the growth defect of the *lmo0866* mutant, suggesting that Lmo0866 is involved in

important cellular processes, it is tempting to speculate that Lmo0866 may also be involved in RNA degradation in *L. monocytogenes*.
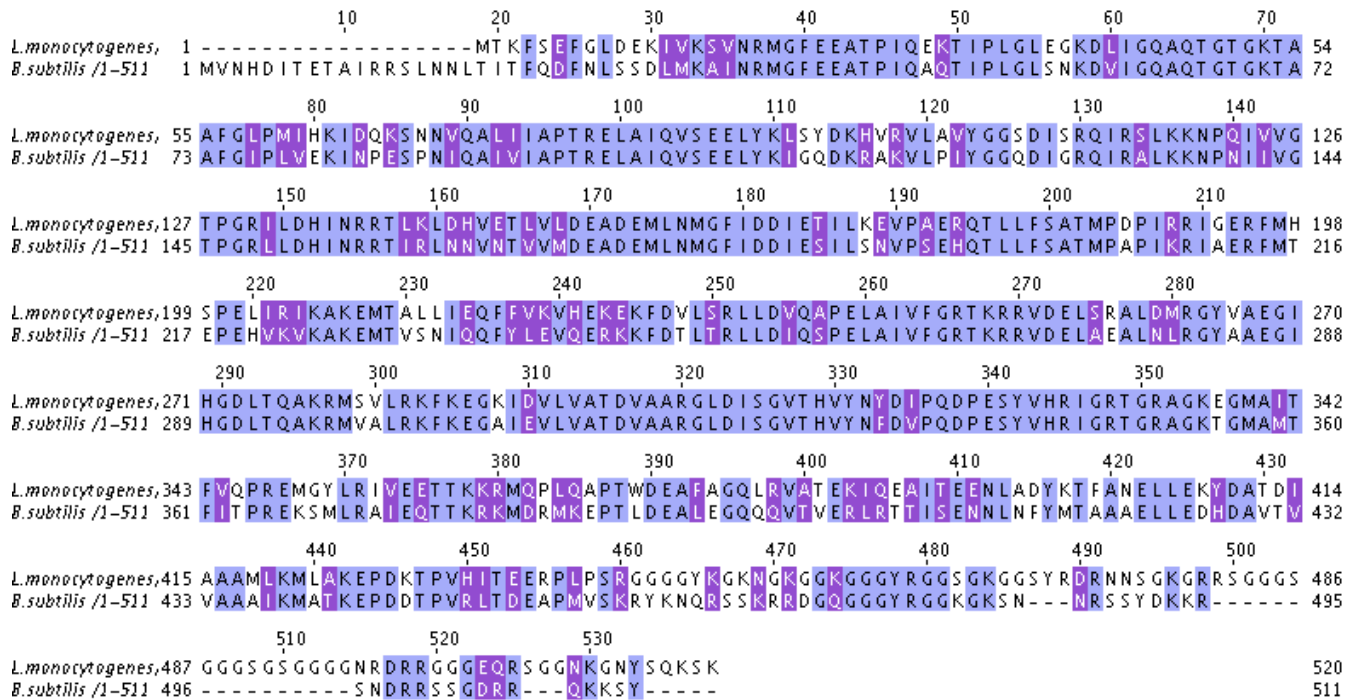

Figure 13: Alignment of amino acid residues of *L. monocytogenes* Lmo0866 and *B. subtilis* CshA proteins. Conserved identical residues are blocked in blue and similar residues are blocked in purple. Alignment was obtained through the Needleman-Wunsch algorithm using matrix BLOSUM62.

Nonetheless, our studies indicate that Lmo0866 is required for transcription of p*fliN-gmaR* and therefore flagellar motility. Since DegU protein levels do not appear to be affected in the *lmo0866* mutant, we hypothesize that Lmo0866 has a direct effect on transcriptional activation of p*fliN-gmaR*. In recent years, there has been increasing evidence showing that some members of the DEAD-box protein family play important roles in transcriptional regulation in eukaryotes (reviewed in 29). DEAD-box proteins can function through interaction with transcriptional coactivators or corepressors, suggesting they may function as bridging factors between other regulators and components of the

transcription machinery, stabilizing the transcription initiation complex by interacting directly with proteins in the complex, or helping recruit transcriptional regulators to the initiation complex. For example, the human DEAD-box protein p68 interacts with the transcriptional coactivators CBP/p300 and RNA pol II, stimulating transcriptional activation mediated by CBP/p300. p68 also acts as a transcriptional coactivator for the nuclear estrogen receptor alpha (Erα). While no DEAD-box protein has been reported to act as transcriptional regulator in prokaryotes, it is possible that Lmo0866 is directly involved in transcriptional activation of p*fliN-gmaR*.

