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Transcriptional and post-transcriptional regulation of the GmaR antirepressor governs temperature-dependent control of flagellar motility in *Listeria monocytogenes*

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Summary

Flagellar motility in *Listeria monocytogenes* (*Lm*) is restricted to temperatures below 37°C due to the opposing activities of the MogR transcriptional repressor and the GmaR antirepressor. Previous studies have suggested that both the DegU response regulator and MogR regulate expression of GmaR. In this report, we further define the role of DegU for GmaR production and flagellar motility. We demonstrate that deletion of the receiver domain of DegU has no effect on flagellar motility in *Lm*. Using transcriptional reporter fusions, we determined that *gmaR* is cotranscribed within an operon initiating with *fliN*. Furthermore, the *fliN-gmaR* promoter ($p_{fliN\text{-}gmaR}$) is transcriptionally activated by DegU and is also MogR-repressed. DNA affinity purification, gel mobility shift and footprinting analyses revealed that both DegU and MogR directly bind *fliN-gmaR* promoter region DNA and that the binding sites do not overlap. Quantitative analysis of *gmaR* transcripts in Δ*mogR* bacteria indicated that transcriptional activation of $p_{fliN\text{-}gmaR}$ by DegU is not inherently temperature-dependent. However, GmaR protein was not detectable at 37°C in Δ*mogR* bacteria, indicating that a temperature-dependent, post-transcriptional mechanism limits GmaR production to temperatures below 37°C. Our findings reveal that flagellar motility in *Lm* is governed by both temperature-dependent transcriptional and post-transcriptional regulation of the GmaR antirepressor.

Introduction

Bacteria can inhabit a diverse range of niches by sensing and adapting to environmental fluctuations through rapid changes in gene transcription and protein expression. *Listeria monocytogenes* (*Lm*) is a Gram-positive bacterium that thrives in a vast range of environments and over a wide spectrum of temperatures (3°C–43°C). As a facultative intracellular pathogen, *Lm* replicates and spreads within the intracellular environment of host cells (Vazquez-Boland *et al.*, 2001). Transition from the extracellular milieu to an intracellular lifestyle requires several environmental signals that lead to the upregulation of virulence factors and the reciprocal downregulation of other genes, such as those required for flagellar motility.

Flagellar motility is a highly advantageous, but energetically demanding survival mechanism utilized by bacteria in the extracellular environment. Flagellar motility allows the bacterium to move towards, or retreat from specific environmental conditions required for optimal bacterial growth (Armitage, 1999). Since flagellar motility in *Lm* increases adherence to abiotic and cellular surfaces, it is important for biofilm formation and cellular invasion (O'Neil and Marquis, 2006; Lemon *et al.*, 2007). However, while flagellar motility can enhance cellular invasion (O'Neil and Marquis, 2006), the continual production of flagella during infection can stimulate innate immune responses that are inhibitory for bacterial survival during infection (Hayashi *et al.*, 2001; Molofsky *et al.*, 2006). Therefore, *Lm* along with several other pathogens downregulate flagellar motility at physiological temperature (37°C) (Peel *et al.*, 1988; Ott *et al.*, 1991; Akerley and Miller, 1993; Kapatral and Minnich, 1995). Temperature is also a signal that co-ordinately regulates virulence factor expression in *Lm*. The master virulence gene activator, PrfA, is regulated by a temperature-dependent secondary structure in the 5′ untranslated region of *prfA* RNA, which inhibits translation of PrfA protein at non-physiological temperatures, and thus limits virulence gene expression to temperatures of 37°C and above (Johansson *et al.*, 2002). Conversely, transcription of flagellar motility genes is repressed at 37°C due to the binding activity of the MogR transcriptional repressor, restricting flagellar motility to low temperatures (30°C and below) (Gründling *et al.*, 2004; Shen and Higgins, 2006).

The bacterial flagellum is a complex substructure requiring the co-ordinate assembly of multiple proteins;

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therefore, most bacterial systems have a three to four tiered hierarchal regulatory cascade controlling the temporal expression and production of flagella (Aldridge and Hughes, 2001; McCarter, 2006). Regulation of flagellar motility in *Lm* differs substantially from other bacterial species as *Lm* lacks conserved master regulators that govern hierarchal regulation of flagella production (Chilcott and Hughes, 2000). In *Lm,* MogR binds directly to flagellar motility gene promoters and represses transcription of all flagellar motility genes in a non-hierarchal manner (Shen and Higgins, 2006). At temperatures below 37°C, the MogR antirepressor GmaR antagonizes MogR repression activity by binding directly to MogR (Shen *et al*., 2006). Temperature-dependent expression of GmaR restricts transcription of flagellar motility genes to low temperatures, however, it is not known how GmaR expression is initiated as temperature decreases below 37°C (Shen *et al*., 2006). Interestingly, transcription of *gmaR* is MogR-repressed and therefore GmaR-regulated; as GmaR is initially produced, transcription of *gmaR* and production of GmaR protein are consequently upregulated (Shen *et al*., 2006). Epistasis and microarray analysis have shown that in addition to GmaR, the DegU response regulator (RR) is also required to relieve MogR repression at low temperatures (Shen *et al*., 2006; Shen and Higgins, 2006). Constitutive expression of GmaR in a Δ*degU* strain restores flagellar motility gene expression (Shen *et al*., 2006), indicating that GmaR acts downstream of DegU and is likely DegU-regulated.

Bacterial two-component signal transduction systems (TCS) enable bacteria to sense environmental changes such as temperature and transduce them into transcriptional responses. A typical TCS consists of a sensor histidine kinase (HK) that detects environmental signals and a cognate RR that binds to DNA to mediate a cellular response. Communication between the HK and RR is controlled by a phosphotransfer event from HK to RR that changes the conformation of the RR DNA-binding domain, thus altering DNA binding specificity of the RR (Bijlsma and Groisman, 2003; Khorchid and Ikura, 2006). Regulation of flagellar motility in several bacterial species is mediated by the activities of TCS that can function as repressors such as the *Bordetella pertussis* BvgAS system or function as activators such as the FlgRS system of *Campylobacter jejuni* (Wosten *et al.* 2004). Interestingly, the *Bacillus subtilis* (*Bs*) DegU RR has the unique ability to either repress or activate transcription of flagellar motility genes depending on the phosphorylation state of the RR receiver domain (Amati *et al*., 2004; Kobayashi, 2007).

The *Bs*DegS sensor kinase alters the DNA binding specificity of *Bs*DegU by mediating phosphorylation of a conserved phosphoryl acceptor site (Asp56) located in the *Bs*DegU N-terminal receiver domain (Mukai *et al*., 1990; Dahl *et al*., 1991). When phosphorylated, *Bs*DegU binds and represses the *fla-che* flagellar motility gene operon (Amati *et al*., 2004). However, transcriptional profiling of a *Bs*DegU deletion strain revealed that *Bs*DegU also activates flagellar motility gene transcription (Kobayashi, 2007). The phosphorylation state of *Bs*DegU is therefore critical for its specific regulatory roles since phosphorylation of the receiver domain alters *Bs*DegU DNA-binding activity (Dahl *et al*., 1992; Kobayashi, 2007). In *Bs*, DegU plays multiple roles in gene regulation for flagellar motility, but also functions in regulating exoprotease production, competence development, biofilm formation and swarming motility (Dahl *et al*., 1992; Hamoen *et al*., 2000; Kobayashi, 2007; Verhamme *et al*., 2007) where *Bs*DegU can function as a transcriptional activator, coactivator or repressor. The *Lm*DegU RR is highly homologous to the *Bs*DegU RR (63% identical, 78% similar). Similar to *Bs*DegU, *Lm*DegU is required for flagellar motility (Knudsen *et al*., 2004). However, unlike *Bs*DegU, *Lm*DegU is an orphan RR since a cognate sensor kinase is absent from the *Lm* genome. Nonetheless, it was recently reported that a phosphoryl acceptor site mutation ($D_{55}N$) in *Lm*DegU reduces flagellin transcription and motility in *Lm* (Gueriri *et al*., 2008; Mauder *et al*., 2008).

In this report, we further define the role of the DegU RR in GmaR production and temperature-dependent flagellar motility in *Lm*. We determined that *Lm*DegU can be phosphorylated *in vitro*; however, the receiver domain is dispensable for DegU-dependent regulation of flagellar motility. We further demonstrated that DegU activates *gmaR* transcription by binding directly to *fliN-gmaR* promoter region DNA; however, DegU-dependent activation of *fliN-gmaR* is temperature-independent. We also determined that a post-transcriptional mechanism limits GmaR protein production to low temperatures, as *gmaR* transcript levels are similar at both 37°C and 30°C in Δ*mogR* bacteria, yet GmaR protein is differentially expressed. Thus, our findings reveal that flagellar motility in *Lm* is governed by both temperature-dependent transcriptional and post-transcriptional control of the GmaR antirepressor.

