Characterization of DegU, a response regulator in *Listeria monocytogenes*, involved in regulation of motility and contributes to virulence

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Abstract

The *degU* (*lmo2515*) gene encodes a putative response regulator in the food-borne pathogen *Listeria monocytogenes*. It has 63% amino acid identity to the DegU response regulator of *Bacillus subtilis*. We have characterized the *degU* gene product in *L. monocytogenes* EGD by generation of a deletion mutant. The Δ*degU* mutant was found to be non-motile in motility plate assay and no flagellin was detected. The mutant was attenuated in challenge of mice. Northern blot analysis suggested that the *degU* gene product is a transcriptional activator of the flagellin gene, *flaA*, at 25 °C. However, the *degU* gene product had no influence on the transcription of *prfA* encoding the major virulence regulator, PrfA. The results indicate that the putative DegU response regulator is a pleiotropic regulator involved in expression of both motility at low temperature and in vivo virulence in mice.
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Keywords: Listeria monocytogenes; Response regulator; DegU; Flagellin; Motility; Virulence

1. Introduction

Listeria monocytogenes is a Gram-positive facultative intracellular motile rod that causes severe food-borne infections in humans and animals. It is ubiquitous in nature and can survive many different hazardous environments, such as low temperature and high osmolarity [1]. Motility and virulence are thermo-regulated in *L. monocytogenes* [2–4]. Cells are motile at 20–25 °C and generally non-motile at 37 °C. The transcription of *flaA* encoding the structural flagella protein is induced at 25 °C, while no transcription is observed at 37 °C [2]. Motility and chemotaxis genes such as *motA*, *cheR*, *cheA* and *cheY* have been found to have a temperature-dependent transcription as well [3,5]. In addition, FlaR regulates the expression of flaA, and the transcription of *flaA* in a *flaR* mutant is decreased at 25 °C; however, at 37 °C the expression of *flaA* is derepressed. The transcription of *flaR* is osmoregulated and the presence of 400 mM NaCl in the growth medium abolishes production of flagellin in *L. monocytogenes* LO28 [6].

The PrfA regulator controls the transcription of the 9-kb *Listeria* pathogenicity island 1 (LIPI-1), which encodes major virulence factors such as listeriolysin, ActA and the two phospholiases [7,8]. The *prfA* gene encoding PrfA is controlled in a temperature-dependent way [9,10]. The PrfA thermo-regulation is due to an untranslated mRNA, which forms a secondary structure, that

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masks the ribosome binding region of the monocistronic *prfA* messenger at temperatures below 30 °C [4]. At 37 °C, the transcription of *prfA* and the PrfA dependent virulence genes can be further induced, when the cells are grown in Brain Heart Infusion broth added activated charcoal [11]. PrfA is the regulator of other virulence genes such as the internalin C and bile salt hydrolase (BSH) encoded by *inlC* and *bsh*, respectively [12,13], and it is involved in regulation of internalin A encoded by *inlA* [14]. In addition, PrfA is found to modify the transcription of the *flaA* and *motA* genes. The amount of *motA* mRNA is twofold higher in a Δ*prfA* mutant compared to the wild-type [5], whereas the expression of *flaA* is downregulated in the P14-A mutant in which *prfA* is constitutively expressed [15].

Microorganisms, including *L. monocytogenes*, have developed sophisticated networks such as two-component signalling systems to adapt to environmental changes [16]. These systems can sense and respond to different stimuli and have been found to be involved in virulence, antibiotic resistance and stress response [16,17]. A typical two-component system consists of a sensor histidine kinase and a cognate, cytoplasmic response regulator. The histidine kinase senses environmental changes and causes an autophosphorylation of the kinase. The phosphate group is transferred to the response regulator, which mediates the adaptive response in the cell [18]. Two-component systems involved in expression of motility and virulence have been identified in *Bordetella bronchiseptica* [19] and *Salmonella enterica* serovar Typhimurium [20]. The BvgAS system in *B. bronchiseptica* and the SirA/BarA system in *S.* Typhimurium are both involved in regulation of a type III secretion system, and mutants in these two-component systems are impaired in motility and virulence [19–21]. The environmental signal for the BvgAS system is among other temperature leading to a temperature-dependent expression of virulence and motility.

