The DegU orphan response regulator of *Listeria monocytogenes* autorepresses its own synthesis and is required for bacterial motility, virulence and biofilm formation

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The Gram-positive intracellular pathogen *Listeria monocytogenes* is endowed with 17 sets of genes encoding two-component systems. *L. monocytogenes* is closely related to the Gram-positive model bacterium *Bacillus subtilis*, in which we have shown previously that the DegS/DegU system plays a central role in controlling stationary phase adaptive responses, including degradative enzyme synthesis and competence. Although an orthologue of the DegU response regulator is present in *L. monocytogenes*, the gene encoding the cognate DegS kinase is conspicuously absent. We have inactivated the *degU* gene of *L. monocytogenes* and shown that DegU negatively regulates its own synthesis. Direct binding of *L. monocytogenes* DegU to its own promoter region was shown *in vitro* by gel mobility shift and DNase I footprinting experiments. DegU was also shown to bind upstream from the *motB* operon, which also encodes the GmaR anti-repressor of flagellar synthesis. In contrast to the situation in *B. subtilis*, DegU was shown to be essential for flagellar synthesis and bacterial motility in *L. monocytogenes* and is cotranscribed with the *yviA* gene located downstream. We also show that DegU is required for growth at high temperatures, adherence to plastic surfaces and the formation of efficient biofilms by *L. monocytogenes*. DegU plays a role in virulence of *L. monocytogenes* as well: in a murine intravenous infection model, an 11-fold increase in LD₅₀ was observed for the *degU* mutant. Taken together, our results indicate that despite the lack of the DegS kinase, DegU is fully functional as an orphan response regulator, and plays a central role in controlling several crucial adaptive responses in *L. monocytogenes*.

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INTRODUCTION

Bacterial survival in the environment is a challenge that requires continuous fine-tuning of genetic expression. One of the most widespread mechanisms used by bacteria in order to successfully adapt to their milieu or host are the so-called two-component systems (TCSs). These sophisticated signal transduction pathways consist of matched

protein pairs, with a histidine kinase, usually membrane-bound with an amino-terminal extracellular sensing loop, and a response regulator, acting as a transcriptional activator (Hoch & Silhavy, 1995). TCSs are involved in a broad range of bacterial responses, including sporulation, virulence, biofilm formation, and synthesis of extracellular enzymes. Many of these systems are known to interact, effectively forming a bacterial sensory transduction network, an aspect that has been particularly well studied in the Gram-positive model bacterium *Bacillus subtilis* (Msadek *et al.*, 1995; Msadek, 1999). *Listeria monocytogenes*, a food-borne Gram-positive facultative intracellular pathogen very closely related to *B. subtilis*, can cause severe diseases in immunocompromised hosts such as pregnant

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Abbreviations: EMSA, electrophoresis mobility shift assay; qRT-PCR, quantitative real-time PCR; TCS, two-component system.

women and neonates. These infections include meningitis, septicaemia and gastroenteritis, with a high degree of mortality. This bacterium has been extensively studied, and because of its ability to escape from the phagosome, to grow in the cytosol and to efficiently invade neighbouring cells, has become an established model for intracellular growth (Cossart & Portnoy, 2000).

Analysis of the *L. monocytogenes* genome sequence reveals the presence of 17 TCSs (Glaser *et al.*, 2001). Some of these have been studied, including the CheA/CheY system that controls chemotaxis and motility, LisK/LisR, involved in tolerance to stress and virulence (Cotter *et al.*, 2002), VirS/VirR, critical for *L. monocytogenes* virulence (Mandin *et al.*, 2005), and CesK/CesR, which responds to the presence of cell wall-acting antibiotics and affects β -lactam resistance (Kallipolitis *et al.*, 2003).

The DegS/DegU TCS of *B. subtilis* was one of the first to be identified in Gram-positive bacteria (Kunst *et al.*, 1988; Msadek *et al.*, 1990), and has been shown to play a central role in the signal transduction network that controls stationary phase adaptive responses (Msadek *et al.*, 1995; Msadek, 1999). Expression of motility and chemotaxis genes requires the secondary sigma factor σ^D , whose synthesis and activity are controlled by several different mechanisms (Helmann, 1991). The DegS/DegU TCS is also involved in controlling motility and chemotaxis, since an excess of the phosphorylated form of the DegU response regulator has been shown to abolish expression of the *sigD* gene encoding σ^D (Dahl *et al.*, 1992; Msadek *et al.*, 1990; Tokunaga *et al.*, 1994).

Carboxy-terminal amino acid sequence similarities place DegU within the FixJ/UhpA response regulator subfamily with a characteristic helix–turn–helix DNA-binding motif (Msadek *et al.*, 1990; Stock *et al.*, 1989). Indeed, DegU has been shown to bind directly *in vitro* to the upstream regulatory regions of the *comK* gene (Hamoen *et al.*, 2000) and the *fla/che* operon (Amati *et al.*, 2004), although a consensus binding site for DegU has yet to be established. Interestingly, although an orthologue of the *degU* gene is present in *L. monocytogenes*, the gene encoding the DegS kinase is absent, raising the question of whether the orphan DegU response regulator is functional in *L. monocytogenes* and if so how signal acquisition occurs. While this work was in progress, DegU of *L. monocytogenes* was independently reported to play a role in motility and virulence (Knudsen *et al.*, 2004; Williams *et al.*, 2005a).

In *L. monocytogenes*, cells do not produce flagella and are non-motile when grown at the mammalian host physiological temperature of 37 °C, with flagellar synthesis and motility only occurring when cells are grown at 30 °C and below (Peel *et al.*, 1988). Regulation of flagellar synthesis in *L. monocytogenes* differs quite markedly from that of *B. subtilis* model. Indeed, no orthologue of σ^D is present, nor are there any secondary sigma factors dedicated to flagellar synthesis (Glaser *et al.*, 2001). Nevertheless, many regulatory proteins are known to control motility gene expression

in *L. monocytogenes*, such as PrfA, the central virulence gene regulator (Michel *et al.*, 1998); CtsR, which represses class III stress response genes (Karatzas *et al.*, 2003; Nair *et al.*, 2000); FlaR, a histone-like osmoregulated protein which positively regulates *flaA* expression at 25 °C and acts as a repressor at 37 °C (Sanchez-Campillo *et al.*, 1995); MogR, a transcriptional repressor of flagellar motility genes (Shen & Higgins, 2006); and GmaR, an anti-repressor of MogR (Shen *et al.*, 2006).

Flagellar motility has also been shown to be important for biofilm formation in *Listeria* (Lemon *et al.*, 2007). Indeed, bacteria in the environment are predominantly sessile, adhering to inert surfaces and developing as multicellular colonies sheathed within an exopolysaccharide matrix, a structure referred to as a biofilm. *Listeria* biofilm development varies greatly depending on the strain, environmental conditions (pH, growth medium composition, and temperature), and surface properties (Tresse *et al.*, 2006).

