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 phospholases [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 ΔprfA 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, ∆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 digests 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 HindIII and BamHI, and cloned into pAUL-A [29]. The plasmid, pAUL-ΔdegU, 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 ΔdegU 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.
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 4,500 g 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 OD600 = 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 (Bomholtgaard Breeding and Research Center, Ltd.) were used. Bacteria were grown at 37 °C in BHI broth until OD600 = 0.6. Mice were infected intragastrically with $2 \times 10^9$ CFU or intraperitoneally with $2 \times 10^4$ 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 (OD600 = 0.6). Cells were harvested by centrifugation at 10,000 g 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 μl 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 $[\alpha-\beta^32P]dCTP$ by using Ready-To-Go DNA Labelling Beads (Amersham). The hybridization was performed at 65 °C in hybridization buffer (0.5 M sodium phosphate, 7% SDS, 1 mM EDTA, 100 μg/ml salmon sperm DNA, 0.5 mg/ml sheared herring sperm DNA, 100 mM NaCl) for 2 h at 65 °C. After hybridization, the membranes were washed once for 20 min in 2x SSC/0.1% SDS at room temperature, then twice for 15 min in 0.1x SSC/0.1% SDS at 42 °C. The membranes were then exposed to X-ray film for autoradiography.

<table>
<thead>
<tr>
<th>Table 1 The sequence of the primers used in the study</th>
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<tr>
<td>Primers used during the experiments</td>
<td></td>
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<tr>
<td>p2515SOE1HindX</td>
<td>5’-TTCCAATAATAAGCTTTGTGTCCATCGTATGGA(^a)</td>
</tr>
<tr>
<td>p2515SOE2</td>
<td>5’-CCGGAACAAACTGATGATC</td>
</tr>
<tr>
<td>p2515SOE3X</td>
<td>5’-GAATCAGGTGTTTTCCGCTAAACGGAATCGACGG(^b)</td>
</tr>
<tr>
<td>p2515SOE4BamX</td>
<td>5’-AATGAAATTCTGAGTACCATTATGGC</td>
</tr>
<tr>
<td>flaA-forward</td>
<td>5’-ACGAAGGCATGACTCAACGG</td>
</tr>
<tr>
<td>flaA-reverse</td>
<td>5’-TAACCAATGGGATCCACAG</td>
</tr>
<tr>
<td>degU-forward</td>
<td>5’-TGCTAACAGCTGAGCTATGTG</td>
</tr>
<tr>
<td>degU-reverse</td>
<td>5’-AAGTGAAGACTAAGCTATCTGC</td>
</tr>
<tr>
<td>prfA-forward</td>
<td>5’-TTATCCCTAACCCAGAAGGC</td>
</tr>
<tr>
<td>prfA-reverse</td>
<td>5’-TTATCGAAGCTCATCCACC</td>
</tr>
<tr>
<td>inlA-forward</td>
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<tr>
<td>inlA-reverse</td>
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<td>dnaK-forward</td>
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<td>dnaK-reverse</td>
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\(^a\) Bold sequence: HindIII restriction enzyme site.

\(^b\) Underlined sequence: sequence complementary to p2515SOE2.

\(^c\) Bold sequence: BamHI restriction enzyme site.
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 rA promoters (TTGACAN17TATAAT) [35] and rD promoters (TAAA-N15GCCGATAT) [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 (AGAAN11TTCAG) [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-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
motility plate assay (data not shown). Western blot analysis revealed that the \( \text{degU} \) mutant did not express flagellin at 25 or 37 °C (Fig. 3). These results suggest that the \( \text{degU} \) 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 \( \Delta \text{degU} \) 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 \( \Delta \text{degU} \) mutant when compared to growth in BHI (Fig. 3).

3.3. The level of virulence is decreased in the \( \Delta \text{degU} \) mutant compared to the wild-type strain

The effect of the mutation in the \( \text{degU} \) gene was examined in a mouse virulence model where groups of five mice were inoculated with EGD or the \( \Delta \text{degU} \) mutant 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 \( \Delta \text{degU} \) 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 \( \text{degU} \) gene product is involved in regulation of \( \text{flaA} \) 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 \( \text{flaA} \) transcript at 25 °C was significantly decreased in the \( \Delta \text{degU} \) mutant when compared to the wild-type (Fig. 5(a)) and at 37 °C a low level of \( \text{flaA} \) transcript was detected in both EGD and the mutant. In the \( \Delta \text{degU} \) mutant similar amounts of transcript were detected at 25 and 37 °C. These data suggest that the \( \text{degU} \) gene product is involved in the expression of \( \text{flaA} \) mRNA at 25 °C.

A single transcript of \( \approx 1.8 \) kb was detected in EGD when using an internal fragment of the \( \text{degU} \) gene as probe (Fig. 5(b)). In the \( \Delta \text{degU} \) mutant, no transcript was detected as expected.

Next, the transcription of the \( \text{prfA} \) gene and the \( \text{inlA} \) 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
found in amount of prfA (Fig. 5(c)) or inlA transcripts (Fig. 5(d)) at neither temperatures when the ΔdegU 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 ΔdegU 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 ΔdegU 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.

Phosphorylated DegU represses the transcription of several motility genes in B. subtilis 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 Ctp 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.

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