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Response Regulator DegU of *Listeria monocytogenes* Controls Temperature-Responsive Flagellar Gene Expression in Its Unphosphorylated State[▽]

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We demonstrate that in *Listeria monocytogenes*, temperature-responsive transcriptional control of flagellar genes does not rely on the phosphorylation of the conserved phosphorylation site (D55) in the receiver domain of response regulator DegU. Furthermore, proper control of DegU-regulated genes involved in ethanol tolerance and virulence is independent of receiver phosphorylation.

In *Listeria monocytogenes*, a facultative intracellular bacterium, the expression of flagellum-based motility is regulated in response to the growth temperature (7, 13), with the permissive temperature being 30°C and below. Temperature-dependent transcriptional regulation of flagellar genes in *L. monocytogenes* relies on three regulatory proteins: the repressor protein MogR, the response regulator DegU, and GmaR, an antirepressor of MogR (3, 4, 9, 10, 13). The MogR protein was shown to bind directly to multiple TTTT-N5-AAAA recognition sites within the promoter regions of the motility genes, thus preventing their transcription at the nonpermissive temperature (3, 9). Response regulator DegU is required to relieve MogR-mediated repression by enabling expression of GmaR at low temperatures. In vitro DNA-binding experiments demonstrated that GmaR is able to both interfere with the binding of MogR to flagellar gene promoters by protein-protein interaction and disrupt preformed repressor-DNA complexes (10). Apparently, DegU is also involved in the regulation of flagellin expression on the posttranscriptional level, since compared to the wild type, a *mogR*- and *degU*-negative mutant of *L. monocytogenes* produced reduced amounts of the FlaA protein, albeit increased transcription of *flaA* (9). Since transcription of *degU* is not responsive to temperature and the DegU protein could be detected at elevated and low temperatures (9, 13), it is expected that the activity state of the transcriptional activator DegU is modulated in response to temperature. Usually the activity of a response regulator is controlled by receiver phosphorylation via a cognate histidine kinase which autophosphorylates in response to the appropriate environmental stimulus (6). However, DegU of *L. monocytogenes* is an orphan response regulator which apparently lacks a cognate histidine kinase (2, 12). Since interaction of a noncognate histidine kinase with DegU is conceivable, we analyzed the contribution of receiver phosphorylation to the transcriptional control of DegU-regulated target genes by complementing a nonmotile in-frame *degU* deletion mutant of *L. monocytogenes* EGD (12) with a mutated *degU* allele carrying a substitution of the putative phosphate-accepting aspartic acid residue (D55) for asparagine. D55 in *L. monocytogenes* DegU corresponds to D56 in the orthologous response regulator protein of *Bacillus subtilis*, which was shown to be the target of phosphorylation by the cognate histidine kinase DegS (1). In fact, recombinant His_6-tagged DegU of *L. monocytogenes* was phosphorylated by DegS of *B. subtilis* in vitro, while phosphoryl group transfer to the mutated response regulator protein DegU(D55N) was not observed (data not shown).

The mutated *degU* allele was integrated into the chromosome of the *L. monocytogenes* Δ*degU* strain by homologous recombination via DNA fragments flanking the *degU* gene by using a two-step pLSV1-based integration-excision procedure described earlier (12). To construct the *degU*(*D55N*) allele with 5′ and 3′ flanking sequences, recombinant PCR was performed by amplification of a 428-bp fragment by using primer pair degU-u5/degU(D55N)1 and a 729-bp fragment by using primer pair degU(D55N)2/degU-d3 and by annealing of the purified PCR products, followed by PCR-based filling-in reactions. After digestion with EcoRI and BamHI, the recombinant PCR fragment was ligated into the mutagenesis plasmid pLSV1 (14), yielding knockout plasmid pLSV-degU(D55N). A control construct, pLSV-degU, was obtained by PCR amplification of the wild-type *degU* gene and its flanking sequences with primer pair degU-u5/degU-d3 and by ligation of the resulting 1,155-bp EcoRI-BamHI fragment into pLSV1 vector DNA. Electrotransformation of the *L. monocytogenes* Δ*degU* strain with pLSV-degU(D55N) and pLSV-degU, respectively, followed by appropriate selection procedures, yielded the *L. monocytogenes degU*(*D55N*) and *L. monocytogenes* Δ*degU** strains. These strains were tested for swimming motility by