In this study, we investigated regulation of *degU* expression in *L. monocytogenes*. We purified the *L. monocytogenes* DegU protein and showed that DegU negatively regulates its own synthesis by binding directly to its promoter region, and that it also binds upstream of the *motB* motility operon, which contains the *gmaR* gene. In addition, we demonstrated that DegU is required not only for flagellar synthesis, motility and virulence, but also for biofilm formation, by complementation of a $\Delta degU$ mutant, restoring the phenotype of the parental EGDe strain. This is, to our knowledge, the first report of *L. monocytogenes* genes directly regulated by DegU and of the involvement of DegU in biofilm formation in this bacterium.

METHODS

Bacterial strains and growth media. *L. monocytogenes* strains used in this study are listed in Table 1. *Escherichia coli* K-12 strain DH5 α [F[−] (ϕ 80d λ lacZAM15) Δ (*lacZ*Y Δ –argF) U169 *recA1 endA1 hsdR17* (r_k^- , m_k^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*] (Invitrogen) was used for cloning experiments, and *E. coli* strain BL21 λ DE3 (Studier & Moffatt, 1986) (Novagen) for protein overproduction and purification. *B. subtilis* strain 168 *trpC2* was used as a Gram-positive cloning host. *E. coli* strains were grown in Luria–Bertani (LB) medium and transformed by electroporation (Sambrook *et al.*, 1989), with selection on plates supplemented with ampicillin (100 μ g ml^{−1}) and kanamycin (25 μ g ml^{−1}) when required. *B. subtilis* cells were grown in LB medium supplemented with 5 μ g chloramphenicol ml^{−1}, and transformed as described previously (Msadek *et al.*, 1998). *L. monocytogenes* strain EGDe and its derivatives were grown in Brain Heart Infusion (BHI) medium (Difco), Modified Welshimer's Broth (MWB) minimal medium (Premaratne *et al.*, 1991) or Roswell Park Memorial Institute (RPMI) 1640 synthetic medium (Sigma-Aldrich) and transformed by electroporation, with selection on BHI plates supplemented with chloramphenicol (10 μ g ml^{−1}) or erythromycin (1 μ g ml^{−1}) when necessary.

DNA manipulations. Oligonucleotides used in this study were synthesized by Sigma-Proligo, and their sequences are listed in Table 2. Chromosomal DNA from *L. monocytogenes* strains was isolated using the MasterPure Gram-positive DNA purification kit (Epicentre

Table 1. *L. monocytogenes* strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
EGDe	<i>L. monocytogenes</i> reference strain	Glaser <i>et al.</i> (2001)
LM1000	EGDe/pMK4	pMK4→EGDe
LM1001	EGDe $\Delta degU$	pMAD $\Delta degU$ →EGDe
LM1002	EGDe $\Delta degU$ /pMK4	pMK4→LM1001
LM1003	EGDe $\Delta degU$ /pMK4 $\Delta degU$	pMK4 $\Delta degU$ →LM1001
LM1010	EGDe $\Delta yviA$	pMAD $\Delta yviA$ →EGDe
Plasmids		
pMAD	Vector for deletion replacement of genes in Gram-positive bacteria	Arnaud <i>et al.</i> (2004)
pMAD $\Delta degU$	pMAD derivative, for deletion of the <i>degU</i> gene	This study
pMAD $\Delta yviA$	pMAD derivative, for deletion of the <i>yviA</i> gene	This study
pMK4	Shuttle vector for Gram-negative and Gram-positive bacteria	Sullivan <i>et al.</i> (1984)
pMK4 $\Delta degU$	Plasmid for expression of <i>degU</i> in <i>L. monocytogenes</i>	This study
pET28/16	pET28a derivative for overproduction of His-tagged proteins	Chastanet <i>et al.</i> (2003)
pETDegULmo	pET28/16 derivative for overproduction of the <i>L. monocytogenes</i> DegU protein	This study

Biotechnologies). DNA fragments were purified following agarose gel electrophoresis using a Qiaquick gel extraction kit (Qiagen). Plasmid DNA was isolated using a QIAprep Spin Miniprep kit (Qiagen), and PCR fragments were purified using the Qiaquick PCR purification kit (Qiagen). T4 DNA ligase and restriction enzymes (New England Biolabs), PCR reagents and *Pwo* thermostable DNA polymerase (Roche) were used according to the manufacturer's recommendations. Nucleotide sequencing of plasmid constructs was carried out by Genome Express-Cogenics.

Plasmid and mutant construction. Plasmid pMAD $\Delta degU$ was used to generate a markerless $\Delta degU$ mutation in *L. monocytogenes* strain EGDe. Two DNA fragments, of 761 and 781 bp, were generated by PCR using oligonucleotide pairs OSA14/OSA15 and OSA5/OSA6, respectively (Table 2), corresponding to the chromosomal DNA regions located directly upstream and downstream from the *degU* gene. These two DNA fragments were cloned in tandem between the *EcoRI* and *BamHI* sites of the pMAD vector (Arnaud *et al.*, 2004) in two consecutive steps, resulting in plasmid pMAD $\Delta degU$. The plasmid was introduced by electroporation into *L. monocytogenes* strain EGDe, and transformants were selected at 30 °C on BHI plates containing erythromycin and X-Gal (50 µg ml⁻¹). Integration and excision of pMAD $\Delta degU$ was performed as described previously (Arnaud *et al.*, 2004) with a non-permissive growth temperature of 42 °C, yielding strain LM1001 ($\Delta degU$), in which the entire *degU* coding sequence was removed. The gene deletion was confirmed by PCR amplification.

The same strategy was used to remove the coding sequence of the *yviA* gene in *L. monocytogenes* strain EGDe. Plasmid pMAD $\Delta yviA$ was constructed by cloning two PCR-generated fragments, of 244 and 229 bp, using oligonucleotide pairs HD119/HD120 and HD121/HD124 between the *EcoRI* and *BamHI* sites of the pMAD vector (Arnaud *et al.*, 2004). Plasmid transformation, integration and excision were then carried out as described above, resulting in strain LM1010 ($\Delta yviA$), and the gene deletion was verified by PCR amplification using oligonucleotides HD118/HD123.

In order to complement the *L. monocytogenes* $\Delta degU$ mutant, the *degU* gene was cloned into the pMK4 shuttle plasmid (Sullivan *et al.*, 1984). It has been reported previously that the presence of the *B. subtilis* *degU* gene is toxic when expressed in *E. coli*, and that the gene can only be cloned in *B. subtilis* (Kunst *et al.*, 1988). We found that this was also the case for *degU* of *L. monocytogenes*, and therefore used *B. subtilis* directly as the cloning host. A 1456 bp DNA fragment

corresponding to the entire coding sequence of *degU* with its upstream native promoter was generated by PCR using oligonucleotides OSA14 and HD125. Following restriction with *EcoRI* and *BamHI*, the fragment was cloned between the corresponding sites of plasmid pMK4. We used a DNA concentration of 115 µg ml⁻¹ (7:1 insert to vector molar ratio) in the ligation mixture, since direct transformation of naturally competent *B. subtilis* cells requires that ligations be performed at high DNA concentrations in order to form multimeric DNA molecules, favouring DNA uptake and subsequent resolution within the bacterial host (Kunst *et al.*, 1988).

