Listeria monocytogenes regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence

Angelika Gründling*, Laura S. Burrack*, H. G. Archie Bouwer†, and Darren E. Higgins*‡

*Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115; and ‡Immunology Research, Veterans Administration Medical Center, and Earle A. Chiles Research Institute, Portland, OR 97239

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Previous studies have shown that Listeria monocytogenes flagellar motility genes, including flaA, encoding flagellin, are transcriptionally down-regulated at 37°C. For some L. monocytogenes strains, temperature-dependent motility gene expression is less stringent. By using flaA-lacZ transcriptional fusions, we identified regions upstream of the −35/−10 promoter elements that are necessary for temperature-dependent expression of flaA in L. monocytogenes strain EGDe. Whereas the sequence of the flaA promoter region was identical in L. monocytogenes strain 10403S, transcriptional activity was only partially down-regulated at 37°C in 10403S. This finding suggested that a transacting regulatory protein with differential expression or activity in EGDe might be involved in temperature-dependent transcription of flaA. Indeed, a protein factor capable of specifically binding to the flaA promoter region was identified in cytoplasmic extracts of EGDe by using affinity purification and MS. Deletion of the factor-encoding gene (imo0674) resulted in loss of temperature-dependent flaA expression and an increase in flaA promoter activity. Expression of other motility genes was also deregulated in the imo0674 deletion. We have designated imo0674 as mogR, indicating its role as a motility gene repressor. In tissue culture models, MogR repression of flaA during intracellular infection was independent of temperature and a deletion of mogR reduced the capacity for cell-to-cell spread. During in vivo infection, a deletion of mogR resulted in a 250-fold decrease in virulence. These studies indicate that regulation of flagellar motility gene expression and/or other genes controlled by MogR is required for full virulence of L. monocytogenes.

L. monocytogenes is a food-borne bacterial pathogen of humans and is best known for its intricate intracellular lifestyle (1). The majority of genes encoding virulence factors required for intracellular infection, such as ActA, which is necessary for actin-based motility and cell-to-cell spread (2, 3), are coordinately regulated by the transcriptional activator protein PrfA (4). L. monocytogenes can also swim by means of flagella-based motility in extracellular environments. Previous studies have shown that flagellar motility gene expression in L. monocytogenes is regulated by temperature. L. monocytogenes strains are highly flagellated and motile at low temperatures, 30°C and below, and are typically not motile at temperatures of 37°C or above (5, 6). Furthermore, bacterial flagellins serve as pattern recognition molecules for Toll-like receptor 5-mediated signaling, leading to activation of innate immune responses to infection (7, 8). Because previous studies (9, 10) have shown that transcription of L. monocytogenes flaA, encoding flagellin, is down-regulated at physiological temperature (37°C), it has been proposed that down regulation of flaA expression during in vivo infection by L. monocytogenes may serve as an adaptive mechanism to avoid host recognition and mobilization of host innate immune responses (6, 11).

Several global regulatory factors have been implicated in the control of motility gene expression in L. monocytogenes, including PrfA, the major transcriptional activator of virulence gene expression (12); FlaR, a histone-like protein (13); and CtsR, a negative regulator of class III heat shock genes (14). However, these proteins influence motility gene expression indirectly. To date, no regulatory protein that controls flagellar motility gene expression by binding to promoter regions of these genes has been identified. In this report, we show that temperature-dependent expression of motility genes in Listeria spp. is independent of PrfA. In addition, we have identified a regulator protein that directly binds to the flaA promoter region and provide evidence that this protein, designated as motility gene repressor (MogR), functions as a repressor of motility gene expression and is required for full virulence of L. monocytogenes.

Materials and Methods

Determination of flaA Promoter and lmo0674 Sequences. The flaA promoter region sequences of L. monocytogenes strains EGDe (obtained from M. Loessner, Institute of Food Science and Nutrition, Zurich) and 10403S (15) were found to be identical to the published sequence of L. monocytogenes strain EGD-e (16). The promoter and coding sequence of lmo0674 from strain EGDe were also identical to that published for EGD-e; however, base changes were found in the coding sequence of lmo0674 from strain 10403S (see Fig. 6, which is published as supporting information on the PNAS web site). The lmo0674 sequence from 10403S was submitted to the GenBank database, accession no. AY590468.