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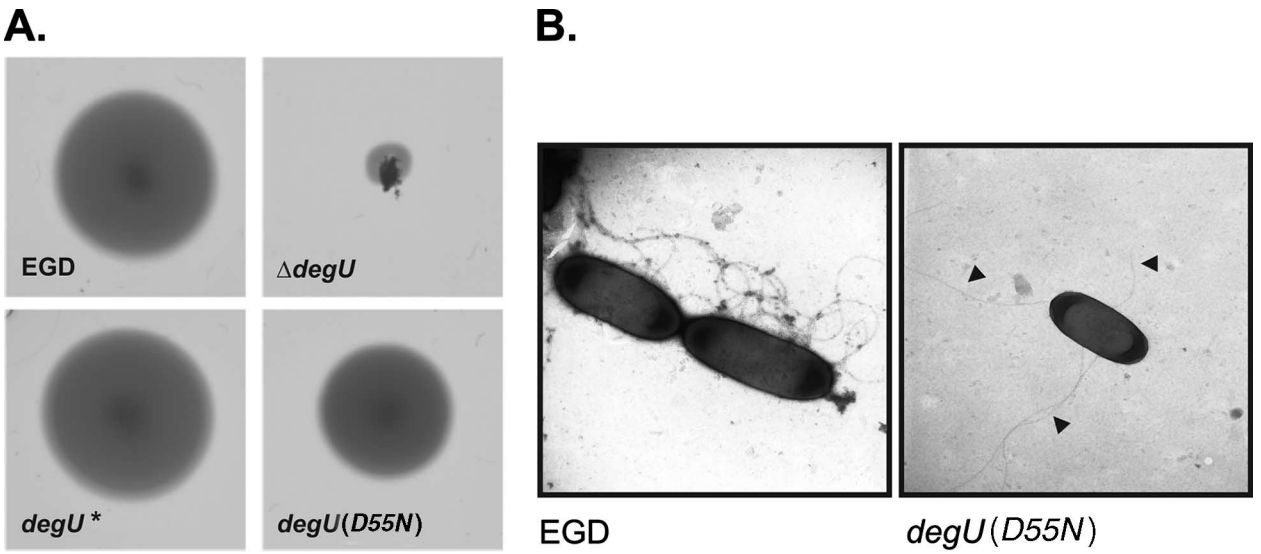

FIG. 1. (A) Swimming motility of the *L. monocytogenes* EGD, Δ*degU*, Δ*degU**, and *degU*(*D55N*) strains. Bacteria from single colonies of the respective strains were stabbed into BHI soft agar, and the plates were incubated for 48 h at 24°C. Swimming halos obtained in a representative experiment are shown. The diameter of halos produced by the *L. monocytogenes* Δ*degU** and *degU*(*D55N*) strains averaged 106% ± 18% and 69% ± 8%, respectively, of the mean diameter of the wild-type strain EGD. (B) Electron micrographs of the *L. monocytogenes* EGD and *degU*(*D55N*) strains. Bacteria were grown without shaking in BHI broth at 24°C and were then examined under a transmission electron microscope (TEM 100; Zeiss) after negative staining with 0.5% uranyl acetate for 1 min. The number of flagella of 50 bacteria were counted per strain. On average, *L. monocytogenes* EGD exhibited seven flagella per cell, while 4.5 flagella were counted in the case of the *degU*(*D55N*) strain. The flagella are indicated by arrowheads. Original magnification, ×20,000.