In *Bacillus subtilis*, the two-component system, DegS–DegU, is involved in a complex network that mediates the transition from exponential to the stationary growth phase. Furthermore, the DegS–DegU system contributes to the regulation of competence for DNA uptake, degradative enzymes and motility [22–24]. In this study we have characterized a putative response regulator DegU in *L. monocytogenes*. The polypeptide encoded by the *degU* gene (*lmo2515*) shows 63% amino acid identity to the *B. subtilis* response regulator DegU. An in-frame deletion mutant, Δ*degU*, was generated in the *degU* gene. The mutant was affected in the expression of *flaA* mRNA and had decreased level of virulence when compared to the wild-type strain. The putative DegU response regulator in *L. monocytogenes* is suggested to be the activator of the *flaA* transcription at 25 °C. However, the mode of action for attenuation is not clear.

2. Materials and methods

2.1. Bacteria and growth conditions

The *L. monocytogenes* wild-strain EGD (BUG600, serotype 1/2a), kindly provided by P. Cossart, was routinely grown in Brain Heart Infusion (BHI) broth (OXOID) at 37 °C overnight with shaking. *Escherichia coli* DH5α was grown in Luria–Bertani broth supplemented with 150 μg ml^{-1} erythromycin at 37 °C overnight with shaking. For expression of virulence genes 0.2% activated charcoal (Merck) was added to BHI (BHIAC) [25].

2.2. Construction of mutant

Gene splicing by overlap extension (gene SOEing) was used to create a recombinant gene fragment for an in-frame deletion mutant in the *degU* gene [26]. Primers were constructed on the basis of the published sequence of *L. monocytogenes* EGD-e [27]. Chromosomal DNA and plasmid extractions, restriction enzyme digestions and DNA ligations were performed according to standard protocols [28]. A 472 bp fragment harbouring the 5′end of the gene and the upstream sequence was amplified with polymerase chain reaction (PCR) from chromosomal DNA of EGD with the p2515SOE1-HindX and p2515SOE2 primers (Table 1). Similar, a 258 bp fragment harbouring the 3′end of the gene and the downstream sequence was amplified with the p2515SOE3X and p2515SOE4BamX primers. These fragments were spliced in a second round of PCR to produce a 730 bp PCR fragment containing an in-frame deletion of 603 bp of the *degU* gene. The PCR fragment was digested with *Hin*dIII and *Bam*HI, and cloned into pAUL-A [29]. The plasmid, pAUL-Δ*degU*, harbouring the 730 bp fragment was isolated and verified by sequencing. The generation of the deletion mutant was performed as described by Guzman et al. [30]. Three presumptive Δ*degU* mutants were obtained and analysed with PCR. One mutant was selected for the characterization of the *degU* gene and this isolate was sequenced to verify the deletion of the 603 bp. For comparison, a 1217 bp fragment of EGD harbouring the *degU* gene was sequenced. The sequencing was performed by DNA Technology (Aarhus, Denmark) and alignments were performed at www.ncbi.nlm.nih.gov using BLASTP and BLASTN.

2.3. Motility plate assay and haemolysis

Swimming of strains was investigated as described by Kathariou et al. [31] with some modifications. A single colony was inoculated with a straight inoculating needle in Tryptic Soy Broth with 0.25% agar (Difco, Becton-Dickinson) and incubated at either 25 or 37 °C for 24–48 h.

Table 1
The sequence of the primers used in the study

Primers used during the experiments	
p2515SOE1HindX	5′-TTTCAATAAT**AAGCTT**GTGTTTTCCATCGCTATGG[a]
p2515SOE2	5′-CGCGAAACAACTGATGATC
p2515SOE3X	5′-<u>GATCATCAGTTGTTTCGCG</u>TAACGGCAATCAAGCACGG[b]
p2515SOE4BamX	5′-AATGAATTC**GGTACC**CGCAGGTTGAGAGGAAGTTG[c]
flaA-forward	5′-ACGAAGGCATGACTCAAGCG
flaA-reverse	5′-TAACCAGCAACTGTAGAACC
degU-forward	5′-GGAGGAGTAGTCATTATGGC
degU-reverse	5′-ACTTCTGGTTGTTGGTAGCC
prfA-forward	5′-TAACCAATGGGATCCACAAG
prfA-reverse	5′-TGCTAACAGCTGAGCTATGTG
inlA-forward	5′-AAGTGACGTAAGCTCACTTGC
inlA-reverse	5′-TGTTGGTGGTGTAGGTTCTTG
dnaK-forward	5′-TTATCCCTAACCCAGAAGGC
dnaK-reverse	5′-TTATCGAAGTCATCTCCACC

[a] Bold sequence: *Hin*dIII restriction enzyme site.
[b] Underlined sequence: sequence complementary to p2515SOE2.
[c] Bold sequence: *Bam*HI restriction enzyme site.