Results

The receiver domain of DegU is dispensable for flagellar motility

Temperature-dependent expression of the GmaR antirepressor restricts flagellar motility gene expression to low temperatures in *Lm* (Shen *et al*., 2006). Although the DegU RR is known to be required for *gmaR* transcription, the regulatory mechanisms governing temperature-dependent production of GmaR remain elusive. We pre-

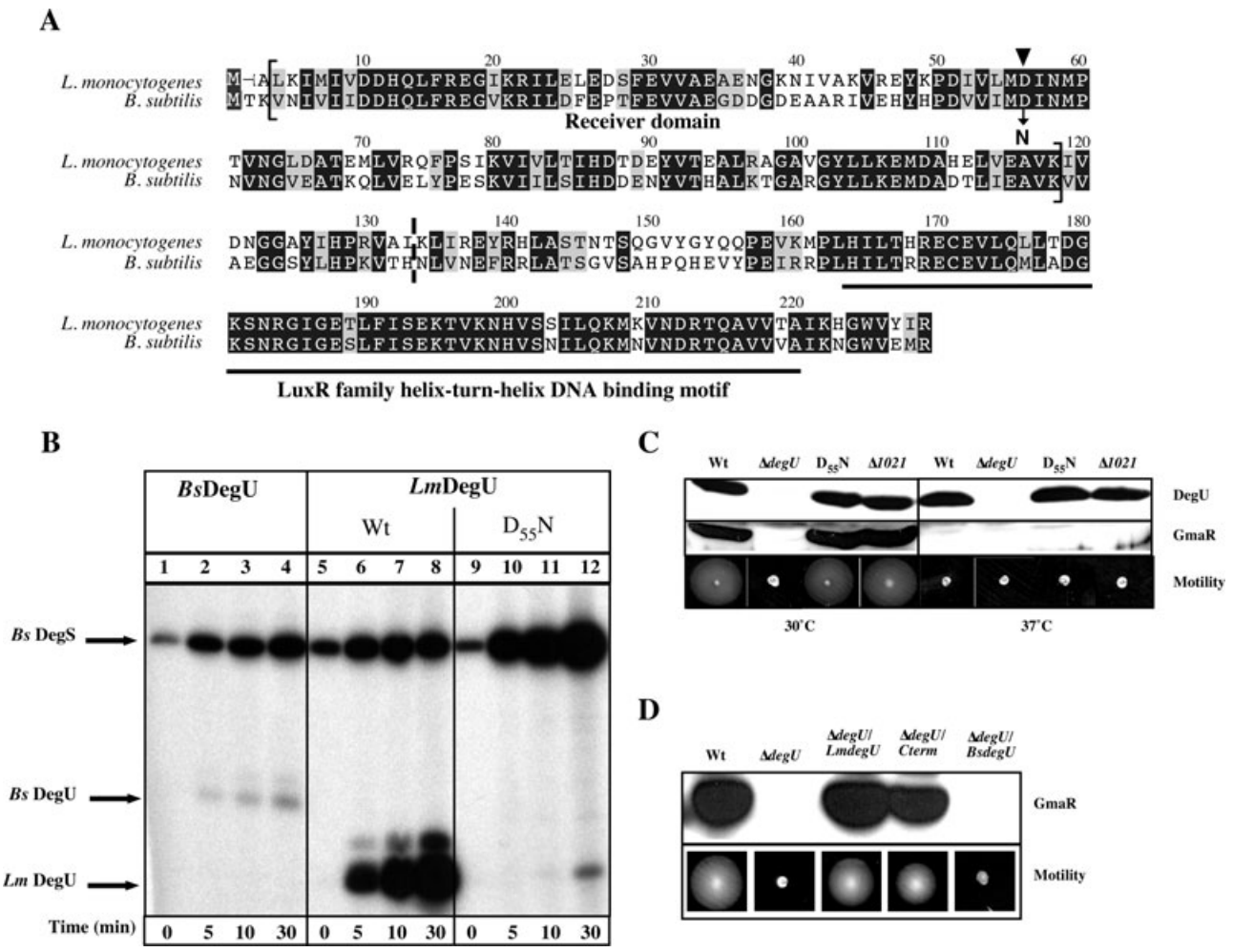

Fig. 1. Phosphorylation of DegU.
A. Alignment of amino acid residues of *Lm*DegU and *Bs*DegU proteins. Conserved identical residues are blocked in black and similar residues are blocked in grey. The *Lm*DegU phosphorylation receiver domain (aa 3–117) is indicated by brackets and contains the conserved phosphoryl acceptor site D55 (▼). The LuxR/FixJ family helix–turn–helix motif (aa 162–219) is underlined in black. The N-terminal truncation site in Δ*degU*/Cterm is marked by a dashed line between aa 132 and aa 133.
B. *In vitro* phosphorylation of DegU. Purified *Bs*DegS sensor kinase was incubated with either *Bs*DegU (lanes 1–4), wild-type (Wt) *Lm*DegU (lanes 5–8), or $Lm\text{DegU}_{D55N}$ (lanes 9–12) in the presence of [γ-^{32}P]-ATP. $Lm\text{DegU}_{D55N}$ has a phosphoryl acceptor site mutation Asp55 to Asn. Reactions were incubated at room temperature (RT) 5, 10 or 30 min Proteins were resolved by SDS-PAGE and phosphorylation was detected using autoradiography.
C. Western blot and motility analysis of DegU_{D55N} and Δ*lmo1021* bacteria. DegU and GmaR protein levels were examined using Western blot analysis of whole-cell lysates prepared from cultures grown at either 30°C or 37°C for 18 h. A GmaR- or DegU-specific polyclonal antibody was used for detection. Motility was examined in low agar (0.3%) motility plates inoculated with a straight needle and incubated for 48 h.
D. Western blot and motility analysis of Δ*degU*/Cterm and Δ*degU*/BsDegU bacteria. A DegU-negative strain was complemented with either full-length *Lm*DegU (Δ*degU*/*LmdegU*), a N-terminally truncated DegU (Δ*degU*/*Cterm*), or full-length *Bs*DegU (Δ*degU*/*BsdegU*). Whole-cell lysates were prepared from cultures grown at 30°C for 18 h. Western blot analysis was used to detect GmaR using a GmaR-specific polyclonal antibody. Motility was also examined at 30°C in low agar (0.3%) motility plates inoculated with a straight needle and incubated for 48 h.

viously reported that DegU protein levels are temperature-independent (Shen *et al*., 2006); therefore, changes in DegU activity may confer temperature specificity for flagellar motility gene transcription. *Lm*DegU is highly homologous to the *Bs*DegU RR, which contains an N-terminal receiver domain and a C-terminal helix–turn–helix DNA binding motif (Fig. 1A). The N-terminal conserved phosphoryl acceptor site Asp56 of *Bs*DegU is phosphorylated by the cognate sensor kinase DegS (Dahl *et al*., 1991). The phosphorylation state of *Bs*DegU is critical for its specific regulatory roles since phosphorylation of the receiver domain alters *Bs*DegU DNA-binding activity (Dahl *et al*., 1992). We therefore hypothesized that phosphorylation of the *Lm*DegU receiver domain may modify *Lm*DegU DNA-binding activity and possibly confer temperature specificity to flagellar motility.

Genetic mutation of the conserved phosphoryl acceptor site Asp56 to Asn in *Bs*DegU (D56N) inhibits phosphorylation *in vitro* and alters *Bs*DegU DNA binding specificity (Dahl *et al*., 1991). It was recently published that the corresponding amino acid change in *Lm*DegU (D55N) results in bacteria that are less motile than wild type and produce less flagellin transcripts (Gueriri *et al*., 2008; Mauder *et al*., 2008). *In vitro* phosphorylation studies of His_6-tagged *Bs*DegS and *Lm*DegU confirmed that the *Bs*DegS HK can phosphorylate *Lm*DegU and that the $D_{55}N$ alteration abolishes the phosphotransfer event (Fig. 1B and Gueriri *et al*., 2008). The low level of detectable ^{32}P-labelled $LmDegU_{D55N}$ at the 30 min time point (Fig. 1B) is likely not physiologically relevant since a similar observation has been previously reported with $CheY_{D55N}$ in *Escherichia coli* (*Ec*) and is the result of a phosphoester group on a heterologous residue (Bourret *et al*., 1990; Lukat *et al*., 1991). To determine if the $D_{55}N$ phosphoryl acceptor site substitution in *Lm* affects *Lm* flagellar motility, Western blot and motility assays were performed using a $LmDegU_{D55N}$ substituted strain. Analysis revealed that temperature-dependent expression of GmaR in $LmDegU_{D55N}$ bacteria was similar to wild type and did not affect flagellar motility in a low-agar-motility assay (Fig. 1C), unlike what has been previously reported (Gueriri *et al*., 2008; Mauder *et al*., 2008). Although *Lm*DegU is an orphan RR, a type II HK similar to *Bs*DegS could potentially trans-phosphorylate *Lm*DegU as seen previously with the NarL RR in *Ec* (Schröder *et al*., 1994). Deletion of *lmo1021* (Δ*1021*), the only putative type II sensor kinase in *Lm*, also did not affect GmaR expression or flagellar motility (Fig. 1C). Furthermore, additional *in vitro* phosphorylation studies did not detect phosphorylation of *Lm*DegU by Lmo1021 (data not shown). Taken together, these results strongly suggest that phosphorylation of the conserved *Lm*DegU phosphoryl acceptor site does not significantly regulate *Lm*DegU activity. If the D55 phosphoryl acceptor site of *Lm*DegU is dispensable, then it is possible that the entire N-terminal receiver domain may be dispensable for DegU-dependent regulation of GmaR. To examine this hypothesis, *Lm*DegU was N-terminally truncated at amino acid 133 and heterologously expressed in a DegU-negative strain (Δ*degU*/*Cterm*). The Δ*degU*/*Cterm* strain, harbouring the *Lm*DegU helix–turn–helix DNA binding domain and lacking the receiver domain, still produced GmaR and was motile at low temperature (Fig. 1D). Collectively, these results demonstrate that *Lm*DegU is able to function to produce GmaR in the absence of phosphorylation of the conserved phosphoryl acceptor site and that only the C-terminal portion of DegU containing the DNA binding domain is sufficient. In addition, whereas *Lm*DegU and *Bs*DegU are highly homologous, *Bs*DegU does not complement *Lm* Δ*degU* (Fig. 1D), reinforcing the contrasting roles DegU plays in these bacterial species.

