Supporting Methods

Determination of *flaA* Promoter and *lmo0674* Sequences. The *flaA* promoter region was amplified from chromosomal DNA preparations of *Listeria monocytogenes* strains EGDe and 10403S by using the primer pair *Bam*HI-flaA-595 and flaA-ext, and the promoter region sequence was determined by automated fluorescence sequencing (for primer sequences see Table 2). The *flaA* promoter sequences were identical to the published sequence of strain EGD-e (1). Primers 5-Bam-withP-lmo0674 and 3-Sal-lmo0674 were used to amplify the *lmo0674* gene and an ≈350-bp upstream region from chromosomal DNA of strains EGDe and 10403S and sequenced by using the same primer pair and an internal *lmo0674* primer (int-seq-lmo0674). The promoter and *lmo0674* sequence of strain EGDe was identical to the published sequence of strain EGD-e, whereas several base changes were found in the sequence from strain 10403S. The 10403S-derived *lmo0674* DNA sequence was submitted to the EMBL/GenBank/DDBJ database, accession no. AY590468.

Bacterial Growth Conditions. Antibiotics were used at the following concentrations: chloramphenicol 20 μg/ml for selection of pPL2 derivatives in *Escherichia coli* and 5-7.5 μg/ml for selection of integrated pPL2 derivatives in *L. monocytogenes*; 1-5 μg/ml erythromycin for selection of integrated *erm* gene containing pPL2 derivatives in *L. monocytogenes*; 1 μg/ml erythromycin and 25 μg/ml lincomycin for selection of strains with Tn917-derived transposon insertions.

Detection of Flagella. *Listeria* strains were grown ≈24 h at RT or at 37°C in 2 ml BHI medium without shaking. Flagella were detected by using a crystal violet stain as described (2). Stained cultures were analyzed by light microscopy. The percentage of bacteria that possessed at least one visible flagellum was calculated by inspecting 200 individual bacteria per culture.

Strain and Plasmid Construction. Pfu polymerase (Stratagene) was used for PCRs when DNA fragments were subsequently used for plasmid construction. All other enzymes were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturer's instructions. All plasmids were initially constructed in *E. coli* strain XL1-Blue. Strain EGDeΔ*flaA*, containing an in-frame deletion in the *flaA* gene, was created by allelic exchange (3) by using plasmid pKSV7ΔflaA (4). Strain EGDe *flaA*::Tn was isolated after transposon mutagenesis by using vector pTV51ts (5) and selected as a nonmotile colony on low-agar plates (0.375% agar). Strain EGDe *flaA*::Tn contains a transposon insertion after 123 bp (41 aa) of *flaA* coding sequence. The transposon insertion resulted in interruption of the *flaA* gene and a *flaA* promoter-*lacZ* reporter gene fusion. The site of transposon insertion was determined by arbitrarily primed PCR performed as described (6).

The site-specific integration vector pPL3 was constructed by inserting tandem copies of the *rrnB* T1 transcription terminator into the unique *Sac*I site of plasmid pPL2 (7). The *rrnB* T1 terminator region of vector pLIV1 (8) was amplified with the primer pair 5' *Sac*I *Dra*II rrnB term and 3' *Sac*I rrnB term (Table 2). The resulting PCR product was digested

with *Sac*I and ligated with *Sac*I-linearized pPL2. Plasmid pHPL3 was constructed in a two-step process. First, the 5' phosphorylated primers 5' *Sac*I Phyperspac (–40, +1) *Eag*I and 3' *Sac*I Phyperspac (–40, +1) *Eag*I, containing the Hyper–SPO1 promoter sequence (9), were annealed and ligated with *Eag*I- and *Sac*I-digested pPL2, resulting in plasmid pHPL2. Next, tandem copies of the *rrnB* T1 transcription terminator were inserted into the unique *Sac*I site of pHPL2, resulting in plasmid pHPL3. Vector pPL3e was constructed by replacing the Gram-positive *cat* gene in vector pPL3 with the Gram-positive *ermC* gene. Primer pair 5' ermC incl –35 and 3' *Pvu*I ermC, was used to amplify the *ermC*-resistance gene from plasmid pHS-LV (10). The PCR product was digested with *Apa*LI and *Pvu*I and ligated with the pPL3 vector that had been digested with the same enzymes.