Haemolysis was investigated on Blood agar base (OXOID) supplied with 10% calf blood and incubated at 25 or 37 °C for 36 h. CAMP test was performed on Blood agar base supplied with 10% sheep blood by streaking *Staphylococcus aureus* S31H6 together with the strains to be investigated and incubated at 37 °C for 36 h.

2.4. Growth experiments

Two millilitre overnight culture was inoculated in 98 ml BHI broth and incubated at 25 or 37 °C with mild agitation (100 rpm). Growth of the culture was followed by measuring optical density at 600 nm (OD_{600}) until stationary phase. Furthermore, the growth was investigated in BHI with 400 mM NaCl.

2.5. SDS–PAGE and Western blotting

For analysis of flagellin expression, cell preparations were prepared by centrifugation of 2 ml overnight cultures at 4.500g at 4 °C for 10 min. Pellet was resuspended in PBS, mixed with sample buffer (Bio-Rad) and dithiothreitol, and boiled for 10 min. For analysis of Clp ATPases, cells were grown to $OD_{600} \sim 0.6$ and lysates were made by sonication. The SDS–PAGE was carried out using Bio-Rad Ready Gels (Bio-Rad), which contained a 4% acrylamide stacking and 12% acrylamide separating gel. Proteins were transferred to nitrocellulose by electrophoresis. Blocking, washing, antibody incubation and colour development were performed as described by Dons et al. [2]. Monoclonal antibodies against *L. monocytogenes* 4b flagellin was kindly provided by W. Donachie, Moredun Research Institute, Edinburgh, Scotland, and polyclonal antibodies directed against ClpE from *Lactococcus lactis* was kindly provided P. Varmanen, Helsinki University.

2.6. Mouse virulence assay

Eight weeks old female BALB/c mice (Bomholtgård Breeding and Research Center, Ltd.) were used. Bacteria were grown at 37 °C in BHI broth until $OD_{600} \sim 0.6$. Mice were infected intragastrically with 2×10^9 CFU or intraperitoneally with 2×10^4 CFU (5 per group). Three days after infection, the mice were sacrificed by cervical dislocation, and the spleen and the liver were dissected. The organs were homogenized with 10 ml PBS containing 0.6% Triton X-100. Tenfold serial dilutions of the lysates were plated on BHI agar plates. Colonies were counted after overnight incubation at 37 °C overnight. The data were analysed statistically by the Student's *t*-test.

2.7. RNA purification and Northern blotting

For transcriptional analysis, RNA was purified from exponential growing cells ($OD_{600} \sim 0.6$). Cells were harvested by centrifugation at 10.000g for 5 min and pellet was frozen in ethanol-dry ice prior to storage at −80 °C. The cells were lysed with a FastPrep FP120 instrument (BIO101, ThermoSavent) for 45 s at speed 6.0 and total RNA was isolated with the RNeasy kit (QIAGEN). For analysis of transcription, 3 µg total RNA was denatured at 65 °C for 5 min, chilled and loaded onto a 1.2% formaldehyde–agarose gel as described by RNeasy Mini Handbook (QIAGEN). The RNA was transferred to a Hybond-N membrane (Amersham) by capillary blotting and UV cross-linked. Hybridization probes were generated by PCR from chromosomal DNA of EGD using specific primers (Table 1) for the *flaA*, *degU*, *dnaK*, *inlA* and *prfA* genes. The probes were labelled with [α-^{32}P]dCTP by using Ready-To-Go DNA Labelling Beads (Amersham). The hybridization was performed at 65 °C in hybridization buffer (0.5 M sodium

phosphate, pH 7.2, 7% (w/v) SDS) overnight as described by Arnau et al. [32]. Washing was performed at 65 °C one time for 15 min with the washing buffer (20 mM sodium phosphate, pH 7.2, 1% (w/v) SDS) followed by two washes for 10 min.

3. Results

3.1. Identification of a DegU homologue in L. monocytogenes

The BvgA response regulator of *B. bronchiseptica* [33] was used in a BLAST search of the *L. monocytogenes* EGD-e genome [27] for putative response regulators involved in motility and virulence. The putative polypeptide encoded by the *lmo2515* gene was identified and the two polypeptides have an amino acid identity of 30%. The polypeptide encoded by the *lmo2515* gene and the DegU protein of *B. subtilis* [34] have 63% amino acid identity. In the newly sequenced *L. monocytogenes* strain F2365 (serotype 4b) (Genbank Accession No. YP_015076) the *lmo2515* gene is annotated *degU*. Thus, the *lmo2515* gene and the encoded polypeptide can be renamed as the *degU* gene and the putative DegU response regulator, respectively.