Temperature-dependent transcription of gmaR *initiates from the* fliN *promoter*

Whereas hierarchal regulation in most bacterial species ensures ordered production and assembly of flagella, MogR repression of all flagellar motility gene promoters in *Lm* results in a non-hierarchal regulatory scheme. Derepression of MogR by the GmaR antirepressor is absolutely necessary for flagellar motility gene transcription to occur at low temperatures. To further understand temperature-dependent expression of GmaR, we sought to determine the mechanism of DegU-dependent regulation of *gmaR* transcription.

gmaR is located in the first operon of the flagellar motility gene locus ~10 kb downstream of the *fliN* promoter (p_{fliN}) (Fig. 2A). To determine if p_{fliN} is the only promoter controlling transcription of *gmaR*, we constructed transcriptional fusions of regions of the *fliN-gmaR* operon to a *gusA* reporter and determined β-glucoronidase activity (Fig. 2B). Transcriptional fusions containing the *fliN* and *cheY* promoter regions fused to *gusA* were used as controls and demonstrated β-glucoronidase activity as expected (Fig. 2B; lanes 1 and 6 respectively), whereas transcriptional fusions containing DNA regions from within the *fliN-gmaR* operon yielded no β-glucoronidase activity (Fig. 2B; lanes 2–5). These results suggest that the MogR-regulated promoter directly upstream of *fliN* ($p_{fliN\text{-}gmaR}$) is the only promoter controlling transcription of *gmaR.* Since GmaR functions as a MogR antirepressor and the *fliN-gmaR* promoter is MogR-repressed (Shen *et al*., 2006), these data indicate that production of *gmaR* transcripts is positively regulated by GmaR.

DegU activates gmaR *transcription by binding directly to* fliN-gmaR *promoter region DNA*

While *gmaR* transcription is repressed by MogR at 37°C, it is unknown how transcription of *gmaR* is initiated as temperatures decrease below physiological levels. Given our previous findings that *gmaR* transcripts are absent in Δ*degU* bacteria and that GmaR functions downstream of DegU (Shen *et al*., 2006), we hypothesized that DegU may activate the $p_{fliN\text{-}gmaR}$ promoter to initiate transcription of *gmaR*. Analysis of $p_{fliN\text{-}gmaR}$ activity by both primer extension and transcriptional fusions revealed that DegU is required for transcriptional activation of the *fliN-gmaR* promoter (Fig. 3A and B). DegU-dependent transcriptional activation was specific for the *fliN-gmaR* promoter and was not required for transcription from other flagellar motility gene promoters (*flaA* shown in Fig. 3A and unpublished data). Surprisingly, DegU-mediated activation of $p_{fliN\text{-}gmaR}$ in a MogR-negative strain (Δ*mogR*) occurred at both 30°C and 37°C (Fig. 3B). This result indicates that

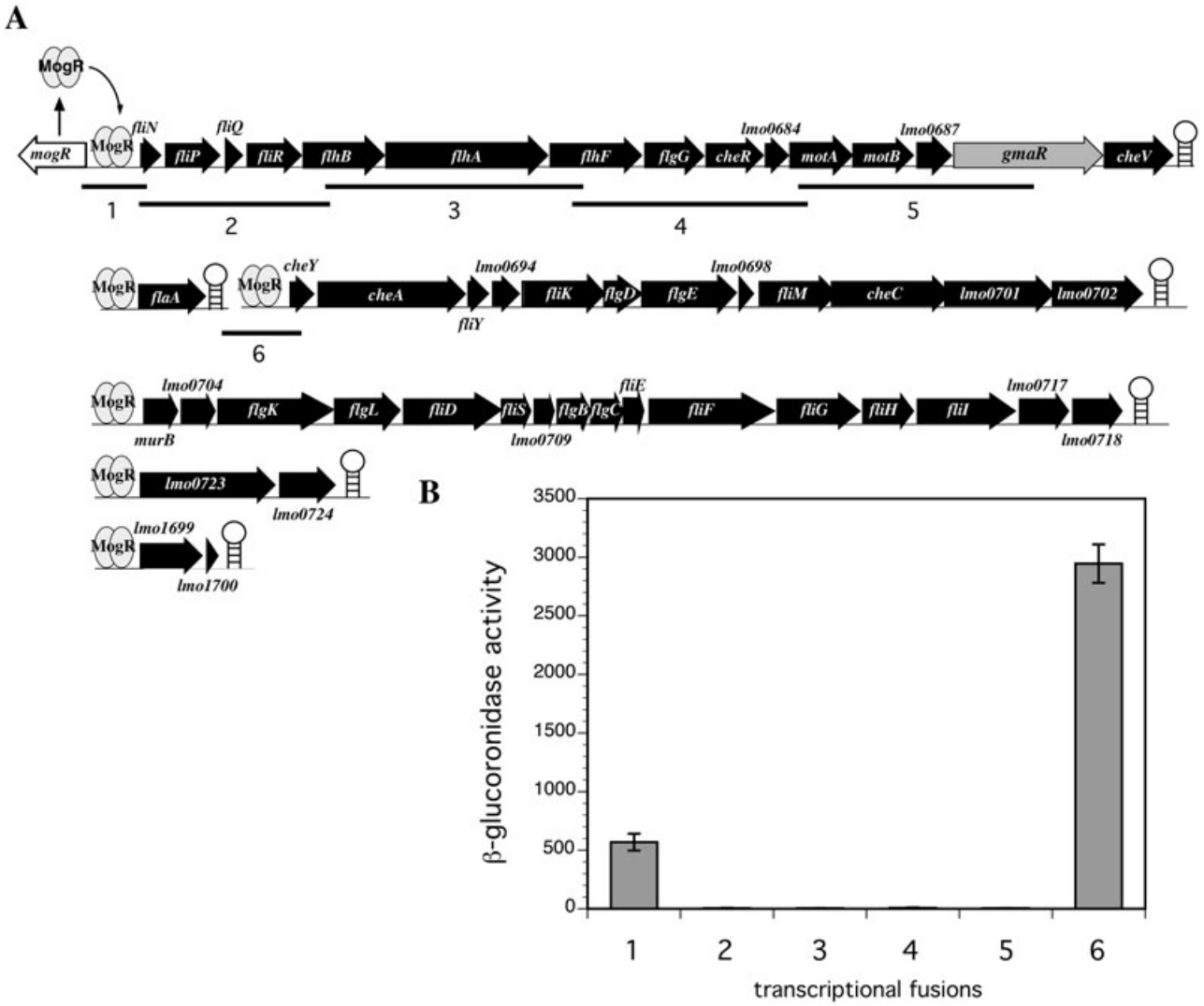

Fig. 2. *gmaR* is transcribed from a promoter upstream of *fliN*.
A. Genetic organization of the *Lm* flagellar motility gene locus. The gene encoding the MogR antirepressor, *gmaR,* is located in the first operon of the flagellar motility gene locus with transcription initiating upstream of *fliN* and terminating at a predicted transcriptional terminator (open circle on ladder). *mogR* is divergently transcribed from *fliN* and MogR functions to repress transcription of all six flagellar motility gene promoters. Numbered underlined fragments correspond to the transcriptional fusions described in Fig. 2B.
B. Analysis of *fliN-gmaR* promoter activity as determined by β-glucuronidase assay. DNA regions from the *fliN-gmaR* operon (labelled 1–5 in Fig. 2A) were fused to a *gusA* reporter and integrated in single copy into the $tRNA^{Arg}$ locus of wild-type *Lm*. As a positive control, the *cheY* promoter region was also fused to a *gusA* reporter (labelled 6 in Fig. 2A). Bacteria were grown at 30°C in BHI broth for 18–20 h. β-Glucuronidase activities represent the means and standard deviations of three independent experiments.

the inherent ability of DegU to activate $p_{fliN\text{-}gmaR}$ transcription is temperature-independent.

To identify the DNA elements within the $p_{fliN\text{-}gmaR}$ region that are required for DegU-dependent activation, promoter region truncations spanning –278, –209 and –173 to +274 nucleotides (nt) relative to the transcriptional start site were examined using reporter fusions to *gusA* (Fig. 3C). The $p_{fliN\text{-}gmaR}$ truncation fusions revealed that DNA elements located between –209 and –173 nt upstream of the predicted *fliN-gmaR* transcriptional start site are required for DegU-dependent activation (Fig. 3B).