Bacterial and Eukaryotic Cell Growth Conditions. Listeria and Escherichia coli strains used in this study are listed in Table 1, which is published as supporting information on the PNAS web site. Listeria strains were grown at temperatures of 30°C, 37°C, or at room temperature (RT; 18–25°C) in brain heart infusion medium (BHI; Difco). E. coli strains were grown in LB medium at 37°C with shaking. The mouse cell lines J774 and L2 were maintained at 37°C in a 5% CO2-air atmosphere (17).

Strain and Plasmid Construction. The construction of the bacterial strains and plasmids and the antibiotic concentrations used in this work are described in Supporting Methods, which is published as supporting information on the PNAS web site. Primer sequences are in Table 2, which is published as supporting information on the PNAS web site.

Analysis of Surface-Extracted and Cyttoplasmic Proteins by SDS PAGE.

Eight-millilitre-aliquots of BHI medium were inoculated with single colonies of Listeria strains and incubated without shaking for ∼24 h at RT or at 37°C. Optical density (OD600) readings were taken.

Abbreviations: RT, room temperature; BHI, brain heart infusion. Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY590468).

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PrfA, and the results in Fig. 1 demonstrate that temperature-dependent motility gene expression is not due to differences in promoter sequences, but rather that the observed temperature-dependent difference in gene expression is due to a transacting factor(s). Because L. monocytogenes flaA transcripts were examined (Fig. 1), we found that when grown at RT, all wild-type strains examined were motile on soft agar plates (Fig. 1A), highly flagellated (Fig. 1B), and expressed high levels of FlaA protein on the bacterial surface (Fig. 1C). Consistent with temperature-dependent FlaA expression, flagella could not be detected on the surface of L. innocua or L. monocytogenes EGDe when grown at 37°C. Of interest, ~2% of L. monocytogenes 10403S had a single detectable flagellum on the bacterial surface when grown at 37°C (Fig. 1B). This single flagellum was randomly distributed on the bacterial surface and not necessarily polar located as depicted in Fig. 1B. This finding is in agreement with a published observation (6) that strain 10403S remains motile at higher temperatures. However, the number of flagella and the amount of FlaA protein on the bacterial surface increased as the temperature was decreased (Fig. 1B and C). Differences in flaA expression between L. innocua and EGDe compared with 10403S were more pronounced when transcript levels were examined (Fig. 1D). Whereas flaA specific transcripts were only detected at RT in L. innocua and L. monocytogenes EGDe, significant levels of flaA transcripts were detected in 10403S, when grown at 37°C (Fig. 1D, lane 5). Because L. monocytogenes strains EGDe and 10403S have identical flaA promoter region sequences, this finding suggested that the observed temperature-dependent difference in gene expression is not due to differences in promoter sequences, but might be due to a transacting factor(s). Because L. innocua lacks PrfA, and the results in Fig. 1 demonstrate that temperature-dependent motility gene expression is observed in nonpathogenic L. innocua, temperature-dependent motility gene expression in Listeria spp. can be PrfA-independent. Furthermore, results depicted in Fig. 1 indicated that temperature-dependent motility and flagellation in Listeria is not only regulated on a transcriptional level but also on a posttranscriptional level. Despite the presence of a significant amount of flaA transcripts in 10403S when grown at 37°C (Fig. 1D, lane 5), there was no FlaA protein detectable on the bacterial surface by Western blot (Fig. 1C, lane 9), and only a few (2%) 10403S bacteria contained a single flagellum on the surface (Fig. 1B).

**Plaque Formation Assay in L2 Fibroblasts and Determination of LD₅₀ Values.** Plaques were formed as described (18). A gentamicin concentration of 40 μg/ml was used in the agarose medium overlay, and plaques were scanned to digital images 5 days after infection. Assays were performed with four independent cultures, and plaque sizes are given as the percentage of wild-type EGDe plaque size. LD₅₀ values were determined as described (19).