An alignment of the nucleotide sequences harbouring the *degU* gene in *L. monocytogenes* EGD-e and *L. monocytogenes* EGD (BUG600) (Genbank Accession No. AY667428) wild-types was made. The two sequences are identical with exception of two nucleotides in position −243 and 164, respectively, upstream of the translational start codon of the *degU* gene (Fig. 1(b)). In BUG600 at position −164 a G to A transition has occurred and in position −243 an A to G transition was found.

An analysis of the region located upstream of the *degU* gene showed that sequences with homology to the consensus sequences for σ^A promoters (TTG-ACAN$_{17}$TATAAT) [35] and σ^D promoters (TAAA-N$_{15}$GCCGATAT) [36] are located upstream of the translational start codon of the *degU* gene (Fig. 1(b)). Next, we analysed the genome of the *L. monocytogenes* EGD-e for the presence of the *B. subtilis* putative DegU target sequence (AGAAN$_{11}$TTCAG) [37] with the pattern search algorithm on the ListiList website [38]. Putative DegU target sequences were identified in the regions upstream of the *degU* and the *flaA* genes (Fig. 1(b) and (c)).

3.2. The ΔdegU mutant is not motile and does not express flagellin

To characterize the putative DegU response regulator in *L. monocytogenes* EGD (BUG600), an in-frame deletion mutant in the *degU* gene was constructed. The in-
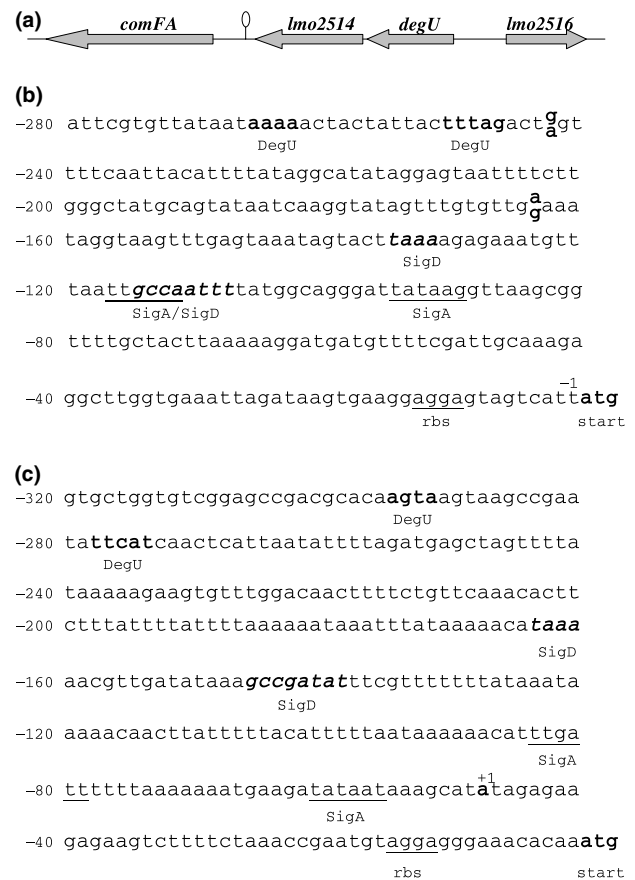

Fig. 1. (a) Genomic organization of the *degU* gene region in *L. monocytogenes*. Arrows indicate the orientation of the genes. Hairpin structure illustrates a putative terminator. The *comFA* gene encodes a polypeptide, which is similar to the late competence protein ComFA [38]. The *lmo2514* gene encodes a putative polypeptide with similarity to the *B. subtilis* DegV protein, which is of unknown function [38]. The *lmo2516* gene encodes a polypeptide with similarity to conserved hypothetical proteins [38]. (b) Nucleotide sequence of the promoter region of the *degU* gene. The transitions in position −164 and −243 are marked with bold with the EGD (BUG600) wild-type being the upper letter and the EGD-e wild-type being the lower letter. (c) Nucleotide sequence of the promoter region of the *flaA* gene. The transcription initiation site of the *flaA* mRNA (+1) found by Dons et al. [2] is indicated. (b) and (c) Sequences in the *degU* and the *flaA* promoter regions with homology to the consensus sequences of σ^A promoters (underlined) [35] and σ^D promoters (italic) [36] as well as the putative consensus target site for DegU (bold) [37] of *B. subtilis* are marked. The putative ribosome-binding sites (rbs) are underlined.