The different-length *flaA*-promoter *lacZ* fusions were constructed in vector pPL3e as follows: The six *flaA* promoter specific primers *Bam*HI-flaA-595 (301 bp), 5-Bam-flaA-full-P (234 bp), *Bam*HI-flaA-724 (172 bp), *Bam*HI-flaA-780 (116 bp), 5-Bam-flaA-35 (89 bp), and 5-Bam-flaA-NO-P (25 bp) were used together with the 3'-specific *lacZ* primer 3-*Kpn*I-LacZ to amplify the *flaA* promoter-*lacZ* region by using chromosomal DNA of strain EGDe *flaA*::Tn as template. The resulting PCR products were digested with *Bam*HI and *Kpn*I and ligated with vector pPL3e that had been digested with the same enzymes. The resulting plasmids were named pPL3e-301-*lacZ*, pPL3e-234-*lacZ*, pPL3e-172-*lacZ*, pPL3e-116-*lacZ*, pPL3e-89-*lacZ* (minimal promoter), and pPL3e-25-*lacZ* (no promoter), where the number preceding *lacZ* within the plasmid name refers to the number of nucleotides of *flaA* promoter region DNA upstream of the *flaA* translational start site included in each construct. Also, the promoterless *lacZ* gene was placed under HyperSPO1 promoter control in plasmid pHPL3. Chromosomal DNA of strain EGDe *flaA*::Tn and the primer pair 5-Bam-flaA-NO-P and 3-*Kpn*I-LacZ were used in a PCR to amplify the promoterless *lacZ* gene. The resulting PCR product was digested with *Bam*HI and *Kpn*I and ligated with vector pHPL3 that had been digested with the same enzymes, resulting in plasmid pHPL3-*lacZ*. The temperature-independent HyperSPO1 promoter-*lacZ* fusion was used as a control in β-galactosidase assays. Plasmids were introduced into *L. monocytogenes* strains EGDe and 10403S by electroporation (11).

The *lmo0674* gene from strain EGDe and 10403S was cloned with its native promoter into vector pPL3 as follows: the primer pair 5-Bam-withP-lmo0674 and 3-Sal-lmo0674 and chromosomal DNA of strain EGDe or 10403S were used to amplify the *lmo0674* gene and an ≈350-bp upstream region. The resulting PCR product was digested with *Bam*HI and *Sal*I and ligated with plasmid pPL3 that had been digested with the same enzymes. The resulting plasmids were named pPL3-*674E* (EGDe) or pPL3-*674S* (10403S).

Strain EGDeΔ*674* containing an in-frame deletion in *lmo0674* was constructed by PCR SOE (12) using the following primers: 5-1kb-Kpn-lmo0674, 5-int-lmo0674, 3-1kb-Bam-lmo0674, and 3-int-lmo0674 and chromosomal DNA of strain EGDe. The resulting PCR product was digested with the restriction enzymes *Kpn*I and *Bam*HI and ligated with the allelic exchange vector pCON1 (13) that had been digested with the same restriction

enzymes, resulting in plasmid pCON1Δ*0674*. This plasmid was used to create strain EGDeΔ*674* by allelic exchange as described (3).

Phage Transduction. The *L. monocytogenes* phage P35 was used to transduce the Tn917 transposon. Phage transduction was performed as described (14), with the exception that transductants were selected directly on BHI plates containing 10 mM sodium citrate and 1 μg/ml erythromycin.