To further support the transcriptional fusion studies, DNA affinity purification was used to determine if DegU binds directly to the *fliN-gmaR* promoter region. Biotin-labelled *fliN-gmaR* promoter region DNA coupled to Strepavidin-beads was incubated with lysates isolated from Δ*degU,* wild type or Δ*mogR* bacteria. Bound proteins were eluted and then separated on an SDS-PAGE gel and analysed by Western blot (Fig. 4A). In support of the requirement for DegU in transcriptional activation of $p_{fliN\text{-}gmaR}$ (Fig. 3A and B), DegU present in both wild-type and Δ*mogR* lysates was shown to bind to the –278 to +100 *fliN-gmaR*

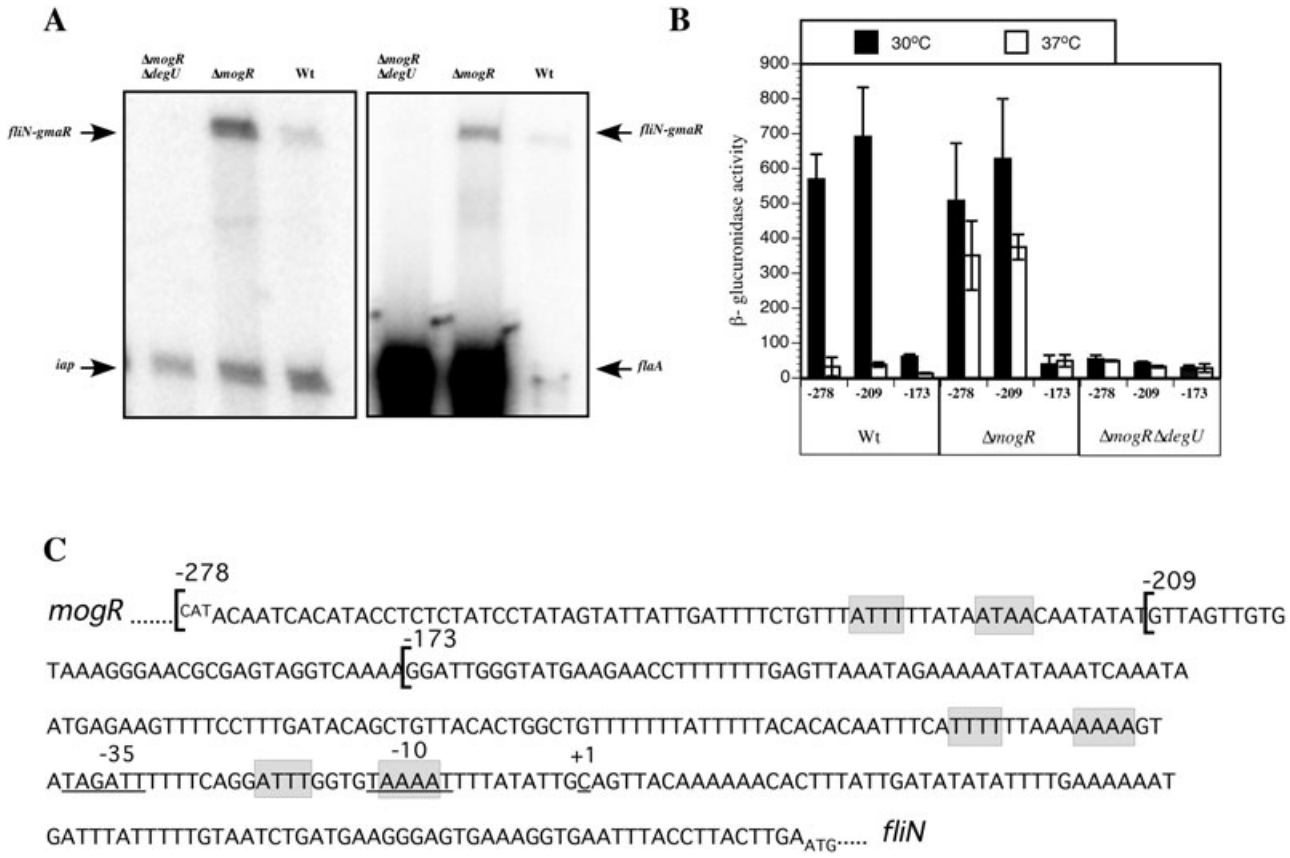

Fig. 3. The *fliN-gmaR* promoter is DegU-activated.
A. Analysis of *fliN-gmaR* transcripts by primer extension. RNA was extracted from Δ*mogR* Δ*degU,* Δ*mogR* and wild-type (Wt) strains grown at 30°C in BHI broth for 6 h. Transcript specific primers were end-labelled with [γ-^{32}P]-ATP. Primer extension products were separated on a 5% denaturing acrylamide gel and detected by a phosphorimager. Primers for *flaA* and *iap* were included in independent experiments as controls.
B. Analysis of *fliN-gmaR* promoter activity as determined by β-glucuronidase assay. Transcriptional fusions of *fliN-gmaR* promoter region DNA to *gusA* were integrated in single copy into the tRNA^{Arg} locus of wild-type (Wt), Δ*mogR* or Δ*mogR* Δ*degU* bacteria. Cultures were grown in BHI broth at 30°C or 37°C for 18–20 h prior to analysis. β-Glucuronidase activities represent the means and standard deviations of three independent experiments. Numbers correspond to fusions harbouring –278, –209 or –173 through +274 relative to the transcriptional start of *fliN-gmaR*.
C. DNA sequence of the *mogR-fliN* intergenic region. The DNA sequence begins with the start codon (negative strand) for *mogR* and ends with the start codon for *fliN*. The transcriptional start site of the *fliN-gmaR* promoter (+1) was identified by primer extension and is underlined along with the predicted –35 and –10 elements. Transcriptional fusions of the *fliN-gmaR* promoter region DNA (–278, –209, –173 through +274 relative to the transcriptional start) are indicated by brackets and were fused to a *gusA* reporter and analysed in Fig. 2B. The predicted MogR binding sites are shaded in grey.

promoter region DNA fragment (Fig. 4A). Since DegU did not bind the –173 to +100 *fliN-gmaR* promoter region DNA, this result strengthens the hypothesis that DegU activates the *fliN-gmaR* promoter region by binding to DNA encompassing sequences –173 to –209 nt upstream of the transcriptional start site (Figs 3B and 4A). As expected, MogR bound both the –278 and –173 to +100 *fliN-gmaR* promoter region DNA fragments (Fig. 4A) since predicted MogR binding sites are contained within both of these DNA fragments (Fig. 3C).

Gel mobility shift analysis was subsequently used to determine if His-tagged purified *Lm*DegU can bind $p_{fliN\text{-}gmaR}$ region DNA *in vitro* in the absence of cell lysates. Increasing concentrations of DegU incubated with radiolabelled *fliN-gmaR* promoter region DNA fragments, spanning –278 and –209 to +100 relative to the transcriptional start site, resulted in the formation of shifted (S) and super-shifted (SS) DNA complexes (Fig. 4B, lanes 1–10), whereas incubation of DegU with the –173 to +100 DNA fragment resulted in only partially shifted DNA complexes (S) at high DegU concentrations (Fig. 4B, lanes 11–15). These results indicated that purified *Lm*DegU was able to bind to *fliN-gmaR* promoter region DNA spanning –278 and –209 to +100, but was unable to efficiently bind to *fliN-gmaR* promoter region DNA spanning –173 to +100 nt relative to the start of transcription. DegU did not require any additional bacterial factors to bind *fliN-gmaR* promoter region DNA since purified DegU alone was