stabbing the bacteria into semisolid brain heart infusion (BHI) agar (0.25% agar; Difco) and by measurement of the diameters of the swimming halos which were formed around the inoculation point after 48 h of incubation at 24°C. In three independent experiments, the diameters of at least 12 swimming halos were determined, and the diameters obtained were normalized to the mean diameter of halos formed by the wild-type strain EGD. In contrast to the completely nonmotile *L. monocytogenes* Δ*degU* parent strain, the Δ*degU** strain exhibited swimming motility which was not significantly different from that of wild-type *L. monocytogenes* EGD (Student's *t* test; $P > 0.05$). Swimming motility was also observed for the *L. monocytogenes* *degU*(*D55N*) strain; however, the swimming halos produced by this mutant showed 31%-reduced diameters compared to those of *L. monocytogenes* EGD ($P < 0.001$) (Fig. 1A). In agreement with this result, in electron microscopical images, fewer flagella per cell were observed, on average, in cases of the *L. monocytogenes* *degU*(*D55N*) strain than in cases of the wild-type strain EGD (Fig. 1B).

In order to analyze the transcript amounts of flagellum-specific genes, RNA was prepared from the *L. monocytogenes* EGD, Δ*degU*, and *degU*(*D55N*) strains grown at 24°C in BHI broth to an optical density of 1.0 at 600 nm (13). RNA slot blot analysis was performed according to the protocol of Pflock et al. (8) by using a *flaA*-specific hybridization probe that was PCR amplified with primer pair flaA-S5/flaA-S3. While no *flaA*-specific transcript could be detected in the *L. monocytogenes* Δ*degU* strain, the complemented *degU*(*D55N*) strain produced *flaA*-specific mRNA; however, the amount of transcript was reduced to about 50% of that of the wild-type strain *L. monocytogenes* EGD (Fig. 2A). In addition, transcription of the flagellar genes *flaA* (lmo0690), *flgK* (lmo0705), *fliF* (lmo0713), *fliN* (lmo0675), and *fliI* (lmo0716) and the chemotaxis genes *cheY* (lmo0691), *cheR* (lmo0683), and *mcp* (lmo0723) was monitored by quantitative real-time PCR (qRT-PCR), which was performed essentially as described by Mertins et al. (5). In accordance with previous results (13), transcription of the flagellar and chemotaxis genes was almost abolished in the *L. monocytogenes* Δ*degU* strain, while the amounts of the *flaA*-, *flgK*-, *fliF*-, *fliN*-, *fliI*-, *cheY*-, *cheR*-, and *mcp*-specific transcripts were found to be modestly reduced in the *L. monocytogenes* *degU*(*D55N*) strain (Fig. 2B; Table 1). Transcription was affected most prominently in cases of *mcp* (65% of wild-type efficiency), *cheR* (75% of wild-type efficiency), and *flaA* (75% of wild-type efficiency). Transcription of the motility genes in the *L. monocytogenes* EGD and *degU*(*D55N*) strains grown at 37°C was also assessed by qRT-PCR, and equal amounts of transcript were observed (data not shown). Moreover, supernatant proteins were isolated from *L. monocytogenes* cultures grown at 24°C, as described previously (13), and were subjected to immunoblot analysis with a polyclonal rabbit antiserum (H-AB; Denka Seiken UK Ltd.) directed against the flagella of *L. monocytogenes* serotype 1/2a. In accordance with reduced transcription of *flaA* (Fig. 2A and B), the amount of flagellin expressed in the *L. monocytogenes* *degU*(*D55N*) strain was decreased to about 55% of the expression level of FlaA in wild-type strain EGD (Fig. 2C). As observed previously (9, 13), no FlaA protein could be detected in the *L. monocytogenes* Δ*degU* strain (Fig. 2C). These results demonstrate that the phosphorylation of DegU at the conserved receiver phosphorylation site is not a prerequisite for its function as a temperature-responsive activator of expression of the antirepressor protein GmaR. However, reduced transcription of *flaA* in the *L. monocytogenes* *degU*(*D55N*) strain indicates that the