Plasmid pET28/16 (Chastanet *et al.*, 2003), a derivative of plasmid pET28a (Novagen), was used for protein overproduction in *E. coli*. DegU of *L. monocytogenes* was overproduced using plasmid pETDegULmo, constructed by cloning a PCR-generated *NcoI/XhoI* DNA fragment corresponding to the *L. monocytogenes* *degU* coding sequence (698 bp; oligonucleotides degUgenF/degUgenR) between the *NcoI* and *XhoI* sites of plasmid pET28/16, replacing the stop codon with a *XhoI* restriction site. This allows the creation of a translational fusion adding six histidine residues to the carboxy-terminus of the protein, while placing expression of the gene under the control of a T7 bacteriophage promoter.

Motility plate assays. Bacterial swimming was investigated on swim plates as described elsewhere (Kathariou *et al.*, 1995; Knudsen *et al.*, 2004). Single colonies were inoculated in Tryptic Soy Broth (TSB) with 0.25 % agar and incubated at either 25 or 37 °C for 48 h.

Overproduction and purification of DegS and DegU. Plasmid pETDegULmo was introduced into a BL21 λ DE3 strain, in which the T7 RNA polymerase gene is under the control of the inducible *lacUV5* promoter, which also carries the pREP4 plasmid, allowing coproduction of the GroESL chaperonin in order to optimize recombinant protein solubility (Amrein *et al.*, 1995). The resulting strain was grown in 2 l LB medium at room temperature, expression was induced during the mid-exponential growth phase by addition of 1 mM IPTG, and incubation was continued for 4 h. The DegU protein was then purified using a two-step procedure as follows. Cells were centrifuged at 10 800 g for 30 min and resuspended in one-fiftieth of the culture volume of buffer A (20 mM Tris/HCl, pH 8, 300 mM NaCl, 0.25 % Tween 20). Cells were disrupted by sonication, and cell debris was removed by two consecutive 30 min centrifugation steps at 17 200 g. *E. coli* crude protein extracts were loaded onto a 0.2 ml Ni-nitrilotriacetic acid (Ni-NTA) agarose column

Table 2. Oligonucleotides used in this study

Name	Sequence	Gene and use
OSA5	ACGCGTCGACGGGGGACTATATGAACG	<i>degU</i> deletion
OSA6	CGGGATCCCGCTTCTAATTGTGCAAGCC	<i>degU</i> deletion
OSA14	CGGAATTCCTCCAAAATAACGAGTAACTAC	<i>degU</i> deletion
OSA15	ACGCGTCGACCTTCGCGAAACAACCTGATG	<i>degU</i> deletion
OSA30	CCGCTTAACCTTATAATCCCTGCC	<i>degU</i> primer extension
degUgenF	CCACCATGGCACTCAAAATCATGATTGTAG	DegU overproduction
degUgenR	CTCCTCGAGGCGAATGTATACCCAGCCGTG	DegU overproduction
degUpF	GAAGAATTCAATAATGATCCAACATAAGGA	<i>degU</i> gel shift
degUpR	GGAGGATCCAATGACTACTCCTCCTCACT	<i>degU</i> gel shift
degUmid	GTAACAGAAGCACTTAGAGCTGGAGCAG	<i>degU</i> RT-PCR
yviAmid	GGATAATCTGGTCCAATGGCTCGTTTGC	<i>yviA</i> RT-PCR
HD4	CGTATTAACAGTTCTCTTGATGACG	<i>flaA</i> qRT-PCR
HD5	ACAAGTCAATACCCATGGATGAGTT	<i>flaA</i> qRT-PCR
HD18	GTGTTGATAATCGGTCTTAATGGTG	<i>flaR</i> qRT-PCR
HD19	GCAACAGCGTTCCAATTTTCCTGCC	<i>flaR</i> qRT-PCR
HD24	ATGATAATCTGTTACAGCTTGAGAA	<i>cheA</i> qRT-PCR
HD25	TCCATTGCATGCGTTAAATCAGCCA	<i>cheA</i> qRT-PCR
HD26	GCAGCTACGAATCAATGTCGTGCCA	<i>yviA</i> qRT-PCR
HD27	GCAGGTTGAGAGGAAGTTGGGAAAT	<i>yviA</i> qRT-PCR
HD111	CGATTGCAGCTGGTGTGCTGAAGTA	<i>gmaR</i> qRT-PCR
HD112	ATCGCGCAATCGGAATGCTTCGGAAA	<i>gmaR</i> qRT-PCR
HD113	TAGGCATACAATCACATACC	<i>lmo0675</i> gel shift
HD114	AAATAAGAAATTCCCGCAAG	<i>lmo0675</i> gel shift
HD115	AACAACAAACCAATGGCGCGGGTTTAT	<i>gmaR</i> internal PCR fragment
HD116	TTTTAGCGGAATTCAGGTACATTGGC	<i>gmaR</i> internal PCR fragment
HD118	CGCGAATTCAGTGTGAACGGACTAGATGCAACTG	<i>yviA</i> deletion
HD119	CGCGAATTCGCCACTACACATCTTAACGCAT	<i>yviA</i> deletion
HD120	ACGCGTCGACGTCCCCTGAGATTCTTTAGC	<i>yviA</i> deletion
HD121	ACGCGTCGACTTGGTCAATAAAATAAAGAAA	<i>yviA</i> deletion
HD123	CGCGGATCCTTGCCAAATAACTGCGCATCCGTA	<i>yviA</i> deletion
HD124	CGCGGATCCCTGTATTACCTCCCCTT	<i>yviA</i> deletion
HD125	CGCGGATCCGTTGTGCTGTCTAGTAACCAC	<i>degU</i> complementation
OP133	CACCTGGAGTAAACCAATTAGTACG	<i>rpoB</i> qRT-PCR
OP134	TAGTGGGTAAAGCATGATATCAACA	<i>rpoB</i> qRT-PCR

(Qiagen) equilibrated with buffer A. The column was then washed with 10 volumes of buffer B (20 mM Tris/HCl, pH 8, 300 mM NaCl), and the protein was eluted using the same buffer with a linear imidazole gradient (30–500 mM). Fractions were analysed by SDS-PAGE, pooled and dialysed against buffer C (20 mM Tris/HCl, pH 8, 1 mM EDTA) and loaded onto a 0.5 ml DEAE-Sepharose (Pharmacia) column equilibrated with buffer D (20 mM Tris/HCl, pH 8, 1 mM EDTA, 0.5 mM DTT). The protein was then eluted with a 0–1 M linear NaCl gradient. Fractions were pooled and dialysed against buffer E (20 mM Tris/HCl, pH 8, 1 mM EDTA, 200 mM NaCl, 50%, v/v, glycerol) to remove salt and concentrate the protein solution approximately fourfold. Protein concentrations were determined using the Bio-Rad protein assay (Bradford, 1976).