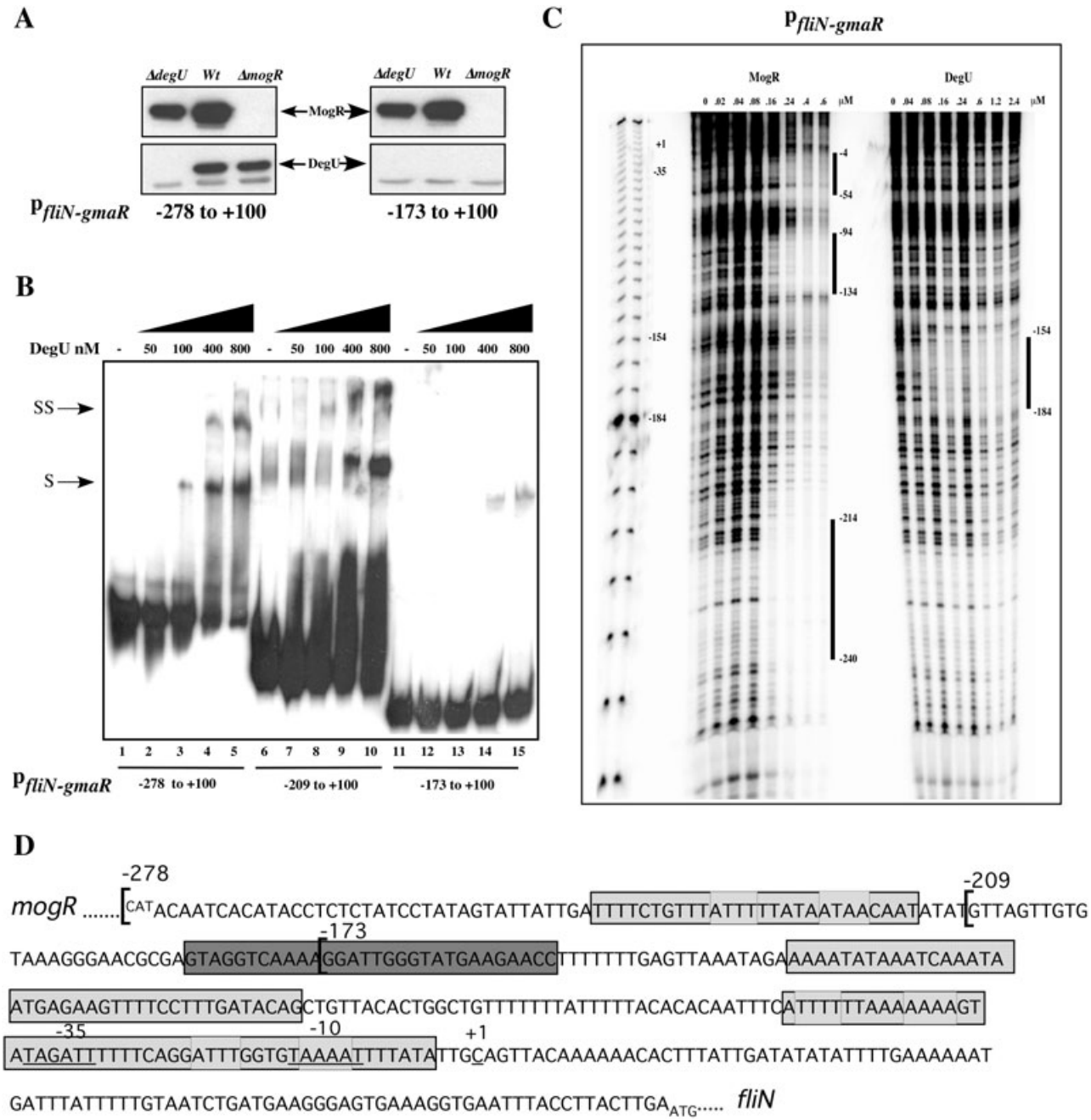

Fig. 4. DegU binds directly to *fliN-gmaR* promoter region DNA.
A. Affinity purification of proteins that specifically bind *fliN-gmaR* promoter region DNA. Whole-cell lysates of wild-type (Wt), Δ*degU* or Δ*mogR* bacteria were incubated at RT with magnetic Dynabeads coupled to *fliN-gmaR*-specific promoter region fragments (–278 to +100 or –173 to +100 relative to the transcriptional start site). DNA–bead complexes were washed, proteins were eluted and then separated by SDS-PAGE. DegU- or MogR-specific polyclonal antibody was used for Western blot detection. Arrows indicate DegU and MogR.
B. Gel shift analysis of DegU binding to *fliN-gmaR* promoter region DNA. Radiolabelled *fliN-gmaR* promoter region DNA fragments spanning –278, –209 or –173 to +100 relative to the transcriptional start site were incubated at RT with increasing amounts of purified His_6-tagged *Lm*DegU. Binding reactions were separated by non-denaturing PAGE and detected by autoradiography. Shifted (S) and Super-shifted (SS) complexes were detected.
C. DNase I footprint analysis of MogR and DegU binding to *fliN-gmaR* promoter region DNA. A radiolabelled DNA probe spanning the *fliN-gmaR* promoter region from –278 to +100 relative to the transcriptional start site was incubated at RT with increasing amounts of purified His_6-tagged *Lm*DegU or MogR and subsequently treated with DNaseI. Samples were run on a denaturing 6% acrylamide gel and the footprint was detected by phosphoimager. Binding sites are labelled with negative numbers relative to the transcriptional start site.
D. DNA sequence of the *mogR-fliN* intergenic region showing DegU and MogR binding sites. The DNA sequences corresponding to the three regions of MogR binding as identified in Fig. 4C by DNaseI footprint analysis are shaded in grey. The predicted MogR binding sites are marked with light grey boxes. The DegU footprint as identified in Fig. 4C is shaded with dark grey.

sufficient for binding and shifting the DNA fragments. Identical results were obtained when the DNA affinity purification and gel mobility shift analyses were performed at 37°C, suggesting that the *in vitro* DNA binding ability of DegU is not affected by temperature (data not shown). Taken together, the DNA affinity purification, gel mobility shift analysis and transcriptional activation data indicate that DegU binding to *fliN-gmaR* promoter region DNA requires sequences within –173 and –209 nt upstream of the transcriptional start site and that DegU binding mediates transcriptional activation of the *fliN-gmaR* promoter.

To further localize the specific DNA binding site(s) for DegU and MogR within the *mogR-fliN* intergenic region, DNase I footprinting analysis was performed using *fliN-gmaR* promoter region DNA and purified His-tagged *Lm*DegU and His-tagged MogR. At low concentrations, MogR bound to three distinct regions of the *fliN-gmaR* promoter region DNA: –4 to –54, –94 to –134 and –214 to –240 relative to the transcriptional start site (Fig. 4C and D), which is similar to MogR binding to *flaA* promoter region DNA (Shen and Higgins, 2006). The DegU footprint spanned nt –184 to –154 relative to the transcriptional start site (Fig. 4C and D). The DegU footprint corresponds to the *fliN-gmaR* promoter region sequences required for DegU binding that were identified by DNA affinity purification (Fig. 4A), gel mobility shift analysis (Fig. 4B), as well as DegU-dependent transcriptional activation using *gusA* reporter fusions (Fig. 3B). Interestingly, the MogR and DegU binding sites do not overlap as determined by DNase I footprinting analysis (Fig. 4C and D).

A post-transcriptional mechanism controls temperature-dependent GmaR production

We have previously shown that both Δ*mogR* Δ*degU* and Δ*degU* constitutively expressing GmaR (Δ*degU*/c*gmaR*) are non-motile at low temperatures (< 37°C) despite evidence that flagellin is produced (Shen *et al*., 2006; Shen and Higgins, 2006). Since DegU is required for transcriptional activation of the first operon in the *Lm* flagellar motility gene cluster (Fig. 3A and B), we hypothesized that the DegU-dependent motility defect of Δ*mogR* Δ*degU* bacteria is due to the absence of essential flagellar biosynthetic components encoded in the *fliN-gmaR* operon that are required for proper flagellar assembly and motility. Effectively, DegU-dependent transcriptional activation of the entire *fliN-gmaR* operon and not just *gmaR* is required for flagellar motility in *Lm* at low temperatures. To confirm this hypothesis, a *Lm* strain was constructed in which a promoter (p_{Hy}) was inserted upstream of the *fliN-gmaR* promoter in a Δ*mogR* Δ*degU* strain to provide constitutive transcription of the *fliN-gmaR* operon in a MogR-negative, DegU-negative background (Fig. 5A, Δ*mogR* Δ*degU*/p_{Hy}). Motility assay analysis revealed that the Δ*mogR* Δ*degU*/p_{Hy} strain was slightly motile at low temperature. While the Δ*mogR* Δ*degU*/p_{Hy} strain was not as motile as MogR-negative bacteria, the lack of increased motility is likely due to placement of the p_{Hy} promoter and promoter strength relative to the native DegU-activated $p_{fliN\text{-}gmaR}$ promoter. However, the fact that constitutive expression of the *fliN-gmaR* operon overcomes the DegU-dependent motility defect, strongly suggests that the requirement for DegU in *Lm* flagellar motility is strictly for transcriptional activation of the *fliN-gmaR* operon. Since we have established that DegU is capable of activating transcription of the *fliN-gmaR* promoter and that this activation does not appear to be temperature-dependent (Fig. 3B), it remained to be determined how GmaR production is restricted to low temperatures (Fig. 1C).

To determine if a post-transcriptional mechanism controls temperature-dependent GmaR production, we analysed *gmaR* transcript and GmaR protein levels in a Δ*mogR* strain, which allows temperature-independent transcription of *gmaR* to occur (Fig. 3B). Quantitative Real-Time PCR analysis revealed that *gmaR* transcript levels in Δ*mogR* bacteria do not change in response to temperature (Fig. 5B). This result correlated with transcriptional fusion studies indicating that in Δ*mogR* bacteria, DegU can activate transcription of $p_{fliN\text{-}gmaR}$ at low temperatures (30°C) and at 37°C (Fig. 3B). Collectively, these results reveal that although DegU is required for activation of the *fliN-gmaR* promoter, transcriptional activation is temperature-independent in the absence of MogR. However, since temperature-dependent expression of GmaR protein is observed in Δ*mogR* bacteria (Fig. 5C), a temperature-dependent, post-transcriptional mechanism must control GmaR production. The data in Fig. 5 indicate that two levels of regulation (transcriptional and post-transcriptional) govern production of GmaR and thus the temperature specificity of *Lm* flagellar motility.

Discussion

Temperature-dependent regulation of flagellar motility in *Lm* is mediated by the activities of the MogR transcriptional repressor, the MogR antirepressor GmaR and the DegU RR. In this study, we determined that DegU-dependent transcriptional activation of the first operon in the flagellar motility gene cluster, which contains *gmaR,* is required for *Lm* flagellar motility at low temperatures. DegU activates transcription of the *fliN-gmaR* operon by binding directly to *fliN-gmaR* promoter region DNA. DegU-dependent activation does not depend on the phosphorylation of the conserved DegU phosphoryl acceptor site (Asp55) and is temperature-independent. Furthermore, in Δ*mogR* bacteria, DegU-dependent activation of *fliN-gmaR* transcription is constitutive. Our data indicate that temperature specificity of flagellar motility in *Lm* occurs