**Additional Methods.** See Supporting Methods for a description of additional methods used for strain and plasmid construction, detection of flagella, primer extension analysis, β-galactosidase assays, and affinity purification of Lmo0674 from bacterial cytoplasmic extracts.

### Results

**Temperature-Dependent Regulation of Motility Gene Expression in Listeria Is PrfA-Independent.** We analyzed motility, flagella production, and flaA gene expression in nonpathogenic Listeria innocua (which lacks the prfA-containing virulence gene cluster) in comparison with the virulent L. monocytogenes strains EGDe and 10403S. We found that when grown at RT, all wild-type Listeria strains examined were motile on soft agar plates (Fig. 1A), highly flagellated (Fig. 1B), and expressed high levels of FlaA protein on the bacterial surface (Fig. 1C). Consistent with temperature-dependent FlaA expression, flagella could not be detected on the surface of L. innocua or L. monocytogenes EGDe when grown at 37°C. Of interest, ~2% of L. monocytogenes 10403S had a single detectable flagellum on the bacterial surface when grown at 37°C (Fig. 1B). This single flagellum was randomly distributed on the bacterial surface and not necessarily polar located as depicted in Fig. 1B. This finding is in agreement with a published observation (6) that strain 10403S remains motile at higher temperatures. However, the number of flagella and the amount of FlaA protein on the bacterial surface increased as the temperature was decreased (Fig. 1B and C). Differences in flaA expression between L. innocua and EGDe compared with 10403S were more pronounced when transcript levels were examined (Fig. 1D). Whereas flaA specific transcripts were only detected at RT in L. innocua and L. monocytogenes EGDe, significant levels of flaA transcripts were detected in 10403S, when grown at 37°C (Fig. 1D, lane 5). Because L. monocytogenes strains EGDe and 10403S have identical flaA promoter region sequences, this finding suggested that the observed temperature-dependent difference in gene expression is not due to differences in promoter sequences, but might be due to a transacting factor(s). Because L. innocua lacks PrfA, and the results in Fig. 1 demonstrate that temperature-dependent motility gene expression is observed in nonpathogenic L. innocua, temperature-dependent motility gene expression in Listeria spp. can be PrfA-independent. Furthermore, results depicted in Fig. 1 indicated that temperature-dependent motility and flagellation in Listeria is not only regulated on a transcriptional level but also on a posttranscriptional level. Despite the presence of a significant amount of flaA transcripts in 10403S when grown at 37°C (Fig. 1D, lane 5), there was no FlaA protein detectable on the bacterial surface by Western blot (Fig. 1C, lane 9), and only a few (2%) 10403S bacteria contained a single flagellum on the surface (Fig. 1B).

**DNA Sequences Upstream of the flaA Promoter Are Required for Temperature-Dependent Gene Expression.** Given the suggestion from data in Fig. 1 that a transacting factor may be required for temperature-dependent motility gene expression, we sought to...
Determine what regions of the flaA promoter are required for temperature-dependent regulation. We used chromosomal DNA from strain EGDe flaA::Tn containing a Tn917-lacZ insertion within the flaA gene to generate flaA promoter-lacZ reporter fusions ranging from 25 bp (lacking the promoter) to 301 bp upstream of the flaA translational start site included in each construct. The −35 and −10 promoter elements and the flaA transcriptional start site (10) are indicated as boxes or an arrowhead, respectively. (B) lacZ fusions were introduced in single copy onto the chromosome of EGDe and β-galactosidase activities were determined from cultures grown at 30°C (light shaded bars) or 37°C (dark shaded bars). (C) Same as in B with lacZ fusions integrated into strain 10403S. β-galactosidase activities represent the means plus SD of four individual experiments.