Biofilm formation assays. Bacterial attachment and surface growth on polystyrene microtitre plates were studied during growth of *L. monocytogenes* in freshly prepared MWB minimal medium (Premaratne *et al.*, 1991). Overnight cultures grown in BHI were used to inoculate MWB medium at OD₆₀₀ 0.1, were vortexed briefly, and 200 µl volumes were dispensed into microtitre plate wells, followed by incubation at either 37 or 25 °C for 40 h. The OD₆₀₀ of each culture was measured to ensure that all cells had reached

stationary phase, and the wells were washed five times with PBS and air-dried for 30 min. Biofilms were stained with 0.1% crystal violet for 30 min (200 µl per well), and the wells were again washed five times with PBS and air-dried. The stained biomass was resuspended for quantification in ethanol/acetone (80:20) and A₅₉₅ was measured. The assay was performed in triplicate.

Extraction of total RNA. Total RNA was extracted from *Listeria* cultures grown at either 25 or 37 °C, as previously described (Chastanet *et al.*, 2001; Glatron & Rapoport, 1972), with some minor modifications. Briefly, cells from 40 ml cultures were centrifuged (2 min, 20 800 g) and the cell pellet was resuspended in 1 ml water-saturated phenol. The cell suspension was added to a 2 ml screw-cap microcentrifuge tube containing 0.4 g glass beads (106 µm, Sigma) and 400 µl 2% Macaloid slurry (Bentone MA, Rheox). Cells were disrupted in a FastPrep cell disintegrator (Bio 101) for 40 s at 4 °C. After centrifugation at 20 800 g for 15 min, the supernatants were extracted twice with 1 volume of phenol/chloroform (1:1, v/v), then with 1 volume chloroform. RNA was then precipitated with 2-propanol in the presence of 0.2 M NaCl and resuspended in 50 µl water. RNA concentrations were determined by measuring A₂₆₀ and samples were stored at –80 °C.

Primer extension reactions. Total RNA was used as a template for primer extension reactions using a radiolabelled *degU*-specific oligonucleotide (OSA30, Table 2), as previously described (Chastanet *et al.*, 2001). The corresponding dideoxy chain termination DNA sequencing reactions were carried out by using the same oligonucleotide primer and a PCR-amplified fragment corresponding to the *degU* (622 bp) upstream region (oligonucleotide pair OSA14/OSA30; Table 2) with the Sequenase PCR product sequencing kit (USB).

RT-PCR reactions. RT-PCRs were used to show that the *degU* and *yviA* genes are cotranscribed, using 22 µg total RNA, oligonucleotides *degU*mid and *yviA*mid (100 pmol of each) and the SuperScript One-Step RT-PCR system with RT/Platinum *Taq* DNA polymerase as recommended by the manufacturer (Invitrogen). The absence of genomic DNA in RNA preparations was verified by omitting the RT/Platinum *Taq* Mix and substituting 2 U Platinum *Taq* DNA polymerase in the control reaction. *L. monocytogenes* genomic DNA was used as a template for the positive control PCR. The cDNA synthesis step was carried out at 50 °C for 30 min, and the subsequent PCR conditions were 5 min at 94 °C for one cycle, followed by 1 min at 94 °C, 30 s at 55 °C, and 1 min at 72 °C for 40 cycles. Agarose gel electrophoresis (1%) was used to visualize RT-PCR products with a Smartladder DNA molecular mass marker (Eurogentec).

cDNA synthesis and quantitative real-time PCR (qRT-PCR). RNA samples for qRT-PCR reactions were treated with DNase I using the TURBO DNA-free reagent (Ambion) in order to eliminate residual contaminating genomic DNA. cDNA synthesis and qRT-PCR were then carried out as described previously (Dubrac *et al.*, 2007), using the *L. monocytogenes* *rpoB* gene as an internal standard (Schmittgen & Zakrajsek, 2000) and specific oligonucleotide pairs for each gene (see Table 2).

Gel electrophoresis mobility shift assays (EMSAs). DNA fragments corresponding to the *degU* (418 bp) and *lmo0675* (446 bp) upstream promoter regions and a *gmaR* intragenic region (463 bp) were generated by PCR with *Pwo* polymerase (Roche) and oligonucleotide pairs *degU*upF/*degU*upR, HD113/HD114 and HD115/HD116, respectively (Table 2). Labelling, DNA binding and gel electrophoresis mobility shift DNA-binding assays were performed as described previously (Derré *et al.*, 1999).

Electron microscopy. *L. monocytogenes* strains were grown for 14–16 h at 25 °C in BHI medium with shaking. A drop of bacterial suspension was placed onto a 300-mesh copper carbon-coated grid. The excess was carefully removed and the preparations were negatively stained in 2% uranyl acetate or in phosphotungstic acid (2%). Samples were examined at 80 kV with a transmission electron microscope (JEOL 1200EXII), and electron micrographs were recorded using a Mega view charge-coupled device camera (Eloise SARL) (original magnification × 20 000).

Virulence assays. LD₅₀s were determined by intravenously injecting 8-week-old BALB/c mice with 0.3 ml of serial dilutions of *L. monocytogenes* EGDe and the otherwise isogenic Δ *degU* mutant (strain LM1001; 10³–10⁷ bacteria). Mortality was checked over a 10-day period.

Database comparisons and sequence analysis. Computations were performed with the SubtiList and ListiList relational databases (<http://genolist.pasteur.fr>) (Moszer *et al.*, 2002). Sequence comparisons with the GenBank database were accomplished using the National Center for Biotechnology Information BLAST2 (Altschul *et al.*, 1997) web server with the default parameter values provided.

RESULTS

The *L. monocytogenes* *degU* and *yviA* genes are cotranscribed

L. monocytogenes EGDe genome sequence analysis revealed the presence of a gene encoding an orthologue of the *B. subtilis* DegU response regulator (Lmo2515; 63% amino acid sequence identity). The sequence of the *L. monocytogenes* EGDe *degU* locus was compared with that of *B. subtilis*, revealing several major differences. Not only is the upstream gene encoding the cognate DegS histidine kinase missing from *L. monocytogenes* and *Listeria innocua*, but the upstream intergenic region containing the major *degS*–*degU* operon promoter characterized in *B. subtilis* (Msadek *et al.*, 1990) is also significantly different, despite the presence of an orthologue of the upstream *yvyE* gene (Fig. 1a). Furthermore, the transcription terminator separating the *B. subtilis* *degU* gene from the downstream *yviA* gene (Kunst *et al.*, 1988) is not present in *L. monocytogenes*, in which the *degU*–*yviA* intergenic region is much shorter (20 bp compared with 97 bp in *B. subtilis*), strongly suggesting that the two genes are cotranscribed in *L. monocytogenes*. To verify that this was indeed the case, total RNA was isolated from *L. monocytogenes* EGDe cells growing exponentially in BHI medium at 37 °C (OD₆₀₀ 0.6) and used in RT-PCR experiments (see Methods) using oligonucleotides *degU*mid and *yviA*mid, designed to