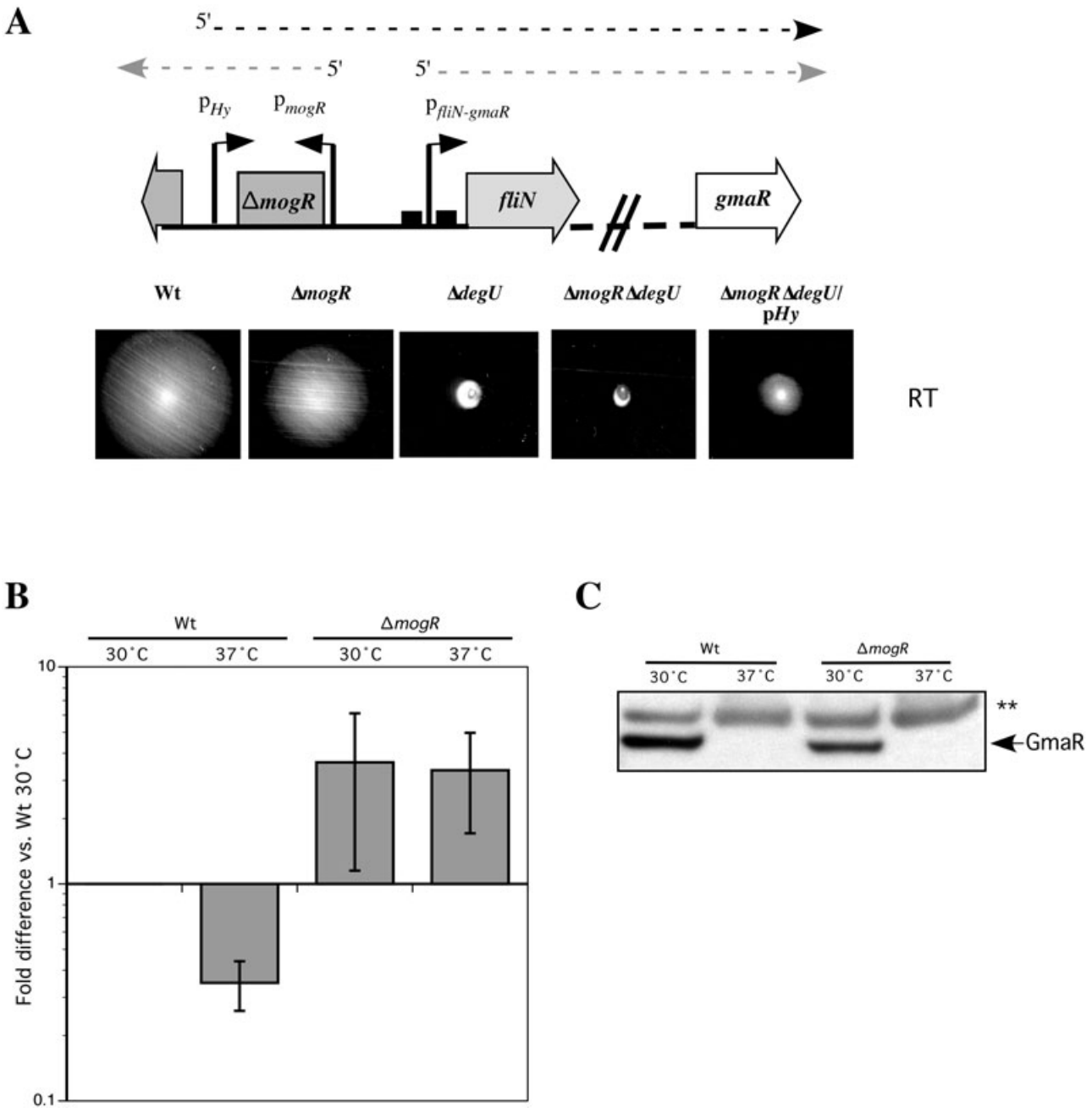

Fig. 5. A post-transcriptional mechanism controls temperature-dependent production of GmaR.
A. Constitutive expression of the *fliN-gmaR* operon in Δ*mogR* Δ*degU*. Native promoters for *mogR* and *fliN-gmaR* are represented by bent arrows (p_{mogR} and $p_{fliN\text{-}gmaR}$). MogR binding sites are marked as boxes overlapping the *fliN-gmaR* promoter. Divergent transcripts initiating from the native promoters are drawn as light dashed arrows. The p_{Hy} promoter was inserted upstream of the native *fliN-gmaR* promoter in a Δ*mogR* Δ*degU* strain to constitutively drive expression of the *fliN-gmaR* operon. Transcription initiating from this promoter is drawn as a dark dashed line. For the motility assay analysis, strains were inoculated into low agar (0.3%) motility plates with a straight needle and incubated at RT for 48 h.
B. Real-time quantitative PCR analysis of *gmaR* transcripts. RNA was extracted from wild-type (Wt) and Δ*mogR* strains grown at either 30°C or 37°C for 24 h. Samples were DNaseI treated and reverse-transcribed with random hexamers to generate cDNA. Relative gene expression was quantified by using real-time PCR and the Pfaffl method ($2^{-\Delta\Delta CT}$). Results represent the average and standard deviation of three independent experiments. The *iap* gene was used as an internal standard and the Wt 30°C sample was set as the calibrator.
C. GmaR protein analysis by Western blot. Total protein samples from cultures used in B were processed for SDS-PAGE and Western blot analysis. A GmaR-specific polyclonal antibody was used for detection. ** indicates a non-specific band that is shown as a loading control.

through a temperature-dependent, post-transcriptional mechanism that limits GmaR expression to temperatures below 37°C. At permissive temperatures, we hypothesize that GmaR production leads to positive auto-regulation of *gmaR* transcription and subsequent transcriptional upregulation of all flagellar motility genes. Therefore, our results demonstrate that flagellar motility in *Lm* is restricted to low temperatures by both MogR transcriptional repression and post-transcriptional regulation of the GmaR antirepressor.

While MogR repression and GmaR antirepression regulate transcription of all flagellar motility gene promoters, DegU is required to activate transcription of $p_{fliN\text{-}gmaR}$ for production of GmaR (Fig. 3A and data not shown). Furthermore, constitutive expression of the *fliN-gmaR* operon in Δ*mogR* Δ*degU* bacteria revealed that DegU-dependent transcription of additional flagellar biosynthetic components within the *fliN-gmaR* operon is necessary for flagellar motility (Fig. 5A). Since the *fliN-gmaR* operon encodes several proteins comprising the flagella export apparatus, the complete lack of flagella in Δ*mogR* Δ*degU* bacteria, despite production of the flagellin subunit (Shen and Higgins, 2006), is likely explained by the absence of proteins necessary for flagellin secretion and flagellum assembly. Therefore, DegU-dependent activation of $p_{fliN\text{-}gmaR}$ is necessary for production of GmaR, but is also required for the proper assembly of flagella.

DegU-dependent activation of $p_{\text{fliN-gmaR}}$

Our results indicated that *Lm*DegU binds directly to *fliN-gmaR* promoter region DNA at −154 to −184 nt upstream of the *fliN-gmaR* transcriptional start site and that binding of DegU to this region is essential for transcriptional activation of the *fliN-gmaR* promoter (Figs 3 and 4). Gel mobility shift and DNaseI footprinting analyses using purified protein confirmed that DegU binds *fliN-gmaR* promoter region DNA independent of additional cellular factors (Fig. 4B and C). Since the gel mobility shift analysis showed a super-shifted complex at higher concentrations of DegU (Fig. 4B), but footprinting analysis only indicated one DNA binding region (Fig. 4C and D), it is possible that DegU binds either as a multimer or that DegU binds multiple sites within the −154 to −184 region. Although the DegU binding site is located 94 nt upstream of the translational start site of MogR (Fig. 4D), MogR expression was found to be constitutive under all conditions examined and unaffected by the deletion of DegU (data not shown). While many transcriptional activators bind close to the −35 region of their regulated promoters, it is not unprecedented for activators to bind promoter region DNA further upstream of the transcriptional start site and still directly activate transcription. DegU binding to the −184 to −154 region upstream of the *fliN-gmaR* promoter may facilitate bending of the DNA to allow contact of DegU with RNA polymerase or may change the conformation of the promoter region DNA to enhance RNA polymerase binding (Rhodius and Busby, 1998). It is also possible that DegU may be an essential coactivator that recruits another protein factor that then makes direct contact with RNA polymerase to activate transcription of *fliN-gmaR* (Rhodius and Busby, 1998). In fact, *Bs*DegU functions as a priming protein in *Bs* competence development. Binding of *Bs*DegU to the *comK* promoter facilitates ComK binding and auto-regulation of *comK* transcription. Therefore, *Bs*DegU is important for initiation of the competence auto-regulatory loop when ComK levels are insufficient to support *comK* transcription (Hamoen *et al*., 2000; Maamar and Dubnau, 2005). Similarly, it is unknown if *Lm*DegU is acting alone at the *fliN-gmaR* promoter or if a coactivator is involved in transcription initiation. However, if *Lm*DegU is interacting with another factor, the interaction domain is presumably contained within the C-terminal portion of DegU, since the N-terminal domain is dispensable for transcriptional activation of *fliN-gmaR* (Fig. 1D).