frame technique was used to prevent polar effects on downstream genes. The ΔdegU mutant and the wild-type had similar growth rates when grown in BHI at 25 or 37 °C as well as with 400 mM NaCl (data not shown). In addition, no difference was found with respect to colony size and haemolytic activity on blood agar and in a CAMP test (data not shown). However, unlike the wild-type EGD, the ΔdegU mutant did not swim at 25 °C in a motility plate assay (Fig. 2). Two other presumptive *degU* mutants, which were isolated during the construction of the ΔdegU mutant, showed the same non-motile phenotype when analysed in

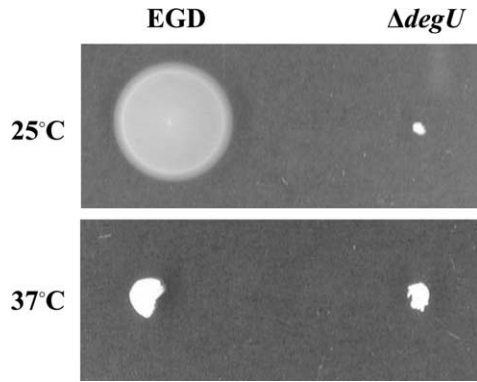Fig. 2. The swimming phenotype of EGD and the Δ*degU* mutant when analysed in motility plate assay (tryptic soy broth with 0.25% agar) at 25 or 37 °C.

motility plate assay (data not shown). Western blot analysis revealed that the *degU* mutant did not express flagellin at 25 or 37 °C (Fig. 3). These results suggest that the *degU* gene product is involved in expression of flagellin either at transcriptional or post-transcriptional level. In addition, the expression of flagellin was investigated of the wild-type and the Δ*degU* mutant grown in BHI with 400 mM NaCl at 25 °C. The expression of flagellin when grown with 400 mM NaCl was neither affected in the wild-type nor in the Δ*degU* mutant when compared to growth in BHI (Fig. 3).

3.3. The level of virulence is decreased in the degU mutant compared to the wild-type strain

The effect of the mutation in the *degU* gene was examined in a mouse virulence model where groups of five mice were inoculated with EGD or the Δ*degU* mu-

tant either intragastrically or intraperitoneally. Both when the mice were infected intragastrically and intraperitoneally the bacterial counts in spleens and livers were significantly reduced for the Δ*degU* mutant (Fig. 4). This indicates that the putative response regulator DegU plays a role in murine listeriosis.

3.4. DegU is involved in regulation of flaA but neither of PrfA-dependent virulence genes nor stress adaptation genes

To investigate if the *degU* gene product is involved in regulation of *flaA* and virulence genes, Northern blot analysis was performed using total cellular RNA from exponential growing cells cultured at 25 or 37 °C. The amount of *flaA* transcript at 25 °C was significantly decreased in the Δ*degU* mutant when compared to the wild-type (Fig. 5(a)) and at 37 °C a low level of *flaA* transcript was detected in both EGD and the mutant. In the Δ*degU* mutant similar amounts of transcript were detected at 25 and 37 °C. These data suggest that the *degU* gene product is involved in the expression of *flaA* mRNA at 25 °C.

A single transcript of ≈ 1.8 kb was detected in EGD when using an internal fragment of the *degU* gene as probe (Fig. 5(b)). In the Δ*degU* mutant, no transcript was detected as expected.

Next, the transcription of the *prfA* gene and the *inlA* gene was investigated using total RNA from cells grown at 25 °C in BHI and at 37 °C in BHI with or without addition of activated charcoal. No differences were