Lmo0674 Protein in EGDe Cytoplasmic Extracts Binds flaA Promoter DNA. To determine whether a factor(s) exists that binds to the flaA promoter region, we coupled biotinylated flaA promoter region DNA to streptavidin-coated magnetic beads (Dynabeads M280) and incubated DNA/magnetic bead complexes with EGDe or 10403S cytoplasmic extracts obtained from cultures grown at RT or at 37°C. Uncoupled beads were also incubated with cytoplasmic extracts as a control. After washing of the magnetic beads to minimize nonspecific binding, proteins that remained bound to the flaA promoter DNA of uncoupled beads were eluted with SDS/PAGE sample buffer, separated on a denaturing polyacrylamide gel, and visualized by silver staining. As shown in Fig. 3, the majority of recovered proteins was found in both EGDe and 10403S extracts, and was eluted from both control and DNA-coupled magnetic beads. However, one prominent protein band with a molecular mass of ~30 kDa was found only in samples containing flaA promoter DNA-coupled beads and EGDe extracts. Comparable amounts of this protein were recovered from EGDe extracts isolated from bacteria grown at RT or at 37°C. This protein band was excised from the gel and was identified by MS as the hypothetical L. monocytogenes protein Lmo0674. Strikingly, the gene encoding Lmo0674 is located immediately upstream of the L. monocytogenes motility gene cluster (16).

Deletion of lmo0674 Results in Increased Motility Gene Expression and Loss of Temperature Dependency. The data depicted in Fig. 3, indicating that Lmo0674 binds to flaA promoter region DNA and the presence of lmo0674 adjacent to the motility gene cluster, suggested that Lmo0674 might play a role in regulation of flagellar gene expression. To gain insight into the function of Lmo0674, we constructed strain EGDeΔlmo0674 containing an in-frame deletion in the lmo0674 gene. A role for Lmo0674 in regulation of flagellar gene expression was first shown by staining of EGDe and EGDeΔlmo0674 for flagella and microscopic examination of bacteria. Although no flagella could be detected on the surface of EGDe when grown at 37°C (Fig. 1B), we found that ~1% of EGDeΔlmo0674 bacteria contained a single flagellum when grown at 37°C (data not shown). This result correlated with the detection of small amounts of FlaA protein on the surface of strain EGDeΔlmo0674, but not EGDe when cultures were grown at 37°C (Fig. 4A, compare lanes 3 and 5). Furthermore, significant levels of FlaA protein were detected in the cytoplasmic fraction of strain EGDeΔlmo0674 at RT and at 37°C (Fig. 4A, lanes 6–9). When grown at RT, similar amounts of FlaA protein were detected on the bacterial surface of strains EGDe and EGDeΔlmo0674 (Fig. 4A, lanes 2 and 4).
strain EGDeΔ674 flaA::Tn. Both primer extension analysis and β-galactosidase assays revealed that flaA promoter activity was significantly altered in the strain lacking Lmo0674. Deletion of lmo0674 abolished temperature regulation of the flaA promoter and a significant increase in flaA transcript levels compared with wild-type EGDe was observed (Fig. 4B, lanes 4 and 5). The increase in flaA promoter activity in the EGDeΔ674 strain indicated that the presence of Lmo0674 led to repression of flaA transcription, even at RT, and is consistent with the affinity purification of Lmo0674 protein from both RT- and 37°C-grown EGDe extracts (Fig. 3). As confirmation for a role of Lmo0674 in repression of flaA transcription, integration of a single copy of the lmo0674 gene from strain EGDe into the chromosome of strain EGDeΔ674 by using plasmid pPL3-674E resulted in restoration of temperature-dependent transcription of flaA, similar to that observed in EGDe (Fig. 4B, lanes 6 and 7). Interestingly, when we introduced a similar construct into EGDeΔ674 containing lmo0674 cloned from strain 10403S (pPL3-674S), only partial complementation of temperature-dependent flaA expression was observed (Fig. 4B, lanes 8 and 9). Sequence analysis of the lmo0674 genes of 10403S and EGDe revealed seven nucleotide changes within the coding region (see Fig. 6). All of the nucleotide changes within the coding region were at the third position of codons and would not lead to an altered Lmo0674 amino acid sequence. However, 10403S contained a G-to-A nucleotide substitution 12 nt upstream of the translational start site, presumably within the ribosome binding site (Fig. 6). This change could cause a decrease in translation efficiency of Lmo0674, resulting in partial complementation and less stringent temperature-dependent flaA expression in strain EGDeΔ674 pPL3-674S (Fig. 4B, lanes 8 and 9) and strain 10403S (Fig. 1D, lanes 4 and 5).