MogR binding to the $p_{\text{fliN-gmaR}}$ *promoter region*

We previously demonstrated that MogR binds specifically to TTTT-N_5-AAAA operator sites found within *Lm* flagellar motility gene promoter regions (Shen and Higgins, 2006). MogR binding to a minimum of two operator sites overlapping the −10 or −35 regions of flagellar promoters results in occlusion of RNA polymerase binding and represses transcription of flagellar motility genes (Shen and Higgins, 2006). Sequence analysis of the *fliN-gmaR* promoter region revealed that a nearly consensus (one mismatch) MogR binding site overlaps the −10 region of the *fliN-gmaR* promoter and a second consensus binding site is located just upstream of the −35 region (Fig. 3C). DNaseI footprinting analysis confirmed that MogR binds the −10 and −35 regions of the *fliN-gmaR* promoter as well as two additional sites further upstream (Fig. 4C and D). A similar MogR footprinting pattern was observed previously with the *flaA* promoter region (Shen and Higgins, 2006); however, it remains to be determined if the additional upstream binding sites aid in MogR repression. It is possible that MogR binding to multiple sites and/or MogR multimerization between sites may produce a higher order DNA-protein structure. The crystal structure of MogR bound to a DNA binding site indicates that MogR functions as a dimer (Shen *et al*., 2009). Therefore, it is possible that multimers of MogR dimers are required for MogR-dependent repression of flagellar motility. Footprinting analysis of the *fliN-gmaR* promoter region indicated that although both MogR and DegU bind within the region, the MogR binding sites do not overlap the DegU binding site

and therefore it is possible that both factors could be bound to the promoter region simultaneously (Fig. 4C and D). Gel shift analysis of simultaneous DegU and MogR binding of $p_{fliN\text{-}gmaR}$ promoter region DNA provides additional support that neither factor occludes the binding of the other and that both factors could potentially bind $p_{fliN\text{-}gmaR}$ DNA concurrently (Fig. S1). Depending on the relative on/off rates for the binding of MogR and DegU to their respective DNA sites, it is therefore possible that DegU-dependent transcriptional activation could occur as a rare event during a MogR-repressed state (i.e. 37°C).

Phosphorylation of LmDegU

In this study, we demonstrated that although the *Lm*DegU RR is capable of being phosphorylated (Fig. 1B), DegU-dependent activation of flagellar motility does not require phosphorylation of the conserved phosphoryl acceptor site (Fig. 1C and D). In fact, DegU-dependent activation of *fliN-gmaR* could be obtained using just the C-terminal DNA-binding domain (Fig. 1D). Since DegU can function without the N-terminal receiver domain that is typically required for signal recognition (Fig. 1A), it is likely that *Lm*DegU is constitutively active. Nevertheless, we cannot rule out the possibility that the C-terminal fragment used in our studies is a constitutively active form of DegU. While there exist several examples of RRs that do not require phosphorylation for activity (Rotter *et al*., 2006; Hong *et al*., 2007), it is surprising that DegU activity for *Lm* flagellar motility appears to be phosphorylation-independent due to the high homology between *Lm*DegU and *Bs*DegU (Fig. 1A). The phosphorylation state of the *Bs*DegU receiver domain alters *Bs*DegU function as either an activator or repressor of flagellar motility (Kobayashi, 2007; Tsukahara and Ogura, 2008). However, despite the similarities between *Bs*DegU and *Lm*DegU, important amino acid differences must exist to confer DNA-binding specificity in *Lm,* as a *Lm* Δ*degU* strain cannot be complemented with *Bs*DegU (Fig. 1D). Since phosphorylation appears not to have a significant role in DegU-dependent activation of flagellar motility in *Lm*, it is not surprising that *Lm*DegU is an orphan RR that lacks a cognate sensor kinase and deletion of the only related type II sensor kinase in *Lm (lmo1021*) had no affect on flagellar motility (Fig. 1C).

Temperature-dependent control of flagellar motility

Although DegU is required for flagellar motility, we have shown that DegU does not regulate flagellar motility in a temperature-dependent manner as previously hypothesized. While DegU-dependent transcription of the *fliN-gmaR* operon is absolutely required for flagellar motility (Fig. 5A), DegU is able to activate transcription of the *fliN-gmaR* operon at both 30°C and 37°C (Fig. 3B), indicating that DegU activity is temperature-independent. In support of this finding, DNA affinity purification and gel mobility shift analyses at 37°C revealed that DegU binding to the *fliN-gmaR* promoter region is unaffected by temperature (data not shown). Whereas analysis of the *fliN-gmaR* promoter in Δ*mogR* bacteria showed that *gmaR* transcription is initiated at 37°C (Fig. 3B), it was unknown if *gmaR* transcripts were stable at 37°C. Quantitative real-time PCR analysis demonstrated that *gmaR* transcripts were present in equivalent levels at both 30°C and 37°C (Fig. 5B). Despite the abundance of *gmaR* transcripts in Δ*mogR* bacteria, GmaR protein was not detectable at 37°C (Fig. 5C). Therefore, a post-transcriptional mechanism regulates temperature-dependent GmaR expression. This temperature-dependent, post-transcriptional mechanism functions in addition to the transcriptional repression of *gmaR* by MogR; therefore, at 37°C both transcriptional and post-transcriptional mechanisms prevent GmaR production. It is likely that the post-transcriptional mechanism involves either translational control or protein stability, since we have already demonstrated that *gmaR* transcripts are stable at elevated temperatures (Fig. 5B). Studies are currently underway to determine the exact mechanism(s) responsible for the temperature-dependent, post-transcriptional control of GmaR, and to identify potential *cis*- and/or *trans*- acting factors involved.

We favour the following model for temperature-dependent regulation of GmaR and flagellar motility in *Lm* (Fig. 6). When flagellar motility is 'OFF' (1), MogR binds and represses all flagellar motility gene promoters at elevated temperatures (37°C and above). DegU can also bind DNA upstream of the *fliN-gmaR* promoter and activate transcription at 37°C, but due to stringent MogR repression, transcription of *fliN-gmaR* is minimized at elevated temperatures. In addition to MogR transcriptional repression of *fliN-gmaR*, a post-transcriptional mechanism prevents GmaR protein production from *gmaR* transcripts at 37°C (Fig. 6, OFF). As temperatures decrease below 37°C (2), the inherent on/off rates of MogR and DegU to their respective binding sites in the $p_{fliN\text{-}gmaR}$ promoter region will generate *gmaR* transcripts that in the absence of the temperature-dependent post-transcriptional regulatory mechanism, will now yield GmaR protein (Fig. 6, Transition). Once GmaR protein is initially produced (3), GmaR can upregulate *gmaR* transcript levels by alleviating MogR repression from the *fliN-gmaR* promoter (Fig. 6, ON). Elevated levels of GmaR protein can remove MogR from all flagellar motility gene promoters permitting flagellar motility gene transcription at low temperatures. If the post-transcriptional mechanism involves GmaR protein instability at higher temperatures, reduced levels of GmaR at 37°C would release MogR protein to reinitiate repression of *gmaR* and other flagellar

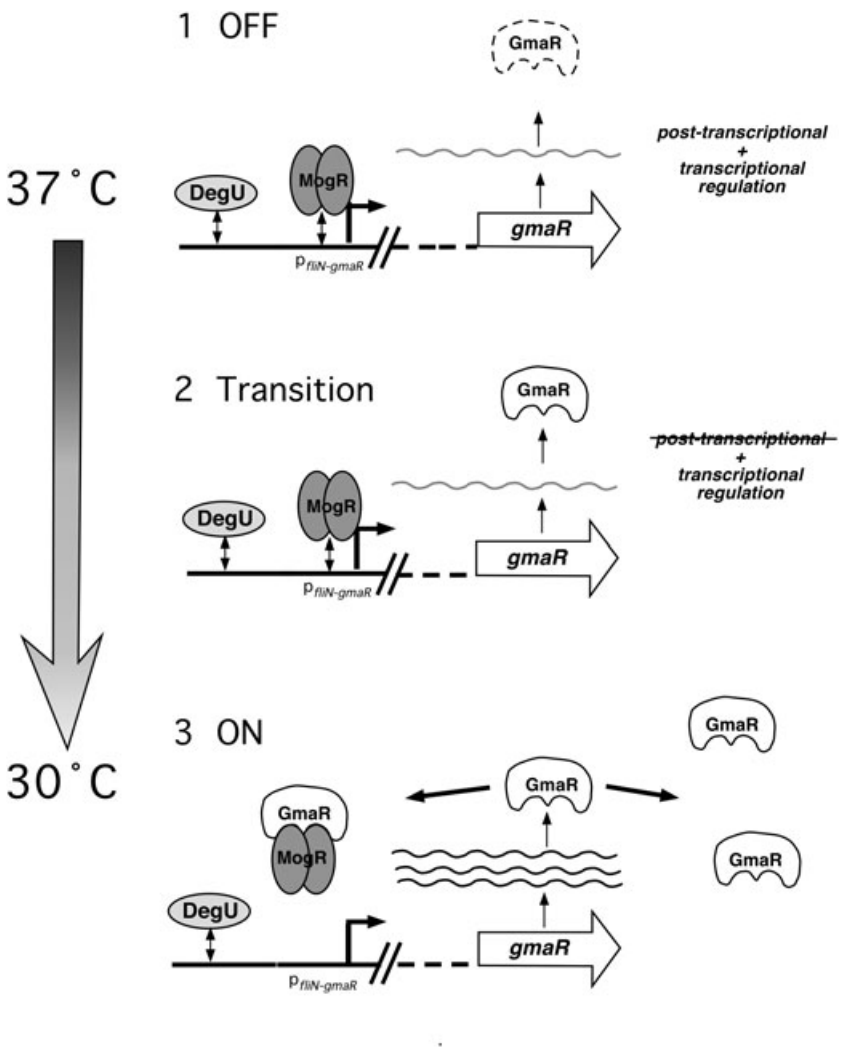

Fig. 6. Model of temperature-dependent regulation of GmaR expression.
1. OFF: At 37°C, when flagellar motility is OFF, the opposing activities of the MogR repressor and the DegU activator at $p_{fliN\text{-}gmaR}$ results in minimal *fliN-gmaR* transcripts. However, a temperature-dependent, post-transcriptional mechanism inhibits GmaR production.
2. Transition: As the temperature decreases below 37°C, the post-transcriptional mechanism is no longer active; therefore, *fliN-gmaR* transcripts result in GmaR protein production.
3. ON: Once GmaR is expressed at low temperatures, GmaR can remove MogR from the *fliN-gmaR* promoter, upregulating transcription of *gmaR*. Elevated levels of GmaR protein results in the relief of MogR repression from all flagellar motility gene promoters, allowing flagellar motility gene transcription and flagellar motility.

motility genes, initiating a transition from an ON to an OFF state.