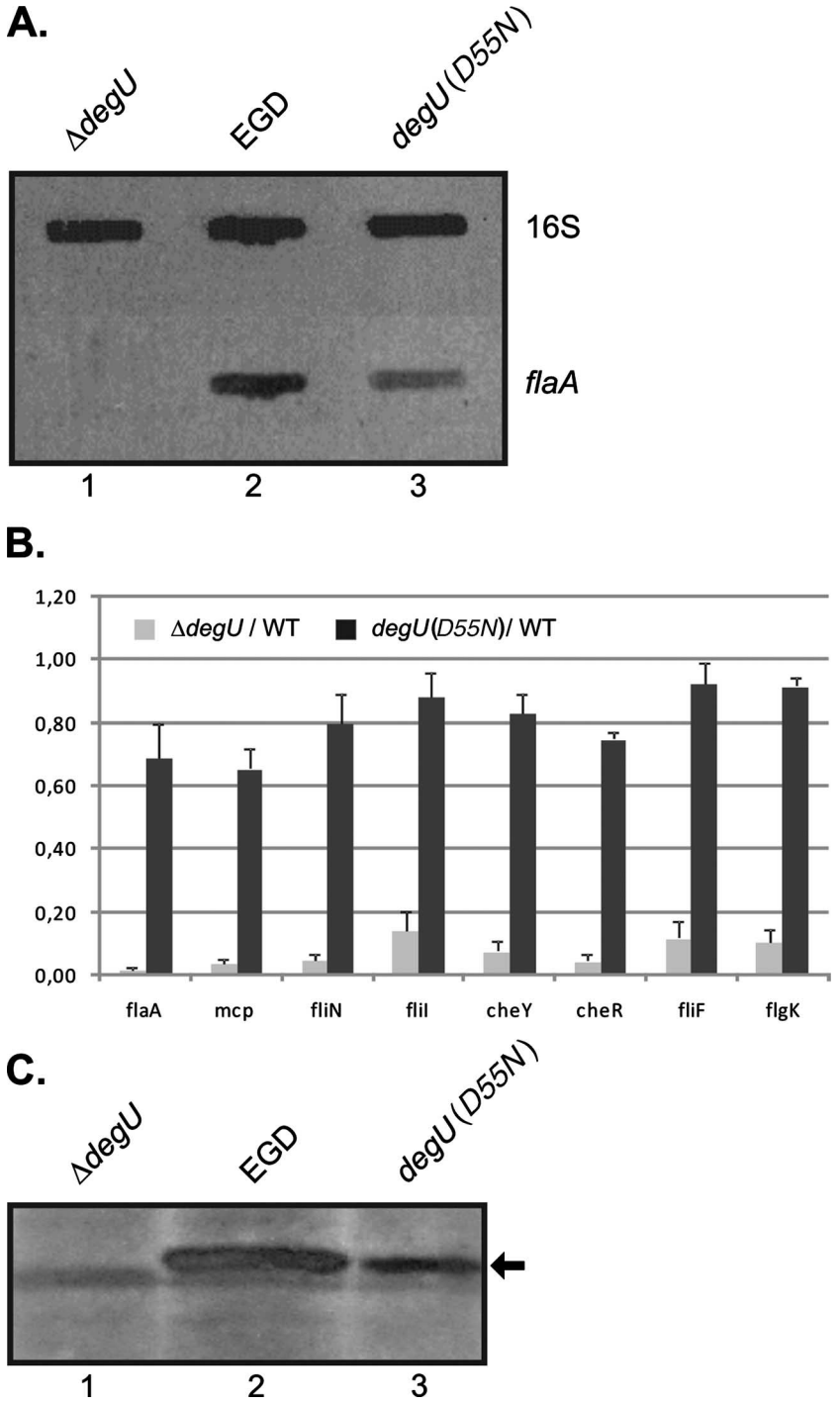

FIG. 2. Expression analysis of motility genes in the *L. monocytogenes* EGD, Δ*degU*, and *degU*(*D55N*) strains. (A) Slot blot hybridization carried out with equal amounts of RNA extracted from the *L. monocytogenes* EGD (lane 2), Δ*degU* (lane 1), and *degU*(*D55N*) (lane 3) strains. Hybridization was performed with *flaA*- and 16S rRNA-specific probes on RNA from three independent preparations. The 16S rRNA-specific hybridization probe was PCR amplified with primer pair 16S-S5/16S-S3. The results of a representative experiment are shown. (B) The relative changes in transcription of *flaA*, *fliN*, *fliI*, *fliF*, *flgK*, *cheY*, *cheR*, and *mcp* in the *L. monocytogenes* Δ*degU* and *degU*(*D55N*) mutant strains, compared to the wild-type strain EGD (WT), were assessed by qRT-PCR. The ratios of the transcript amount detected in the respective mutant to that of the wild type (ratio, mutant/WT) are depicted. The indicated ratios represent the means of results of three qRT-PCR experiments performed in duplicate with cDNA which was reverse transcribed from independent RNA preparations extracted from bacteria grown at 24°C. The relative transcription of the genes under study was normalized to that of the housekeeping gene *rpoB*, as described by Mertins et al. (5). The primer pairs generating the respective amplicons are listed in Table 1. Error bars indicate the standard deviations from the means. (C) Western blot analysis was performed with equal amounts of supernatant proteins prepared from liquid cultures of the *L. monocytogenes* EGD (lane 2), Δ*degU* (lane 1), and *degU*(*D55N*) (lane 3) strains grown at 24°C using a polyclonal rabbit antiserum directed against flagella of *L. monocytogenes* serotype 1/2a. FlaA is indicated by an arrow. The results of one of three independent experiments are shown.