Primer Extension Analysis. Twelve-milliliter aliquots of BHI medium were inoculated with single colonies of *Listeria* strains and incubated at RT (18-25°C) or at 37°C for ≈24 h without shaking. RNA was isolated by using the FastRNA Pro Blue Kit (Q-BIOgene) according to the manufacturer's instructions and RNA concentrations were determined from OD_{260} readings. Ten picomoles of *flaA*-specific primer flaA-ext, *cheY*-specific primer cheY-ext, or *lmo0675*-specific primer lmo0675-ext were end-labeled in a 10-μl reaction with 40 μCi (γ-^{32}) ATP by using 10 units of T4 polynucleotide kinase (New England BioLabs) and incubated at 37°C for 30 min. Forty microliters of TE were added after heat inactivation of the enzyme and unincorporated nucleotides were removed by using G25-Sephadex quick spin columns (Roche Diagnostics). Twelve micrograms of total RNA and 0.2 pmol of end-labeled primer specific for *flaA* (flaA-ext), *cheY* (cheY-ext), or *lmo0675* (lmo0675-ext) were used for primer extension reactions. One mircoliter (0.2 pmol) of end-labeled primer was mixed with 12 μg of total RNA in 1× binding buffer (supplied with the SuperScript III reverse transcriptase; Invitrogen) in a final volume of 10 μl. The primer/RNA mixture was heated for 1 min at 95°C, then for 2 min at 55°C, and immediately placed on ice for 15 min. Extension reactions were performed in a final volume of 15 μl in 1× binding buffer in the presence of 10 mM DTT and 1 mM dNTPs by using the annealed primer/RNA mixture, and 150 units of SuperScript III reverse transcriptase. Reactions were incubated for 45 min at 55°C and stopped by the addition of 5 μl of Stop buffer (95% formamide/20 mM EDTA/0.05% Bromophenol blue/0.05% Xylene Cyanol FF). Samples were heated for 3 min at 95°C and 5 μl were then separated on 5% denaturing polyacrylamide gels. Gels were dried after electrophoresis and exposed to film (Kodak, Rochester, NY) to visualize primer extension products.

β-galactosidase Assays and Measurement of Intracellular *flaA* Promoter Activity. Three-milliliter aliquots of BHI medium were inoculated with single colonies of *L. monocytogenes* strains and incubated for 20-24 h without shaking, at the indicated temperatures. OD_{600} readings were taken of these cultures to normalize β-galactosidase values for bacterial density. Bacteria were pelleted in 1-ml aliquots at 16,000 × *g* for 10 min and resuspended in 100 μl of ABT buffer (60 mM K_2HPO_4/40 mM KH_2PO_4/100 mM NaCl/0.1% Triton X-100, pH 7.0, filter sterilized) and snap-frozen in a dry ice/ethanol bath and stored at –20°C until use. For each assay, samples were thawed and 1:10 and 1:100 dilutions in ABT buffer were prepared. Reactions were set up in a 96-well plate format by mixing 10 μl of MUG substrate (0.4 mg/ml 4-methyl-umbelliferyl-β-D-galactopyranoside in DMSO) and 50 μl of sample (undiluted, 1:10, or 1:100 diluted), using ABT buffer as a control. After incubation for 60 min at RT, 20 μl from each

reaction were removed and diluted into 180 μl of ABT buffer in a black 96-well plate and fluorescence values were determined by using a SpectraMAX GeminiXS instrument (Molecular Devices) at excitation and emission wavelengths of 366 and 445 nm, respectively. Known concentrations of the fluorescent 4-methylumbelliferone (MU) product ranging from 25 to 4000 pmol were used to obtain a standard curve. The fluorescent value of the sample dilution in the linear range of the standard curve was used for β-galactosidase unit calculation. Units were calculated as (picomoles of substrate hydrolyzed × dilution factor)/(ml culture in final sample × OD_{600} x minute) where pmol of substrate hydrolyzed is calculated from the standard curve as (emission reading – y intercept)/(slope).

For intracellular *flaA* promoter activity assays, 5.0×10^6 J774 cells were seeded into 100-mm-diameter Petri dishes and incubated for 12-15 h in DMEM (Mediatech, Herndon, VA) at 37°C in a 5% CO_2-air atmosphere. Two-milliliter aliquots of BHI medium were inoculated with a single colony of each *L. monocytogenes* strain and grown 12-15 h at 37°C. The following day, J774 cells were placed at 27°C in a 5% CO_2-air atmosphere for 1 h. Following washing with PBS, pH 7.1, J774 cells were infected with 5 μl of a PBS-washed bacterial culture ($\approx 1.0 \times 10^7$ bacteria) in 10 ml DMEM. One hour after infection, cells were washed twice with PBS, pH 7.1, and 10 ml of DMEM with 50 μg/ml gentamicin was added. Sixteen hours after infection, cells were washed once with PBS, pH 7.1, and resuspended in 1 ml ABT buffer to lyse J774 cells. Lysed cell samples were centrifuged at 16,000 × *g* for 8 min at 4°C to pellet bacteria. The supernatant was removed and bacteria were resuspended in 25 μl of ABT buffer per dish. A total of four dishes were infected per strain and samples were pooled, resulting in a total volume of 100 μl. Five-microliter aliquots were removed, diluted in PBS and plated on LB plates to determine colony forming units (CFU). The remaining sample was used to perform β-galactosidase assays as described above, with the exception that MUG units were normalized to CFU rather than OD_{600}.