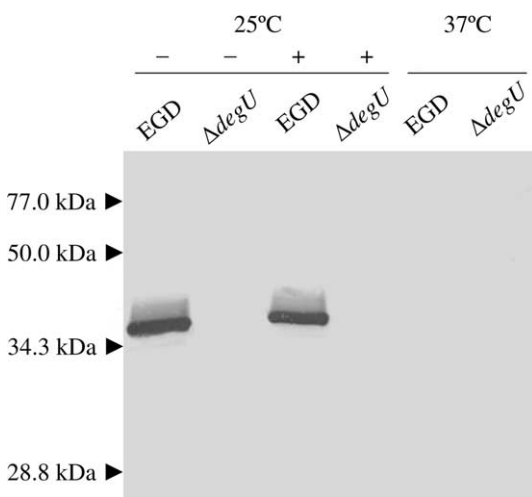

Fig. 3. Western blot analysis of boiled cell preparations made from fresh overnight cultures of EGD and the Δ*degU* mutant grown at 25 °C in BHI without additional NaCl (−) and with a total of 400 mM NaCl (+) and at 37 °C without additional NaCl. Protein extracted from $\approx 5 \times 10^7$ CFU were loaded on the SDS–PAGE gel and incubated with a monoclonal antibody, specific for *L. monocytogenes* 4b flagellin.
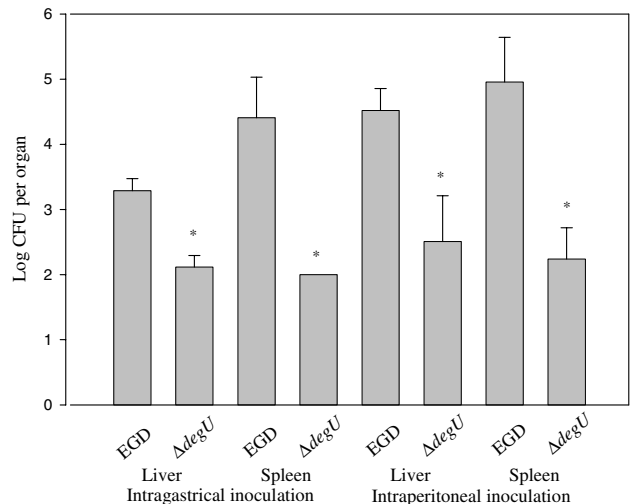