The DegU binding site in the p*fliN-gmaR* region is located -184 to -154 bp upstream of the transcriptional start site, however it is unclear how the binding of DegU to the p*fliN-gmaR* region is transmitted downstream to RNA polymerase to allow activation of transcription. In this instance, Lmo0866 could act as a bridging factor between the DegU response regulator and RNA polymerase. As the C-terminal domain of CshA allows for dimerization and interaction with specific proteins, it is tempting to speculate that the C-terminal domain of Lmo0866, which shares significant homology to the CshA C-terminal domain, may enable dimerization of Lmo0866 and interaction with factors such as DegU or RNA polymerase. Pull-down experiments to determine if Lmo0866 is able to interact with DegU would provide insights into a potential Lmo0866/DegU interaction. Alternatively, interaction between DegU and Lmo0866 may be dependent on DegU binding to the p*fliN-gmaR* region, therefore a gel mobility shift analysis with p*fliN-gmaR* DNA, and purified DegU and Lmo0866 proteins should be performed. These studies would also provide insights into the ability of Lmo0866 to bind to p*fliN-gmaR* DNA. However, it is less likely that Lmo0866 binds DNA since no DNA-binding domain was identified in the Lmo0866 protein sequence and no DEAD-box protein previously identified to be involved in transcriptional regulation has been shown to bind DNA.

Alternatively, another model for Lmo0866 regulation of p*fliN-gmaR* transcription could involve Lmo0866 helicase activity. Lmo0866 could bind to DegU and mediate unwinding of p*fliN-gmaR* DNA to favor transcriptional activation. It would therefore be interesting to determine if the helicase activity of Lmo0866 is required for transcription of p*fliN-gmaR* activity by mutating the DEAD box motif to a helicase inactive NEAD sequence (30). This would provide additional insights into the mechanism of Lmo0866-mediated transcriptional activation of p*fliN-gmaR*. Nonetheless, we cannot exclude the remote possibility that because of the potential role of Lmo0866 in the RNA degradosome complex, a mutation in *lmo0866* may cause a more general metabolic defect that results in poor flagellar production. Therefore, further analysis is needed to determine how Lmo0866 is involved in regulating transcription initiating at p*fliN-gmaR* and as a result flagellar motility.

CodY is involved in the regulation of MogR protein levels at 37°C

We were able to isolate multiple *mogR* mutants from the mutagenesis screen for factors involved in repression of p*fliN-gmaR* at 37°C. Furthemore, only *mogR* mutants demonstrated high β-glucuronidase activity, suggesting that MogR is the primary repressor of p*fliN-gmaR* and does not require any additional factor(s) to function as a repressor, this is in accordance with previously published data. We were however able to identify CodY as being involved in the repression of p*fliN-gmaR,* but a *codY* mutant had an intermediate β-glucuronidase activity suggesting that the CodY effect on transcription of p*fliN-gmaR* may be indirect. CodY is a transcriptional regulator controlling genes involved in carbon and nitrogen assimilation. CodY is able to monitor the energetic and nutritional status of the cell by sensing the levels of GTP and branched chain amino acids (BCAA). Interestingly, the *codY* regulon, as defined by microarray analysis, identified the flagellar motility gene cluster as being derepressed in a Δ*codY* mutant (25). Moreover, in *B. subtilis* CodY was shown to directly repress the flagellin gene in a nutrient rich environment by binding to the flagellin gene promoter

(31). Since, we hypothesized that the role of CodY for repression of p*fliN-gmaR* was indirect, we investigated MogR protein levels in a *codY* mutant and found that MogR levels are indeed decreased. To further investigate if CodY regulates *mogR* transcription or MogR protein levels, a q-RT PCR for *mogR* should be performed. As *mogR* was not identified in the *codY* regulon, it is tempting to speculate that CodY is involved in the regulation of MogR protein levels by the action of another CodY regulated protein, such as a protease.

6. CONCLUSION

Flagellar motility is an essential mechanism allowing bacteria to move and to survive in very diverse environments. *L. monocytogenes* is a facultative intracellular pathogen that represses transcription of flagellar motility genes at physiological temperatures (37°C and above). Interestingly, regulation of flagellar motility genes in *L. monocytogenes* differs significantly from other bacterial species as homologs to the master regulators are lacking. In *L. monocytogenes*, the MogR transcriptional repressor represses transcription of all flagellar motility genes in a non-hierarchal manner at temperatures of 37°C and above. GmaR, the MogR anti-repressor binds to MogR preventing MogR repression of flagellar motility genes at temperatures below 37°C. The response regulator DegU is required for transcription of all flagellar motility genes and acts by transcriptionally activating *fliN-gmaR*, the first operon of the flagellar gene cluster. In this study, we identified a novel factor, Lmo0866, as being required for p*fliN-gmaR* transcriptional activation and therefore flagellar motility. Moreover, we identified CodY as being involved in p*fliN-gmaR* repression at 37°C. Given the multiple environments *L. monocytogenes* can encounter during its extracellular and intracellular existence and the importance of proper regulation of flagellar motility genes for bacterial survival, *L. monocytogenes* has evolved a complex regulatory system involving multiple factors that work together to fine tune flagellar motility gene expression in response to environmental conditions.

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