Lmo0674 Modulates Expression of Other Motility Genes. We next determined whether Lmo0674 affects expression of other genes involved in motility of L. monocytogenes. Transcript levels for cheY (Fig. 4C), a gene encoding a chemotaxis protein and lmo0675, a gene showing homology to the Bacillus cereus flIN flagellar switch protein (data not shown), were both dramatically...
for EGDe was $3 \times 10^3$, whereas the LD$_{50}$ for EGDeΔ674 was $\sim 1 \times 10^6$, which equates to a 250-fold decrease in virulence for EGDeΔ674. Confirmation that the virulence defect was attributed to the lack of MogR expression was obtained, because the LD$_{50}$ for the EGDeΔ674 pPL3-674E strain was $\sim 5 \times 10^3$. These results indicate that MogR is required for full virulence of *L. monocytogenes*.

### Discussion

In this report, we identified a regulatory protein (MogR, formerly *Lmo0674*) that represses expression of flagellar motility genes in *L. monocytogenes*. MogR was identified by affinity binding purification using flagellar promoter region DNA and MS (Fig. 3). Deletion of the gene encoding MogR in *L. monocytogenes* strain EGDeΔ674 dramatically altered flaA gene expression such that transcription of *flaA* was no longer regulated by temperature and increased in comparison with wild-type EGDe. Transcription of at least two other motility genes, *cheY* and *lmo0675*, was also deregulated in a similar manner in the mogR deletion strain (Fig. 4). The biochemical and genetic data presented in this report demonstrate that MogR binds to the *flaA* promoter region and represses gene expression of *flaA* and other motility genes. MogR is not only required for temperature-dependent motility gene repression during extracellular growth but also appears to function during intracellular infection to down-regulate motility gene expression independent of temperature (Fig. 5). Misregulation of gene expression upon deletion of mogR resulted in a decreased ability of bacteria to spread from cell to cell as well as a 250-fold increase in LD$_{50}$ during infection of BALB/c mice. Therefore, coordinate regulation of motility gene expression or other genes regulated by MogR contributes to full virulence of *L. monocytogenes*.

To gain further insight into the mechanism of MogR regulation, we used the PROFILESAN analysis program (20) to search for known motifs within the amino acid sequence of MogR. A weak helix–turn–helix motif similarity, indicative of a DNA-binding protein, was found within MogR, suggesting that MogR might bind directly to DNA without the need of an adaptor protein. However, MogR does not show striking homology to any previously characterized protein in known databases. Nonetheless, uncharacterized ORFs that show homology to MogR are found in *L. innocua*, *Bacillus cereus*, and *Bacillus anthracis*. All genes encoding for MogR homologs are located adjacent to a cluster of genes displaying homology to flagellar-based motility genes. Indeed, in this report, we have shown that motility gene expression in nonpathogenic *L. innocua* is regulated by temperature in a similar manner as in *L. monocytogenes* EGDe (Fig. 1). Therefore, we believe that the MogR homolog in *L. innocua* has a similar function as in *L. monocytogenes* in regulating motility gene expression. Moreover, the finding that temperature-dependent motility is observed in *L. innocua* negates a direct role for PrfA, a transcriptional regulator of virulence gene expression in *L. monocytogenes*, in regulating temperature-dependent motility gene expression.