Fig. 1. The *degU*–*yviA* operon of *L. monocytogenes*. (a) Comparison of the *degU* loci of *B. subtilis* and *L. monocytogenes*. The circle indicates a *B. subtilis* transcriptional terminator. (b) Cotranscription of *degU* and *yviA* in *L. monocytogenes*. Lanes: 1, Smartladder (Eurogentec) DNA molecular mass markers (400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 6000, 8000 and 10 000 bp); 2, RT-PCR of total RNA; 3, PCR of total RNA (no reverse transcriptase); 4, PCR of genomic DNA.

hybridize within the *degU* and *yviA* coding sequences and amplify a 879 bp fragment encompassing the intergenic region (Table 2). As shown in Fig. 1(b), the corresponding DNA fragment was amplified successfully either from total RNA following treatment with reverse transcriptase (lane 2) or from genomic DNA (lane 4), but was not amplified from RNA samples that were not treated with reverse transcriptase (lane 3), indicating that *degU* and *yviA* form an operon in *L. monocytogenes* although they are not cotranscribed in *B. subtilis*. In order to investigate the function of the *degU* and *yviA* genes, we constructed the LM1001 ($\Delta degU$) and LM1010 ($\Delta yviA$) mutant strains of *L. monocytogenes* EGDe, in which the entire coding sequences of the genes were removed (see Methods). While this work was in progress, two independent reports described the inactivation of *degU* in *L. monocytogenes* strain EGD (Knudsen *et al.*, 2004; Williams *et al.*, 2005a). However, there are significant differences between strain EGD and the strain whose genome sequence is available, EGDe. Indeed, strain EGDe is more virulent than strain EGD and also differs significantly in its surface protein profile (O. Dussurget, unpublished observations).

DegU autorepresses its own synthesis

Primer extension experiments were used to follow *degU* expression. Total RNA was extracted from strains EGDe and LM1001 ($\Delta degU$) during mid-exponential growth in BHI medium at 37 °C and used for primer extension experiments. The nucleotide sequence of the region preceding the *degU* transcription start site revealed appropriately spaced potential -10 and -35 regions sharing strong similarities with the consensus sequences of promoters recognized by the vegetative form of RNA polymerase, $E\sigma^A$ (Fig. 2a). Interestingly, the *degU* gene transcript of *L. monocytogenes* is preceded by a fairly long untranslated region (UTR) of 261 nt, whereas the UTR of the *B. subtilis degS–degU* operon is only 116 bases. We did not detect any potential ORFs within this UTR.

Several response regulators are known to control their own synthesis. We therefore examined transcription from the *degU* promoter in strain LM1001 ($\Delta degU$), in which the *degU* coding sequence had been removed. Transcription from the *degU* promoter was found to be increased systematically in the $\Delta degU$ mutant, indicating that DegU negatively regulates its own synthesis (Fig. 2b). This was confirmed by qRT-PCR analysis (data not shown).

Purification of DegU

In order to determine whether DegU binds directly to its own promoter region, the *L. monocytogenes* DegU protein was overproduced by cloning its coding sequence in plasmid pET28/16, placing the gene under the control of an inducible T7 bacteriophage promoter and creating a translational fusion that adds a carboxy-terminal extension containing six histidine residues, and was purified by immobilized metal affinity chromatography (IMAC) using

Fig. 2. Primer extension analysis of *degU* expression. (a) Total RNA was extracted from *L. monocytogenes* strain EGDe during mid-exponential growth in BHI medium at 37 °C. Primer extension experiments were performed using a *degU*-specific primer (OSA30) (lane 1). Sanger dideoxy chain termination sequencing reactions (GATC) were carried out on a PCR fragment corresponding to the *degU* upstream region (OSA14/OSA30). The nucleotide sequence of the *degU* upstream region is indicated. The transcription initiation site is labelled +1, and the -35 and -10 sequences are boxed. (b) Primer extension analysis of *degU* transcription in *L. monocytogenes* strains EGDe and LM1001 ($\Delta degU$). Total RNA was extracted from strains EGDe (lane 1) or LM1001 (lane 2) during mid-exponential growth in BHI medium at 37 °C, and primer extension experiments were performed using a *degU*-specific primer (OSA30).

an Ni-NTA agarose column (see Methods). As described for the *B. subtilis* DegU regulator (Hamoen *et al.*, 2000), DegU of *L. monocytogenes* was found to be associated with *E. coli* chromosomal DNA following purification by IMAC, which interfered with its ability to bind DNA in EMSA experiments (data not shown). The IMAC step was therefore followed by a second affinity chromatography purification using DEAE Sepharose (see Methods). As shown in Fig. 3, the *Listeria* DegU protein was obtained with a purity greater than 95 % and displayed the expected apparent molecular mass of ~26.8 kDa (Fig. 3, lane 4).

DegU binds specifically to the *degU* promoter region

As shown above, DegU autorepresses its own synthesis in *L. monocytogenes*. An *in vitro* approach was used to

Fig. 3. Purification of *L. monocytogenes* DegU. SDS-PAGE analysis of crude cytosolic extracts from *E. coli* BL21 λ DE3 carrying pET28/16 (lane 2) or pETDegULmo (lane 3). Purified DegU was loaded in lane 4. Molecular mass standards were loaded in lane 1.

demonstrate a direct interaction between DegU and the *degU* promoter region. Purified DegU was used in EMSAs with a 418 bp radiolabelled PCR-generated DNA fragment corresponding to the *degU* promoter region. This DNA fragment, extending from positions -139 to $+261$ relative to the translation initiation site, was incubated with increasing amounts of purified DegU in the presence of an excess of non-specific competitor DNA [$1 \mu\text{g}$ poly-(dI-dC)]. As shown in Fig. 4(a), DegU bound specifically to the radiolabelled fragment, leading to progressive displacement of the probe to a higher-molecular-mass protein–DNA complex (Fig. 4a, lanes 1–4). As DegU concentrations were progressively increased, two additional larger protein–DNA complexes were observed (Fig. 4a, lanes 5 and 6), suggesting the existence of up to three binding sites with different affinities in this promoter region. These results demonstrate that DegU of *L. monocytogenes* represses its own synthesis by binding directly to the *degU* promoter region.