Preparation of Bacterial Cytoplasmic Extracts. Thirty-six milliliter aliquots of BHI medium were inoculated with a single colony of *L. monocytogenes* strains and incubated for 24 h at RT or at 37°C without shaking. Bacteria were collected by centrifugation, washed once with lysis buffer (10 mM Tris•HCl, pH 7.5/50 mM NaCl) and resuspended in 0.4-0.6 ml of lysis buffer supplemented with Complete protease inhibitor mixture (Roche). Samples were placed in FastProtein Blue tubes (Q-BIOgene) and bacteria were lysed in a FastPrep apparatus FP120 for 30 sec at setting 6.0. Bacterial cell extracts were recovered by pelleting the lysis matrix by centrifugation at 16,000 × *g* for 7 min. Next, bacterial membranes were removed from extracts by ultracentrifugation at 100,000 × *g* for 60 min at 4°C. Protein concentrations of the resulting extracts were determined by using the Bradford assay (Sigma). Typically, protein concentrations between 2 and 4 mg/ml were obtained. Bacterial cell extracts were stored in 20% glycerol at –20°C.

Affinity Purification of Lmo0674 From Bacterial Cytoplasmic Extracts. The 5' biotinylated primer (327-biotin) and primer 3' *Eco*RV shift with P were used to amplify the *flaA* promoter region by using plasmid pPL3e-301-*lacZ* as template. The resulting PCR product was gel-purified (1.5% agarose gel) by using the Qiagen gel extraction kit

(Qiagen, Valencia, CA). Two-hundred fifty microliters of Streptavidin-coupled Dynabeads M-280 (10 mg/ml) (Dynal, Lake Success, NY) were washed 2 times with 1 ml of binding and wash buffer (B&W buffer: 1 M NaCl/5 mM Tris•HCl, pH 7.5/0.5 mM EDTA) according to the manufacturer's instructions. Next, 5 μg of biotinylated PCR product was added to the Dynabeads in 1 ml of B&W buffer and incubated for 15 min at RT with gentle shaking. Afterward, beads were washed 3 times with 1 ml B&W buffer and resuspended after the last wash in 250 μl of lysis buffer (10 mM Tris•HCl, pH 7.5/50 mM NaCl). A second aliquot of Dynabeads was treated the same as described above except that no DNA was added to the beads. These beads were used for the no DNA control-binding reactions. Binding reactions were performed as follows: 300 μl of 5× binding buffer (50% glycerol/5 mM $MgCl_2$/25 mM, Tris•HCl, pH7.5/250 μg/ml BSA/1.15 M NaCl/62.5 μg/ml sonicated salmon sperm DNA/2.5 mM DTT/20 mM EDTA) were mixed with 40 μl of washed *flaA* promoter DNA coupled Dynabeads (≈800 ng DNA) or uncoupled Dynabeads and 750 μl of bacterial cell extracts at a concentration of 1.65 mg/ml in lysis buffer containing the Complete protease inhibitor mixture (Roche). Samples were then taken up to a final volume of 1.5 ml with ddH_2O. Binding reactions were incubated 30 min at RT (18-25°C) on a rotisserie. Samples were washed four times with 1 ml of 1× binding buffer and resuspended after the last wash in 50 μl of 2× protein sample buffer. Samples were boiled for 5 min, placed on a magnet for 2 min, and 40 μl of the supernatants were separated on a 10% SDS polyacrylamide gel. Proteins were visualized by silver staining using the silver staining kit for proteins from Amersham Pharmacia (Piscataway, NJ). One protein band that was apparent only with EGDe extract samples and DNA-coupled Dynabeads was excised and placed in fixing solution (50% methanol and 5% acidic acid) for subsequent MS analysis (Taplin Biological Mass Spectrometry facility, Harvard Medical School, Boston).

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