Data from our group and others (6, 21) have shown that temperature-dependent motility gene expression is less stringent in *L. monocytogenes* strain 10403S. It is very plausible that the molecular basis for this difference is on the level of MogR expression. Indeed, a MogR deletion strain could be complemented when mogR, under the control of its native promoter, was cloned from strain EGDe, but was only partially complemented when mogR was cloned from strain 10403S (Fig. 4). Sequence analysis revealed several nucleotide changes in mogR of strain 10403S compared with EGDe. One of these changes is predicted to be within the ribosome-binding site, presumably resulting in decreased translation of MogR in strain 10403S. Preliminary results from our laboratory show that MogR from strain 10403S, when expressed from a constitutive promoter with
an altered ribosome binding-site sequence, can effectively repress flaA promoter activity (data not shown).

A deletion of mogR decreases the virulence of L. monocytogenes in a mouse model of infection, as well as its ability to spread from cell to cell during in vitro infection of mouse L2 fibroblasts. We envision multiple possibilities for the requirement of MogR for full virulence of L. monocytogenes. Flagellin proteins from several bacteria have been shown to possess potent proinflammatory activity and to activate host inflammatory responses through Toll-like receptor 5 (7, 8). Indeed, the L. monocytogenes FlaA protein has been shown to activate host cells by means of Toll-like receptor 5 signaling (6, 8). However, no differences were observed in virulence, bacterial clearance, or induction of L. monocytogenes-specific T lymphocyte responses between strain 10403S and an isogenic flaA deletion strain in the mouse infection model (6). This study concluded that L. monocytogenes flagellin is not essential for pathogenesis or an essential trigger for innate or adaptive immunity to L. monocytogenes infection. However, data presented here indicated that only a small percentage of bacteria in a 10403S population actually possessed flagella at elevated temperature (Fig. 1B). We further showed that despite high flaA transcript levels generated at elevated temperature, no FlaA protein could be detected on the bacterial surface of strain 10405S by Western blot (Fig. 1C), yet small amounts of FlaA protein could be detected on the surface of EGDeΔflaA (Fig. 4A). This result indicates that both transcriptional and posttranscriptional levels of regulation play a role in temperature-dependent flagellation in L. monocytogenes. Furthermore, we detected FlaA protein within the cytoplasmic fraction of strain EGDeΔflaA (Fig. 4A), which is in contrast to strain 10403S (data not shown). Thus, an increase in the total amount of FlaA produced in the mogR deletion strain EGDeΔflaA might be sufficient to elicit a more robust innate immune response, resulting in faster clearance of infection, in contrast to what was observed for 10403S (6). Alternatively, increased expression of one or more MogR-regulated motility genes might compromise the integrity of L. monocytogenes, leading to the observed increase in LD50 and reduced plaque size in the in vitro cell-to-cell spreading assay. Finally, MogR might be necessary for the regulation of genes other than those required for flagellar motility. This latter possibility would be of great interest, in light of the fact that a mogR homolog is found in the genome of the nonmotile bacterial pathogen Bacillus anthracis.

Several questions regarding MogR remain to be answered and include: how are temperature and other environmental signals integrated through MogR to affect gene expression in L. monocytogenes? What is the full complement of genes regulated by MogR? How does MogR repress gene expression on a molecular level? Regions upstream of the minimal promoter sequence are required for repression of flaA promoter activity, as determined by β-galactosidase activity using different-length flaA promoter-lacZ fusions (Fig. 2). This finding could indicate that MogR binds to a region upstream of the RNA polymerase-binding site, potentially interacting with components of RNA polymerase. Indeed, by using gel shift analysis, we observed weak binding of a protein or proteins from cytoplasmic extracts of EGDe, but not 10403S, to a flaA-specific DNA fragment containing sequences upstream of the RNA polymerase-binding site (data not shown). However, significantly stronger binding was observed to a flaA-specific DNA fragment, including both upstream sequences and the flaA promoter region sequence (data not shown), indicating that MogR might require RNA polymerase or part of the RNA polymerase-binding site for its action. Future studies may clarify how MogR functions in regulation of gene expression and virulence and will further our understanding of how gene expression is coordinately regulated in bacterial pathogens.

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