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)[a]	Restriction recognition site	Strand[b]	Position[c]
degU(D55N)1	AGTTGGCATATTAATgTtCATTAAAAC		+	2678814–2678842
degU(D55N)2	GATATTGTTTTAATGaAcATTAATATGCC		−	2678814–2678842
degU-u5	taatatggatccAATTACATTTTATAGGCATATAGG	BamHI	−	2679202–2679225
degU-d3	GTGAATGAATTCTCCTGGGGC	EcoRI	+	2678064–2678084
16S-S5	GGAAACTGGAAGACTGGAGT		+	238114–238134
16S-S3	GCTGATCCACGATTAGTAGCG		−	238817–238838
flaA-S5	TTAGATGCAGCAAGCAAAAAC		+	725070–725091
flaA-S3	AGTTGCGATGGATTGATTGTT		−	725589–725610
flaA-A5	CGTGAACAATCAATCCATCG		+	725585–725605
flaA-A3	ACATTTGCGGTGTTTGGTTT		−	725717–725737
fliF-A5	TGCAAGAAAAAGTTGGCACA		+	744264–744284
fliF-A3	TTCCTGCAGCGGTTCCTTTT		−	744264–744284
fliI-A5	CCAAAAAGGGAAAATCCACA		+	747278–747298
fliI-A3	GCTTTGATTAAACGGGAGCA		−	747418–747438
fliN-A5	GGCAGTACTTGCGGGAATTT		+	711162–711182
fliN-A3	CAACACCTTTTCCCCGTCT		−	711303–711322
flgK-A5	CTGGCAAATGGATCCTGAAT		+	739350–739370
flgK-A3	GTGGCAACCTCGGTAATGAT		−	739490–739510
cheY-A5	CGGAAATGGATGGCTTAGAA		+	726203–726223
cheY-A3	CGCTTGGAAAGGTTTTACGA		−	726335–726355
cheR-A5	ACGCAAATGAAACGTCGAAT		+	718565–718585
cheR-A3	ATTGCGATTACGGAAAAACG		−	718701–718721
mcp-A5	AGTGGCGACCTATCATACCG		+	754186–754206
mcp-A3	ACGTTGTCGCTCGAAGATTT		−	754318–754338
rpoB-A5	CACCCTGAAGCTCCATTTGT		+	274959–274979
rpoB-A3	ACACGACGAACCCAGATTTC		−	275070–275090

[a] Sequences in uppercase letters are derived from the genome sequences of *L. monocytogenes* EGD-e (2). Sequences introduced for cloning purposes are shown in lowercase letters, and restriction recognition sequences are underlined.
[b] +, positive; −, negative.
[c] Nucleotide positions refer to the genome sequence of *L. monocytogenes* EGD-e (2).

DegU(D55N) protein is less efficacious as a transcriptional activator, which might be due to a conformational distortion caused by the receiver mutation.

