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*.

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...
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, LssK/LssR, involved in tolerance to stress and virulence (Cotter 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 exopoly saccharide 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 ΔdegU 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 DH5α [F− (λodhaZΔM15) Δ(lacZΔM15lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+)] 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 μg ml−1) and kanamycin (25 μg ml−1) when required. B. subtilis cells were grown in LB medium supplemented with 5 μg 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 Welchimer’s Broth (MBW) minimal medium (Pembratine 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 μg ml−1) or erythromycin (1 μg ml−1) when necessary.

DNA manipulations. Oligonucleotides used in this study were synthesized by Sigma-Prolog 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**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGDe</td>
<td>L. monocytogenes reference strain</td>
<td>Glaser et al. (2001)</td>
</tr>
<tr>
<td>LM1000</td>
<td>EGDe/pMK4</td>
<td>pMK4—EgEgD</td>
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<tr>
<td>LM1001</td>
<td>EGDe AdegU</td>
<td>pMADAdegU—EGDe</td>
</tr>
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<td>LM1002</td>
<td>EGDe AdegU/pMK4</td>
<td>pMK4—LM1001</td>
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<tr>
<td>LM1003</td>
<td>EGDe AdegU/pMK4degU</td>
<td>pMKAdegU—LM1001</td>
</tr>
<tr>
<td>LM1010</td>
<td>EGDe AyviA</td>
<td>pMADAyviA—EgEgD</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMAD</td>
<td>Vector for deletion replacement of genes in Gram-positive bacteria</td>
<td>Arnaud et al. (2004)</td>
</tr>
<tr>
<td>pMADAdegU</td>
<td>pMAD derivative, for deletion of the degU gene</td>
<td>This study</td>
</tr>
<tr>
<td>pMADAyviA</td>
<td>pMAD derivative, for deletion of the yviA gene</td>
<td>This study</td>
</tr>
<tr>
<td>pMK4</td>
<td>Shuttle vector for Gram-negative and Gram-positive bacteria</td>
<td>Sullivan et al. (1984)</td>
</tr>
<tr>
<td>pMKAdegU</td>
<td>Plasmid for expression of degU in L. monocytogenes</td>
<td>This study</td>
</tr>
<tr>
<td>PET28/16</td>
<td>pET28a derivative for overproduction of His-tagged proteins</td>
<td>Chastanet et al. (2003)</td>
</tr>
<tr>
<td>PETDegULmo</td>
<td>pET28/16 derivative for overproduction of the L. monocytogenes DegU protein</td>
<td>This study</td>
</tr>
</tbody>
</table>

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 two 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 pMADAdegU was used to generate a markerless ΔdegU 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 of 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 pMADAdegU. 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 pMADAdegU was performed as described previously (Arnaud et al., 2004) with a non-permissive growth temperature of 42 °C, yielding strain LM1001 (AdegU), 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 pMADAyviA 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 (AyviA), and the gene deletion was verified by PCR amplification using oligonucleotides HD118/HD123.

In order to complement the L. monocytogenes ΔdegU 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 Ncol/Xhol DNA fragment corresponding to the L. monocytogenes degU coding sequence (698 bp; oligonucleotides degugenF/degugenR) between the Ncol and Xhol sites of plasmid pET28/16, replacing the stop codon with a Xhol 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 GroEL 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-fifth 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

<table>
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<th>Sequence</th>
<th>Gene and use</th>
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<td>OSA5</td>
<td>ACGGCTCGACGGGAACTATATGAACG</td>
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<td>CGGGATCCCGTTTACATTTTGCAAGCC</td>
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</tr>
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<td>OSA15</td>
<td>ACGGCTCGACCTGAGGAAAAAATCATGATG</td>
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<tr>
<td>OSA30</td>
<td>CGGTCTAACCTTATATGCCCGTCC</td>
<td>degU primer extension</td>
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<tr>
<td>degUgenF</td>
<td>CAACATGGCAGCTTAAATCATGATTGAG</td>
<td>DegU overproduction</td>
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<tr>
<td>degUgenR</td>
<td>CTCCCTGAGGAAAAATGCATACGCCGTTG</td>
<td>DegU overproduction</td>
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<td>degUapF</td>
<td>GAAAGATTCTAAATATGACATCAGAAAGAA</td>
<td>degU gel shift</td>
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<td>degUupF</td>
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<td>degUmid</td>
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<td>degU RT-PCR</td>
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<td>yviAmid</td>
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<td>flaA qRT-PCR</td>
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<td>HD115</td>
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<td>gmnlq internal PCR fragment</td>
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<td>HD116</td>
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<td>yviA deletion</td>
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<td>yviA deletion</td>
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<td>OP133</td>
<td>CACCCGAGGCTAACAATAAACATTAATGACG</td>
<td>rpoB qRT-PCR</td>
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<tr>
<td>OP134</td>
<td>TAGGCGTGAACATGATGATCACCAAA</td>
<td>rpoB qRT-PCR</td>
</tr>
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</table>