In this report, we have defined the requirement of DegU for flagellar motility in *Lm*, but the question still lingers as to why a RR has evolved to activate a complex bacterial system constitutively. Moreover, we have uncovered a post-transcriptional layer of regulation through which temperature-dependent control of flagellar motility occurs. Since flagellar motility is an energetically demanding process, it is not surprising that several layers of regulation would be needed to ensure a committed 'OFF' regulatory state at physiological temperatures (37°C). Similarly, as temperatures fluctuate, RNA and protein levels within the bacterium also fluctuate until a steady-state commitment (ON or OFF) for flagellar motility can be reached (Fig. 6). Inherent in this model, auto-regulation of *gmaR* transcription by GmaR protein allows for an adaptive temporal response to temperature fluctuations. Therefore, by incorporating both transcriptional and post-transcriptional responses, *Lm* has the ability to stringently regulate expression of flagellar motility in response to environmental temperatures.

Experimental procedures

Bacterial strains and growth media

The *Lm* and *Ec* strains used in this study are listed in Tables S1 and S2 respectively. Specific details for construction of bacterial strains are located in Supporting information. Primers used in this study are listed in Table S3. *Ec* strains were grown in Luria–Bertani (LB) media for plasmid isolation and protein purification. All *Lm* strains are in the EGDe background and were grown in Brain Heart Infusion (BHI) broth. Antibiotics were used at the following concentrations: chloramphenicol at 20 μg ml^{-1} for selection of pPL3 derivatives in *Ec*, 5 μg ml^{-1} for selection of integrated pPL3 derivatives in *Lm* and 7.5 μg ml^{-1} for pCON1 derivatives in *Lm*; 100 μg ml^{-1} carbenicillin for pCON1 derivatives in *Ec*; 30 μg ml^{-1} kanamycin for pET28a vectors in *Ec*. All plasmid constructs were confirmed by automated DNA sequencing. Plasmids were isolated from XL1-Blue or CLG190 strains and

transformed into SM10 for conjugative transfer into *Lm* or electroporated directly into *Lm*.

Purification of His$_6$-tagged proteins

Ec strains DH-E1446, DH-E1451, DH-E1521 and DH-E1523 expressing *Lm*DegU-His$_6$, *Lm*DegU D$_{55}$N-His$_6$, *Bs*DegU-His$_6$ and *Bs*DegS-His$_6$ were grown in LB medium at 37°C to OD_{600} = 0.5, and protein expression was induced with 100 μM IPTG. The induced culture was grown at 30°C for 4 h. Bacteria were pelleted, and the His-tagged proteins were purified using Ni-NTA spin columns (Qiagen) according to the manufacturer's instructions. Purified His-tagged proteins used for *in vitro* phosphorylation were dialysed in buffer E [100 mM Tris HCL (pH 8.0), 200 mM KCl, 4 mM $MgCl_2$, 4 mM $CaCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 5% glycerol] (Dahl *et al*., 1991).

In vitro *phosphorylation*

In vitro phosphorylation assays were performed by mixing DegU and DegS proteins together in a final volume of 40 μl. The phosphorylation reaction was initiated by the addition of 5 μl of ATP containing 20 μCi [γ-^{32}P]-ATP diluted in cold ATP. The final concentration of reaction components were 0.5 μM DegS, 2.7 μM DegU, 1 μM cold ATP and 0.15 μM [γ-^{32}P]-ATP. Reactions were stopped at the time points indicated by removing 10 μl of the reaction and placing it into a tube containing 10 μl of 2× FSB [0.0625 M Tris (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromophenol blue]. The *in vitro* phosphorylation reactions were boiled for 2 min at 95°C and resolved by SDS-PAGE. The resulting gel was dried and then analysed by autoradiography.

Western blot analysis

Bacterial cultures were grown standing at either 30°C or 37°C for 18–20 h. 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, 3 mg ml^{-1} lysozyme] and incubated at 37°C for 1 h. After the 1 h incubation at 37°C, an equal volume of 2× FSB was added. Samples were boiled for 5 min at 95°C and then centrifuged for 1 min at 16 000 *g*. Sixty microlitres of the boiled sample was loaded onto a 6% SDS-PAGE gel for analysis of GmaR or a 12% SDS-PAGE gel for analysis of DegU. Western blot analysis was performed as previously described (Shen *et al*., 2006) using a polyclonal antibody specific for GmaR or DegU.

Motility assay analysis

A single colony was inoculated with a straight needle into BHI agar (0.3%). Plates were incubated at 30°C or 37°C for 24 h.

β-Glucuronidase assay

β-Glucuronidase assays were performed as previously described for β-galactosidase assays except that 4-methylumbelliferyl-β-D-glucuronide was used in the place of 4-methylumbelliferyl-β-D-galactoside (Gründling *et al*., 2004).

Primer extension

Oligonucleotide primers #375, #133 and #326 were used for primer extension analysis of *fliN*, *iap* and *flaA* transcripts respectively. Primer extension was performed as previously described (Gründling *et al*., 2004).

Affinity purification

To generate bacterial cell lysates, 500 ml of a 16–18 h culture of Δ*degU*, Δ*mogR* or wild-type *Lm* were pelleted and resuspended in 20 ml of lysis buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl] supplemented with Complete protease inhibitor mixture (Roche). Cultures were passed 3× through a French press at 4°C and stored at −80°C. The 5′ primers #715 or #718 and the 3′ biotinylated primer #731 were used to amplify the $p_{fliN\text{-}gmaR}$ DNA region from EGDe genomic DNA. Affinity purification was performed as previously described (Gründling *et al*., 2004) with the following exceptions. A low salt binding buffer was used [10% glycerol, 2 mM $MgCl_2$, 10 mM Tris-HCL (pH 7.5), 100 mM NaCl, 0.5 mM DTT, 4 mM EDTA]. Binding reactions consisted of 100 μl of Dynabead-DNA complex (1 mg of beads, 40 ng of DNA), 0.9 ml of 5× BB, and 3.5 ml of bacterial cell lysate. Binding reactions were incubated 3 h at room temperature (18–25°C) on a rotisserie. Proteins were visualized by Western blot analysis using a DegU or MogR specific antibody.

DNaseI footprinting analysis

DNaseI footprinting analysis was performed as previously reported (Shen and Higgins, 2006) with the following exceptions. The *fliN-gmaR* promoter region DNA was amplified with primer pair #715 and #736 to generate a DNA fragment spanning −278 to +100 relative to the transcriptional start site of $p_{fliN\text{-}gmaR}$. The resulting PCR product was digested with EagI and radiolabelled using Klenow enzyme as previously described (Shen and Higgins, 2006). Reactions were terminated by adding 200 μl of cold Stop solution [6.45 ml of 200% ETOH, 18 μg ml^{-1} of Bovine tRNA (R4752 Sigma), 500 μl of NH_4OAc] to each sample, and placed immediately in an ETOH dry ice bath for 20 min to precipitate the DNA. Samples were centrifuged at 16 000 *g* for 15 min at 4°C, washed with 70% ETOH and then resuspended in formamide loading buffer. Samples were loaded onto a denaturing 6% acrylamide gel and analysed with a phosphoimager.

Real-time quantitative PCR

Wild-type *Lm* EGDe and Δ*mogR* strains were grown 16–18 h at 30°C or 37°C and RNA was extracted as previously reported (Gründling *et al*., 2004). A total of three RNA samples were collected for each strain from three independent experiments. Approximately 10 μg of each RNA sample was digested with 8 units of DNaseI (NEB) in a final volume

of 100 µl for 70 min at 37°C to eliminate any trace genomic DNA contamination. cDNA were synthesized from 8 µl (~800 ng) of DNaseI-treated RNA using random hexamers with a SuperScript III First-Strand Synthesis System Kit (Invitrogen) following manufacturer's instructions. No Reverse Transcriptase samples were included and treated identically with the exception that the reverse transcriptase was eliminated from the protocol. The cDNA samples were used in qRT-PCR to quantify mRNA levels of *gmaR* and *iap* by using iQ SYBR Green Supermix (Bio-Rad) and a MiQ cycler (Bio-Rad). Gene-specific primers #732 and #733 were used to amplify a 151 nt fragment of *gmaR* spanning nt 1517 to nt 1667. Gene-specific primers #734 and #735 were used to amplify a 121 nt fragment of *iap* spanning nt 757 to nt 877. The specificity of each primer pair was monitored through melting curves and the primer pair efficiencies were calculated using a standard curve (102% for *gmaR* and 110% for *iap*). Relative gene expression was quantified by using the Pfaffl method of data analysis. The amount of *gmaR* mRNA was normalized using *iap* as an internal standard (*iap* is known to be transcribed equivalently at both 30°C and 37°C) and then compared with the calibrator condition (*gmaR* levels in the wild-type 30°C sample). Therefore, the relative level of *gmaR* mRNA at 30°C in a wild-type strain thus corresponded to 1. mRNA quantification was performed in duplicate from two independent cDNA pools generated from total RNA extracted from three independent experiments.

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