Fig. 4. Colony forming units in the spleen and liver of BALB/c mice. Groups of five mice were inoculated intragastrically with $\approx 2 \times 10^9$ CFU or intraperitoneally with $\approx 1 \times 10^4$ CFU. The experiments were performed twice with similar results. The detection limit of the experiment was 100 CFU equal 2 log CFU per organ and means and standard deviation (SD) are shown. An asterisk indicates a significant difference in the log CFU of mutant when compared to the wild-type ($P = <0.001$, as determined by the Student's t-test).
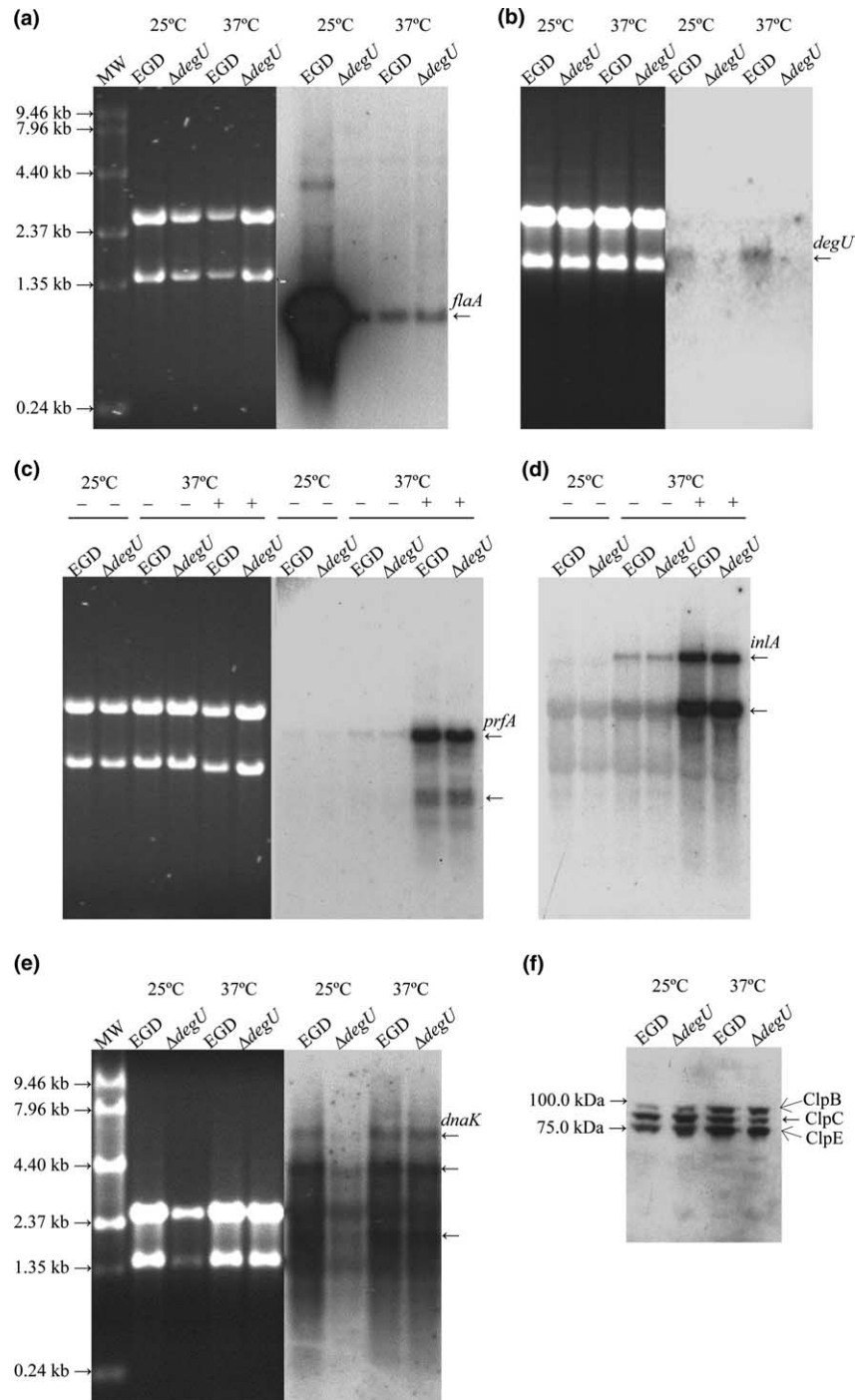
Fig. 5. Northern and Western blot analysis of expression in EGD and the Δ*degU* mutant. (a)–(e) For Northern blot analysis total RNA was isolated from cells grown to mid-exponential phase in BHI at 25 or 37 °C. (c, d) In addition, for studying expression of the virulence genes cells were grown at 37 °C in BHI without (−) or with activated charcoal (+). Total RNA (∼3 μg pr lane) was separated by electrophoresis on a 1.2% agarose–formaldehyde gel. (a) The ethidium bromide stained gel and Northern blot probed with an internal fragment of the *flaA* gene. (b) The ethidium bromide stained gel and Northern blot probed with an internal fragment of the *degU* gene. (c, d) The ethidium bromide stained gel and Northern blot probed with an internal fragment of the *prfA* gene (c) and the *inlA* gene (d). (e) The ethidium bromide stained gel and Northern blot probed with an internal fragment of the *dnaK* gene. (f) For Western blot analysis, total protein was isolated from cells grown in BHI at 25 or 37 °C to mid-exponential phase. Whole-cell lysates from ≈1 × 10⁸ CFU were loaded on the SDS–PAGE gel and incubated with a polyclonal antibody directed against ClpE from *L. lactis*. The three bands are based on the molecular weigh assumed to be ClpB, ClpC and ClpE, respectively.

found in amount of *prfA* (Fig. 5(c)) or *inlA* transcripts (Fig. 5(d)) at neither temperatures when the Δ*degU* mutant was compared with the wild-type, EGD.

The expression of the stress adaptation gene, *dnaK*, was investigated using total RNA from cells grown at 25 or 37 °C. No significant difference was found in the

transcription between the wild-type strain and the *degU* mutant (Fig. 5(e)). All three transcripts were detected in relative smaller amounts for the *degU* mutant at 25 °C as a smaller amount of RNA was loaded. In addition, the expression of Clp ATPases in cells grown at either 25 or 37 °C in BHI was investigated by Western blot analysis with polyclonal antibodies directed against ClpE from *L. lactis*. No difference was found in the protein pattern when the Δ*degU* mutant was compared with the wild-type (Fig. 5(f)).

4. Discussion

In this work, we characterized the *degU* gene putatively encoding a response regulator DegU in *L. monocytogenes*. The results, obtained in this study, are achieved with the constructed Δ*degU* mutant, however, prior to this work was constructed an insertion mutant in the *degU* gene using the pAUL-A plasmid. The *degU* insertion mutant was found to be non-motile at 25 °C. When tested in a mice model with intragastrical inoculation the *degU* insertion mutant was attenuated as the $\log_{10}$ CFU recover of the spleens was 2.1 ± 0.1 for the *degU* insertion mutant and 3.7 ± 1.4 for the EGD wild-type. To exclude polar effects on downstream genes, an inframe deletion mutant was constructed. During the generation of the *degU* mutant three independent mutants were obtained and these were all found to be non-motile. This strongly indicates that no secondary mutation has occurred during the generation of the deletion mutant.