(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) and loaded onto 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 20 000 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 volumes phenol/chloroform/20% sodium acetate (1:1:10, v/v/v) and air-dried for 30 min. RNA concentrations were determined by measuring A260 and A280 and samples were stored at −80°C.

**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, 20 000 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 20 000 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 A280 and A595 was measured. The assay was performed in triplicate.
**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-PCR reactions were used to show that the degU and yviA genes are cotranscribed, using 22 μg total RNA, oligonucleotides degUmid and yviAmid (100 pmol of each) and the reverse transcriptase 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 & Zakrzajsek, 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 gmaIR intragenic region (463 bp) were generated by PCR with Pwo polymerase (Roche) and oligonucleotide pairs degUupF/degUupR, HD113/HD114 and HD115/HD116, respectively (Table 2). Labelling, DNA binding and gel electrophoresis mobility shift DNA-binding assays were performed as described previously (Derre 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 ΔdegU mutant (strain LM1001; 10^2–10^7 bacteria). Mortality was checked over a 10-day period.

**Database comparisons and sequence analysis.** Computations were performed with the SubtilList 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.
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 (*AdegU*) and LM1010 (*AyviA*) 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 (*AdegU*) 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, EρA (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 region systematically in the *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 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
DNA 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 -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,
known to control flaA expression, was abolished in strain LM1001 (ΔdegU). As expected, expression of flaA and cheA was abolished in the ΔdegU 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 amounts of purified DegU. Lanes: 1, no protein; 2, 0.02 μM; 3, 0.05 μM; 4, 0.1 μM; 5, 0.5 μM; 6, 1 μM; 7, 1.5 μM; 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.

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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 μM; 2, 0.3 μM; 3, 0.5 μM; 4, 0.7 μM; 5, 1 μM; 6, 3 μM; 7, no protein. (b) DNase I footprting 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 μM; 3, 0.05 μM; 4, 0.1 μM; 5, 0.5 μM; 6, 1 μM; 7, 1.5 μM; 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.

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I. Gueriri and others
DegU is involved in virulence of L. monocytogenes

We examined virulence of an L. monocytogenes ΔdegU mutant in a murine model. Virulence of the ΔdegU mutant was assayed by intravenous injection as described in Methods, and compared with that of the wild-type EGDe strain. The LD$_{50}$ of the ΔdegU mutant was $4.2 \times 10^4$ c.f.u., whereas that of EGDe was $3.9 \times 10^3$ c.f.u. The ΔdegU 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 (ΔdegU) 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 ΔdegU mutant displays many phenotypes, including, as shown here, growth deficiency in RPMI 1640 synthetic medium, inability to grow at high temperatures (44 °C),
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 ΔyviA 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

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**Fig. 6.** DegU is required for flagellar and motility gene expression and flagellar synthesis. (a) Electron micrographs of strains EGDe, LM1001 (ΔdegU) and LM1003 (ΔdegU/pMK4degU), showing loss of flagella in the ΔdegU 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 EGDe and LM1001 (ΔdegU). (c) qRT-PCR analysis of motility (flaA) and chemotaxis (cheA) gene expression at 25 °C in strains LM1000 (EGDe/pMK4), LM1002 (ΔdegU/pMK4) and LM1003 (ΔdegU/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.
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 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 EGDe parental strain and the 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* 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_2^ 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 σD 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 ΔdegU 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 ΔdegU 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 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 ΔdegU 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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