DNase I footprinting assays were performed on *L. monocytogenes* DNA fragments corresponding to the *degU* promoter region to precisely determine the location of the DegU-binding site. When the non-template strand of the *degU* promoter region DNA fragment was end-labelled, DegU protected three distinct regions extending from positions -2 to $+19$, $+35$ to $+74$ and $+98$ to $+116$ (Fig. 4b) with respect to the transcription initiation site, in agreement with the different sized protein–DNA complexes observed in the gel mobility shift DNA-binding assay (Fig. 4a). All three binding sites either overlapped or were downstream from the transcription initiation site, in agreement with the role of DegU in repressing expression from this promoter. DegU appears to display low affinity for region three, as it only interacted with this sequence at high DegU concentrations, as seen in the EMSA and DNase I experiments (Fig. 4a, lane 6, b, lanes 5–7). We

observed the appearance of DNase I hypersensitive sites within the regions protected by DegU, suggesting that the DNA undergoes bending once the regulator is bound (Fig. 4b).

Close inspection of the three regions protected by DegU failed to reveal any direct or inverted repeat sequences; however, all three binding sites contained a conserved pentanucleotide motif GTAA T/G on either strand, which may be involved in recognition by DegU (Fig. 4b). These results indicate that DegU negatively regulates *degU* expression by binding directly to its operator sequence in the promoter region. These data constitute the first demonstration, to our knowledge, of direct binding of the DegU regulator to a promoter region in *L. monocytogenes*.

DegU is required for growth in RPMI 1640 synthetic medium, growth at high temperature, and motility and flagellar synthesis

DegU is known to be a highly pleiotropic regulator in *B. subtilis*, playing a central role in a signal transduction network that controls stationary phase adaptive responses, including motility, competence and degradative enzyme production (Msadek, 1999). A phenotypic analysis of the ΔdegU strain LM1001 was therefore undertaken to verify that DegU is also a pleiotropic regulator in *L. monocytogenes*, and the mutant was found to be deficient for growth in RPMI 1640 synthetic medium and in BHI when grown at 44°C (Fig. 5a, b). DegU is known to control motility in *B. subtilis* and has also been reported to affect motility in *L. monocytogenes* strain EGD (Knudsen *et al.*, 2004; Msadek *et al.*, 1995; Williams *et al.*, 2005a, b). As shown in Fig. 5(c) using a soft agar swim plate assay, DegU is also required for motility of strain EGDe.

DegU controls flagellar synthesis and the expression of several motility and chemotaxis genes

In order to verify that the observed phenotypes were due to the absence of DegU, the ΔdegU mutant strain was complemented by the introduction of an intact copy of the gene on a multicopy plasmid, resulting in strain LM1003 (ΔdegU pMK4-*degU*). Strains EGDe, LM1001 and LM1003 were observed by transmission electron microscopy, revealing the loss of flagella in the LM1001 ΔdegU mutant and restored flagellar synthesis in the complemented strain LM1003 (Fig. 6a).

In order to determine which motility and chemotaxis genes were controlled specifically by DegU, qRT-PCR experiments were carried out using RNA from cultures grown at 25°C until mid-exponential phase. As shown in Fig. 6(b), expression of the *flaR* gene, encoding a repressor of flagellar synthesis, was not lowered markedly in the ΔdegU mutant, whereas expression of *motB* as well as *gmaR*,

Fig. 4. Gel mobility shift assay and DNase I footprinting analysis of DegU binding to the *degU* promoter region. (a) DegU binds specifically to the *degU* promoter region. DNA-binding reactions were performed with a radiolabelled DNA fragment (10 000 c.p.m.) corresponding to the *degU* promoter region and increasing amounts of purified DegU. Lanes: 1, 0.1 μ M; 2, 0.3 μ M; 3, 0.5 μ M; 4, 0.7 μ M; 5, 1 μ M; 6, 3 μ M; 7, no protein. (b) DNase I footprinting analysis of DegU binding to the *degU* promoter region. Each lane contains 50 000 c.p.m. radiolabelled DNA fragment corresponding to the non-template strand of the *L. monocytogenes degU* promoter region (–139 to +261). Fragments were incubated with increasing amounts of purified DegU. Lanes: 1, no protein; 2, 0.02 μ M; 3, 0.05 μ M; 4, 0.1 μ M; 5, 0.5 μ M; 6, 1 μ M; 7, 1.5 μ M; G + A, Maxam and Gilbert reactions of the corresponding DNA fragment. Brackets indicate regions protected by DegU from DNase I cleavage (1, 2 and 3, respectively) and asterisks indicate the positions of DNase I hypersensitive sites. The nucleotide sequence of the *degU* promoter region is shown with the DNase I-protected areas boxed and arrows indicating a conserved sequence. Positions are numbered relative to the transcription initiation site. The –35 and –10 promoter sequences are overlined and the transcriptional start site is indicated by +1.

known to control *flaA* expression, was abolished in strain LM1001 (Δ *degU*). As expected, expression of *flaA* and *cheA* was abolished in the Δ *degU* mutant and fully restored in the complemented strain LM1003 (Fig. 6c).

DegU binds specifically to the *gmaR* promoter region

GmaR was described recently as an antirepressor of MogR, playing an important role in the control of flagellar synthesis and motility (Shen *et al.*, 2006). GmaR is the

14th gene in the *motB* operon. In order to test whether DegU controls *gmaR* expression directly, a 446 bp radiolabelled DNA fragment corresponding to the *gmaR* promoter region, upstream from the *lmo0675* gene, was generated by PCR using oligonucleotides HD113/HD114 (Table 2). The radiolabelled *gmaR* promoter fragment extends from positions –378 to +68 relative to the translational start site of *lmo0675*, and was incubated with increasing concentrations of DegU in the presence of an excess of non-specific competitor DNA [1 μ g poly-(dI-dC)]. As shown in Fig. 7(a), DegU bound specifically to the radiolabelled fragment, leading to progressive displacement of the probe to a higher-molecular-mass protein–DNA complex (Fig. 7a, lanes 1–4). As DegU concentrations were progressively increased, two additional larger protein–DNA complexes were observed (Fig. 7a, lanes 5 and 6), suggesting the existence of multiple binding sites, as observed for the *degU* promoter region. DegU did not bind, however, under the same conditions and even at the highest concentrations, to a DNA fragment of approximately the same size (463 bp) corresponding to a region internal to the *gmaR* gene (Fig. 7b, lanes 1–6), indicating that DegU binding to the *gmaR* promoter region is specific. This was further demonstrated by cold competitor chase experiments. Indeed, binding of DegU to the *gmaR* promoter was lost when a 30- to 50-fold excess of unlabelled specific competitor probe DNA was added to the reaction (Fig. 7a, lanes 7 and 8), but not in the presence of a 50-fold excess of unlabelled DNA fragment corresponding to the internal fragment of the *gmaR* gene (Fig. 7a, lane 9). Thus, DegU binds specifically to its own promoter region as well as to that of the *motB gmaR* operon.

Fig. 5. DegU is required for growth in RPMI 1640, growth at high temperature and motility. (a) Growth of *L. monocytogenes* strains was carried out in RPMI 1640 at 37 °C: EGDe, (◆); LM1001 ($\Delta degU$), (■); LM1003 ($\Delta degU/pMK4degU$), (▲). (b) Growth defect of the $\Delta degU$ mutant at 44 °C. (c) Motility defect of the $\Delta degU$ strain on soft agar swim plate assays after 48 h at 25 °C.