In addition to the lack of flagellar gene expression, the *L. monocytogenes* Δ*degU* strain exhibited increased sensitivity toward ethanol and reduced virulence in mice after intravenous infection (12). Attenuation of virulence cannot be attributed to the nonmotile phenotype of the *degU*-deficient mutant, since the virulence of *L. monocytogenes* 10403S, an aflagellate mutant lacking *flaA*, was not affected when the bacteria were administered orogastrically or intravenously to mice (11). The DegU-regulated genes responsible for both mutant pheno-

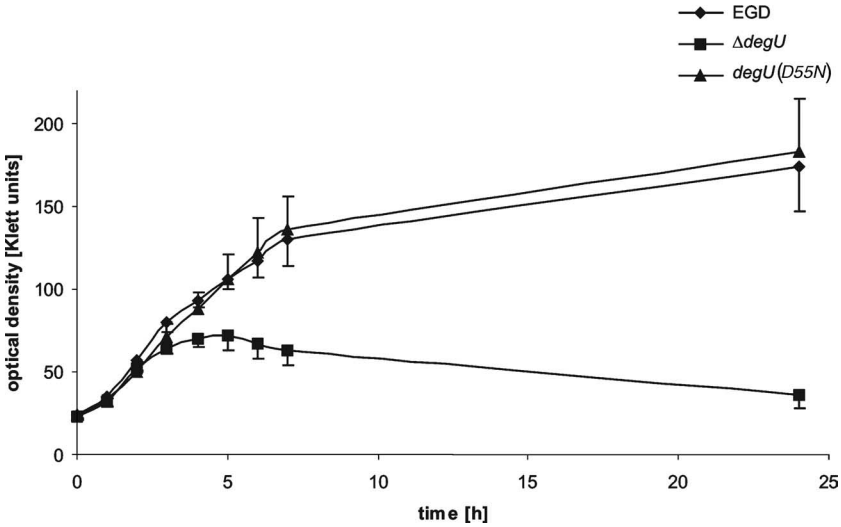

FIG. 3. Growth of the *L. monocytogenes* EGD, Δ*degU*, and *degU*(*D55N*) strains at 37°C in BHI broth in the presence of 5% ethanol. Overnight cultures of the respective strains were diluted 1:50 in BHI broth supplemented with 5% ethanol. The optical densities (in Klett units) were determined at the indicated time points. The growth curve represents the means of results of four independent experiments. Error bars indicate the standard deviations from the means.

types have not been identified yet. The *L. monocytogenes* EGD, Δ*degU*, and *degU*(*D55N*) strains were grown at 37°C in BHI broth supplemented with 5% ethanol. While the *L. monocytogenes* Δ*degU* strain is unable to grow in the presence of 5% ethanol (12), no significant difference was observed in growth of wild-type and *L. monocytogenes degU*(*D55N*) mutant bacteria, suggesting that the D55N substitution in the response regulator DegU does not affect the expression of genes involved in ethanol tolerance (Fig. 3). To investigate whether receiver phosphorylation of DegU is a prerequisite for the proper regulation of virulence-relevant genes, BALB/c mice were intravenously infected with the *L. monocytogenes degU*(*D55N*) strain according to standard protocols (12). The experiment was performed three times, independently, with groups of five animals. At day 3 of infection, no significant differences in the bacterial loads in the livers and spleens of mice infected with the *L. monocytogenes degU*(*D55N*) strain were observed compared to the loads in the respective organs of animals to which the wild-type strain *L. monocytogenes* EGD had been administered. As a control, the *degU* gene was PCR amplified from chromosomal DNA of the *L. monocytogenes degU*(*D55N*) strain reisolated from mice, and the presence of the D55N mutation was confirmed by sequence analysis. Therefore, we reason that the D55N mutation in the receiver sequence of DegU does not interfere with the expression control of virulence-relevant genes.

In conclusion, we have shown that phosphorylation of response regulator DegU at the conserved phosphorylation site is not crucial for the control of target genes whose differential expression is responsible for the complex phenotype of the *L. monocytogenes* strain lacking DegU. The mechanism of temperature-responsive, DegU-dependent regulation of GmaR expression, which might require an additional regulatory protein acting in concert with DegU, remains to be elucidated.

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