The polypeptide encoded by *degU* has a high level of amino acid identity to the DegU response regulator of the DegU–DegS two-component system in *B. subtilis* [34]. Immediately upstream of the *degU* gene in *B. subtilis* is located the *degS* gene encoding the cognate histidine kinase to DegU, DegS. However, in *L. monocytogenes* EGD-e no DegS homologue is found. Thus, based on the genome sequence it appears that DegU is an orphan response regulator in *L. monocytogenes*. No gene is located directly upstream of the *degU* gene and the upstream gene is transcribed in the opposite direction (Fig. 1(a)). The size of the *degU* transcript (1.8 kb, Fig. 5(b)) indicates that *degU* is probably transcribed together with a downstream putative gene, *lmo2514*, encoding a polypeptide with similarity to the DegV polypeptide in *B. subtilis* [38].

By examination of motility of the *degU* mutant at 25 °C, the putative response regulator DegU in *L. monocytogenes* was found to be involved in the regulation of motility and based on Northern blot analysis the *degU* gene product is suggested to be a transcriptional activator of *flaA* at 25 °C. However, motility and the *flaA* expression was not induced at 37 °C in the *degU* mutant like Sanchez-Campillo et al. [6] found in the *flaR*

mutant. FlaR is osmoregulated and represses motility at 400 mM NaCl in the wild-type *L. monocytogenes* LO28, however, the expression of flagellin in the EGD (BUG600) wild-type was not affected with addition of 400 mM NaCl (Fig. 3). So far, we have obtained no indications that DegU and FlaR are involved in the same regulatory mechanism. Instead of, there seems to be a difference between the wild-types LO28 and EGD with regard to osmoregulation of flagellin.

Phoshorylated DegU represses the transcription of several motility genes in *B. sublitis* through repression of *sigD* [24]. Indeed, in *L. monocytogenes* motifs, which share homology with the consensus sequence for σ^D promoters were identified upstream of the *degU* (Fig. 1(b)) and *flaA* (Fig. 1(c)) genes and the *cheY–cheA* operon [2,3] as well as the virulence gene, *inlA* [9]. However, no σ^D homolog has been identified in *L. monocytogenes* though a weak signal was detected from the putative σ^D promoter of the *inlA* gene by Dramsi et al. [9]. In *B. subtilis*, a σ^A motif is present in the promoter region controlling the *degS–degU* operon [39] and a similar putative promoter sequence is present upstream of the translational start codon of *degU* in *L. monocytogenes* (Fig. 1(b)). Furthermore, we have searched for open reading frames which harbour a putative DegU target sequence upstream of the translational start site [37]. Several open reading frames were identified including the *flaA* and *degU* genes (Fig. 1(b) and (c)). These findings suggest that DegU may act similarly in *B. subtilis* and *L. monocytogenes*.

Flagella and chemotaxis have been reported to be required for association and invasion of *L. monocytogenes* when the bacteria are cultured at 24 °C prior infection of Caco-2 cells [40]. However, flagella and chemotaxis are not required for in vivo virulence in a mouse model and indeed, a non-flagellated mutant has increased virulence [40]. As the non-motile *degU* mutant was impaired in virulence, it could indicate either of the two situations. The mutation in the *degU* gene might cause expression of flagellin in vivo, which influences on the virulence. The other possibility is that the *degU* gene product is a pleiotropic regulator and involved in regulation of virulence genes. The Northern blot analysis showed neither transcriptional changes for the *prfA* or *inlA* genes nor was the activity of listeriolysin affected. Several non-PrfA regulated virulence factors have been identified in *L. monocytogenes* as well as different environmental stress factors, which are also involved in virulence. The heat shock protein, DnaK, is implicated in transcription of both *flaA* and *lmaA* encoding a protein involved in the induction of delayed-type hypersensitivity [41,42]. Similar a mutation in the repressor, CtsR, of the genes encoding the Clp heat shock proteins leads to a decrease in *flaA* mRNA [43]. However, the expression of *dnaK* and the Clp ATPases were not affected in the *degU* mutant.

A putative response regulator DegU involved in expression of motility and virulence has been characterized in this study similar to that is reported for the BvgA and SirA response regulators in *B. bronchiseptica* and *S.* Typhimurium, respectively. However, further genetic and biochemical studies are required to elucidate the involvement of DegU in the motility and virulence expression of *L. monocytogenes*.

Acknowledgements

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