DegU is involved in biofilm formation

DegU has recently been described as playing a major role in biofilm formation in *B. subtilis* (Kobayashi, 2007a, b; Verhamme *et al.*, 2007). In order to examine whether this is also true in *L. monocytogenes*, strains EGDe, LM1001 ($\Delta degU$) and LM1003 ($\Delta degU/pMK4degU$) were grown on microtitre plates in MWB medium at 25 or 37 °C. Adherent cells were washed with PBS and stained with crystal violet (see Methods), and the mean OD₅₉₅ values were calculated from replicate experiments. As shown in Fig. 8, the $\Delta degU$ mutant formed less biofilm at 25 °C than the parental strain EGDe, and biofilm formation was restored in the complemented strain LM1003.

DegU is involved in virulence of *L. monocytogenes*

We examined virulence of an *L. monocytogenes* $\Delta degU$ mutant in a murine model. Virulence of the $\Delta degU$ mutant was assayed by intravenous injection as described in Methods, and compared with that of the wild-type EGDe strain. The LD₅₀ of the $\Delta degU$ mutant was 4.2×10^4 c.f.u., whereas that of EGDe was 3.9×10^3 c.f.u. The $\Delta degU$ mutant thus displays a significant decrease in virulence (11-fold). We followed the survival of mice over a 10-day post-inoculation period. Mice infected with the EGDe strain began to die after 2.5 days and were all dead after 4 days, whereas 80 % of the animals infected by strain LM1001 ($\Delta degU$) were still alive after 10 days (Fig. 9). These results clearly show that *degU* plays a significant role in the pathogenicity of *L. monocytogenes*.

DISCUSSION

The *B. subtilis* DegS/DegU TCS is highly pleiotropic, affecting degradative enzyme synthesis, competence gene expression and antibiotic production, as well as motility and chemotaxis (Msadek *et al.*, 1995). During a systematic investigation of *L. monocytogenes* TCSs, our interest was prompted by the fact that although an orthologue of DegU (63 % amino acid sequence identity) is present in this intracellular pathogen, the gene encoding the cognate DegS kinase is missing. Since the *B. subtilis* system is known to function as a molecular switch, with the phosphorylated and unphosphorylated forms of the response regulator required for expression of distinct sets of genes, the absence of the DegS kinase in *L. monocytogenes* was intriguing, and suggested that this orphan response regulator was also active in its unphosphorylated form.

An investigation of the role of DegU in *Listeria* allowed us to show that, in contrast to the situation in *B. subtilis*, DegU negatively regulates its own synthesis by binding to its own promoter. Indeed, in *B. subtilis* DegU is known to positively regulate its own synthesis (Kobayashi, 2007b). DNase I footprinting experiments allowed us to define three binding sites for DegU which overlap the promoter region. Although no clear consensus sequence could be determined from the binding sites, they each contain a conserved pentanucleotide sequence 5'-GTAA T/G-3' in either orientation, which may be involved in recognition by DegU. Interestingly, a clear-cut consensus sequence for DegU binding has yet to be defined in *B. subtilis* as well, in which DNase I footprinting was performed on the *comK* promoter (Hamoen *et al.*, 2000). Since the DNA recognition helix of the DNA-binding helix–turn–helix domain is invariant between DegU of *Bacillus* and *Listeria*, it is likely that they bind to the same DNA sequence, as shown for other orthologous TCSs (Dubrac & Msadek, 2004).

DegU is equally pleiotropic in *L. monocytogenes*. Indeed, the $\Delta degU$ mutant displays many phenotypes, including, as shown here, growth deficiency in RPMI 1640 synthetic medium, inability to grow at high temperatures (44 °C),

Fig. 6. DegU is required for flagellar and motility gene expression and flagellar synthesis. (a) Electron micrographs of strains EGD, LM1001 ($\Delta degU$) and LM1003 ($\Delta degU/pMK4degU$), showing loss of flagella in the $\Delta degU$ mutant and restored flagellar synthesis in the complemented strain. Cells were grown for 14–16 h at 25 °C in BHI medium with shaking and prepared for electron microscopy as described in Methods. (b) qRT-PCR analysis of motility (*flaR*, *gmaR*) and chemotaxis (*motB*) gene expression at 25 °C in strains EGD and LM1001 ($\Delta degU$). (c) qRT-PCR analysis of motility (*flaA*) and chemotaxis (*cheA*) gene expression at 25 °C in strains LM1000 (EGD/pMK4), LM1002 ($\Delta degU/pMK4$) and LM1003 ($\Delta degU/pMK4degU$). Total RNA was isolated from cultures in mid-exponential phase and treated with reverse transcriptase, and specific cDNAs were quantified by qRT-PCR. The results are expressed as the means and SDs (error bars) of six experiments using specific primers for each gene and *rpoB* as the reference gene, and are shown as relative amounts of PCR product.

loss of motility and flagellar synthesis, and a deficiency in biofilm formation. Although we have shown that the *degU* and *yviA* genes are cotranscribed in *Listeria*, in contrast to the situation in *Bacillus*, YviA does not appear to play a role

in the DegU regulatory pathway, since the $\Delta yviA$ mutation (strain LM1010) did not affect growth in RPMI 1640 or at high temperature, flagellar synthesis and motility, or biofilm formation (data not shown), in agreement with

Fig. 7. Gel mobility shift assay analysis of DegU binding to the *lmo0675* promoter region. (a) DegU binds specifically to the *lmo0675* promoter region. DNA-binding reactions were performed with a radiolabelled DNA fragment (10 000 c.p.m.) corresponding to the *lmo0675* promoter region and increasing amounts of purified DegU. Lanes: 1, no DegU; 2, 0.46 μM; 3, 0.92 μM; 4, 1.85 μM; 5, 3.7 μM; 6, 7.4 μM. Lanes 7–9 correspond to reaction mixtures containing 1.85 μM DegU and unlabelled competitor DNA: lanes 7 and 8, 30-fold excess (0.057 μM) and 50-fold excess (0.142 μM) specific competitor (*lmo0675* promoter region), respectively; lane 9, 50-fold excess (0.136 μM) unspecific competitor DNA (internal *gmaR* DNA fragment). (b) DegU does not bind to a DNA fragment internal to the *gmaR* gene. Lanes: 1, no DegU; 2, 0.46 μM; 3, 0.92 μM; 4, 1.85 μM; 5, 3.7 μM; 6, 7.4 μM.

results for *B. subtilis* in which a *yviA* mutant has no obvious phenotype (Henner *et al.*, 1988).

Many factors influence biofilm formation, and we noted that biofilms formed in rich media (BHI) were loosely attached and as a result not easily reproducible, whereas when cells were grown in MWB minimal medium adherence was much stronger. It has been reported recently

Fig. 8. DegU is required for efficient biofilm formation by *L. monocytogenes*. Bacteria were grown overnight in BHI medium and used to inoculate MWB medium at OD₆₀₀ 0.1, and 200 μl of the culture was dispensed into microtitre plate wells. The plates were incubated for 40 h at 25 °C. Attached bacteria were stained with 0.1 % crystal violet solution and the A₅₉₅ was measured.

Fig. 9. DegU plays a role in virulence of *L. monocytogenes*. Survival curves of BALB/c mice after intravenous inoculation with 10⁵ bacteria of the wild-type strain EGDc (■) or the mutant strain LM1001 (Δ degU) (◆).

that flagella are essential for biofilm formation in *L. monocytogenes* (Lemon *et al.*, 2007). Nevertheless, our results suggest that the role of DegU in biofilm formation is not only due to its effect on flagellar synthesis. Indeed, *Listeria* is capable of forming biofilms at 37 °C, even though flagella are not expressed at the host temperature. Whereas no difference was observed in biofilm formation at 37 °C between the EGDc parental strain and the Δ degU mutant when cells were grown in MWB minimal medium, we observed lowered biofilm formation by the mutant strain when cells were grown at 37 °C in BHI medium, although cells are loosely attached when grown in this medium (data not shown). This observation could suggest a role for DegU in biofilm formation that is at least in part distinct from its role in controlling flagellar synthesis. Indeed, the *L. monocytogenes* Δ degU mutant is unable to grow in the presence of 5 % ethanol (Knudsen *et al.*, 2004) or in RPMI 1640 minimal medium as shown here, suggesting that DegU is also involved in other cell processes.

Regulation of flagellar motility in *Listeria* is quite different from that in *Bacillus*, particularly since there is no σ^D secondary sigma factor dedicated to the expression of motility, chemotaxis, flagellar synthesis and autolysin genes in *Listeria*. FlaR is a regulator known to control expression of *flaA*, which encodes flagellin, activating its expression at 25 °C and repressing it at 37 °C (Sanchez-Campillo *et al.*, 1995). However, it appears that DegU does not have a significant effect on *flaR* expression at 25 or 37 °C, suggesting that it does not act through this regulator. It has been shown recently that DegU controls expression of *gmaR*, which encodes a bifunctional O-GlcNAc transferase that regulates flagellar motility by acting as an anti-repressor

of MogR, a repressor of most chemotaxis and motility genes (Grundling *et al.*, 2004; Shen & Higgins, 2006; Shen *et al.*, 2006). We have shown by qRT-PCR that transcription of *mogR* is not regulated by DegU at either 24 or 37 °C (data not shown). It has been suggested that DegU either directly or indirectly activates *gmaR* expression, which antagonizes MogR repression activity to restore the expression of flagellar synthesis and motility genes (Shen & Higgins, 2006; Shen *et al.*, 2006).

We show here that DegU binds directly to the promoter region of the *motB-gmaR* operon, indicating that it likely controls *gmaR* expression directly. In *B. subtilis*, DegU has been shown to bind directly to the promoter region of the *fla-che* operon, which also contains the *sigD* gene encoding the σ^D secondary sigma factor (Amati *et al.*, 2004). An excess of phosphorylated DegU represses expression of *sigD* in *B. subtilis*, and thus leads to loss of flagellar synthesis and motility, yet expression of *sigD* is only lowered approximately twofold in a $\Delta degU$ mutant and cells are fully motile, in contrast to *L. monocytogenes* (Msadek *et al.*, 1993; Tokunaga *et al.*, 1994). The situation in *Listeria* is reminiscent of that described in the so-called undomesticated strains of *B. subtilis*, known to differ significantly in their phenotypes and regulatory pathways from laboratory strains that derive from strain 168, which was originally subjected to repeated rounds of UV and X-ray mutagenesis (Branda *et al.*, 2001; Burkholder & Giles, 1947). Indeed, in contrast to laboratory strains, a $\Delta degU$ mutation in the undomesticated strain of *B. subtilis* ATCC 6051, also known as NCIB 3610, has been reported to lead to loss of flagellar synthesis, much as in *Listeria* (Kobayashi, 2007a). However, this finding is contradicted by a recent report in which the authors claim that the same mutation in the same strain background does not affect flagellar-based motility (Verhamme *et al.*, 2007).

There are several interesting parallels between the DegU regulatory pathways of *Bacillus* and *Listeria*. Indeed, in *B. subtilis*, DegU acts in the competence regulatory pathway by assisting the ComK regulator to act as an antirepressor of the Rok and CodY repressors to allow expression of its own gene, leading into a positive feedback loop, which allows expression of competence genes and formation of the competence bistability state (Hamoen *et al.*, 2000; Smits *et al.*, 2005, 2007). Interestingly, GmaR acts as an antirepressor of MogR, and DegU both activates GmaR synthesis and acts as an indirect antagonist of MogR (Shen & Higgins, 2006; Shen *et al.*, 2006). It is therefore intriguing to note that the ComK-binding site sequence is AAAA-N5-TTTT (Hamoen *et al.*, 1998, 2000), which is identical to the sequence on the antiparallel strand that is bound by MogR, TTTT-N5-AAAA (Shen & Higgins, 2006; Shen *et al.*, 2006). It is therefore tempting to speculate that DegU may be acting in a similar manner in the two systems, by assisting in an antirepressor mechanism, which will likely be the subject of further investigation. Although many orthologues of *Bacillus* competence genes are present in *Listeria* (Borezée *et al.*, 2000), no link could be

established between competence gene expression and DegU.

In agreement with previous results obtained with an *L. monocytogenes* $\Delta degU$ mutant in the EGD background and administered orally or intraperitoneally (Knudsen *et al.*, 2004; Williams *et al.*, 2005a, b), we show here that in the EGDe background virulence is attenuated when the mutant is injected intravenously into mice. This virulence defect cannot be attributed solely to the lack of flagellar synthesis, since the *flaA* and *cheA* mutants have little or no effect on bacterial virulence in the murine model (Bigot *et al.*, 2005; Dons *et al.*, 2004; Way *et al.*, 2004), although it has been reported that flagella influence *Listeria* pathogenicity soon after oral ingestion in a murine model (O'Neil & Marquis, 2006). However, since flagellar synthesis genes are not expressed at the host temperature of 37 °C, the specific role of DegU in virulence remains to be established.

It has been shown recently that DegU is required for biofilm and pellicle formation as well as multicellular behaviour in *B. subtilis* undomesticated strains (Kobayashi, 2007a, b; Verhamme *et al.*, 2007), and that gradual increases in DegU phosphorylation levels are critical in the transition from motile to sessile biofilm-forming cells. DegU is also known to affect biofilm formation in some *B. subtilis* laboratory strains (Stanley & Lazazzera, 2005). Given our results that link DegU not only with motility but also with biofilm formation in *L. monocytogenes*, and the fact that the DegS kinase is lacking in this bacterium, we are currently investigating the role of DegU phosphorylation in *Listeria* and the importance that it may have in the many different phenotypes linked to this pleiotropic regulator.

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