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Role of Flagellin and the Two-Component CheA/CheY System of *Listeria monocytogenes* in Host Cell Invasion and Virulence

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The flagellum protein flagellin of *Listeria monocytogenes* is encoded by the *flaA* gene. Immediately downstream of *flaA*, two genes, *cheY* and *cheA*, encoding products with homology to chemotaxis proteins of other bacteria, are located. In this study we constructed deletion mutants with mutations in *flaA*, *cheY*, and *cheA* to elucidate their role in the biology of infection with *L. monocytogenes*. The Δ*cheY*, Δ*cheA*, and double-mutant Δ*cheYA* mutants, but not Δ*flaA* mutant, were motile in liquid media. However, the Δ*cheA* mutant had impaired swarming and the Δ*cheY* and Δ*cheYA* mutants were unable to swarm on soft agar plates, suggesting that *cheY* and *cheA* genes encode proteins involved in chemotaxis. The Δ*flaA*, Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants (grown at 24°C) showed reduced association with and invasion of Caco-2 cells compared to the wild-type strain. However, spleens from intragastrically infected BALB/c and C57BL/6 mice showed larger and similar numbers of the Δ*flaA* and Δ*cheYA* mutants, respectively, compared to the wild-type controls. Such a discrepancy could be explained by the fact that tumor necrosis factor receptor p55 deficient mice showed dramatically exacerbated susceptibility to the wild-type but unchanged or only slightly increased levels of the Δ*flaA* or Δ*cheYA* mutant. In summary, we show that listerial *flaA*, *cheY*, and *cheA* gene products facilitate the initial contact with epithelial cells and contribute to effective invasion but that *flaA* could also be involved in the triggering of immune responses.

Listeria monocytogenes is a motile, facultatively intracellular bacterium that causes food-borne infections in humans and animals, with symptoms of septicemia, meningitis, and meningoencephalitis (57). This gram-positive bacterium is widely distributed in the environment and is able to grow over a wide range of temperatures (1 to 45°C), pHs, and osmotic pressures (51). *L. monocytogenes* is unusual among pathogens in being able to grow at refrigeration temperatures. Contamination of cold-stored foods with *L. monocytogenes* has been implicated in several outbreaks of epidemic and sporadic disease (20, 50). Entry into the host normally occurs in the gut; in animal models, bacteria pass the gastrointestinal barrier and possibly penetrate the intestinal epithelial cells overlaying Peyer's patches (37, 44). The organism then disseminates to the brain and to the spleen, liver, and other lymphatic systems. The virulence of *L. monocytogenes* is due to its capacity to invade and multiply within host cells, including macrophages, hepatocytes, and epithelial, endothelial, and neuronal cells (10, 57). Early after internalization, bacteria disrupt the phagosomal membrane and access the cytoplasm, where they polymerize actin and spread from cell to cell (11, 55). Each step of the infectious process is dependent on the production of virulence factors, including invasion proteins (InlA and InlB), listeriolysin O, phospholipases, and ActA, which are controlled by the pleiotropic transcriptional activator PrfA (34, 39). The expression of several of the virulence genes is thermoregulated, with a higher expression at 37°C than at 20°C (17, 33). The flagellar filament of *Listeria* is composed of one major subunit, flagellin, which is produced and assembled at the cell surface when *L. monocytogenes* is grown between 20 and 25°C, whereas its production is markedly reduced at 37°C (42). Flagellin is encoded by the *flaA* gene, which is transcribed as a monocistronic unit (15). Immediately downstream of the *flaA* gene are two genes, *cheY* and *cheA*, encoding polypeptides with high homology to the chemotaxis proteins CheY and CheA of *Bacillus subtilis* and *Escherichia coli*, located in a bicistronic unit (16). Analysis of transposon-generated mutants with insertions in the promoter region of this operon suggest that *cheY* and *cheA* are involved in chemotaxis (21). The transcription of *flaA, cheY*, and *cheA* is pronounced at 25°C but nondetectable at 37°C (15, 16).

B. subtilis and *E. coli* CheY and CheA constitute a two-component regulatory system involved in signal transduction of chemotaxis (23, 53). In *B. subtilis* the autophosphorylating activity of CheA increases by binding of attractants to transmembrane receptors (24). Phosphorylated CheA donates the phosphate to the response regulator CheY (4, 24), and phosphorylated CheY interacts with the flagellar motor switch complex to induce counterclockwise (CCW) rotation of the flagella, resulting in smooth swimming behavior (4). The default clockwise (CW) rotation of the flagella in the absence of interaction of CheY-P with switch proteins is associated with tumbling (5).

Flagella and motility contribute to virulence in several bacteria, such as *Campylobacter jejuni* (25), *Legionella pneumo-*

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phila (13), *Clostridium difficile* (54), *Helicobacter pylori* (18), *Aeromonas caviae* (45), *Salmonella enterica* serovar Typhi (36), *Vibrio cholerae* (46), and *V. anguillarum* (41). Further, the flagella of bacteria such as *S. enterica* serovar Enteritidis and *Pseudomonas aeruginosa* are involved in the triggering of innate immune responses in the host, and flagellin up-regulates tumor necrosis factor alpha (TNF-α) expression (9, 58). Toll-like receptors (TLRs) are a family of cell surface receptors that are identified by a conserved cytoplasmic signaling domain. TLRs recognize pathogen-associated molecular patterns and mediate the production of cytokines necessary for the development of effective immunity (31). Flagellin from *L. monocytogenes* binds to TLR5 (26), which is expressed by intestinal epithelial cells, monocytes, and dendritic cells (6, 40). Likewise, TLR5 stimulates the production of TNF-α (26), a cytokine of importance in host resistance to enteric listeriosis (3). To study the role of the flagella and chemotaxis of *L. monocytogenes* in virulence, we constructed and phenotypically characterized defined *flaA*, *cheY*, and *cheA* mutants. We show that flagella and chemotaxis facilitate the adhesion to and invasion of eukaryotic host cells. Furthermore, diminished virulence of Δ*flaA* and Δ*cheYA* mutants relative to the wild type (WT) was observed in TNFR-p55$^{-/-}$ but not in WT mice, suggesting that flagella and *cheYA*-encoded polypeptides participate in the triggering of TNF secretion, leading to protection against infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *L. monocytogenes* WT strain 12067, described previously (40), and its isogenic mutants (described below) were routinely grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 37 or 24°C. *E. coli* DH5α was used as the host for recombinant plasmids and was grown in Luria-Bertani broth. Strains harboring pAUL-A or pAT19 derivatives were grown in the presence of erythromycin (150 μg/ml for *E. coli* and 5 μg/ml for *L. monocytogenes*).

Genetic manipulations. Chromosomal DNA and plasmid extractions, restriction enzyme digestions, and DNA ligations were performed by standard methods (48), using enzymes supplied by New England Biolabs. PCRs were performed with custom primers (DNA Technology, Aarhus C, Denmark) and AmpliTaq DNA polymerase from Perkin-Elmer (Branchburg, N.J.). Nucleotide sequencing was carried out using the Thermo Sequenase fluorescence labeled primer cycle-sequencing kit as specified by the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, England) on an A.L.F. sequencer (Amersham Pharmacia Biotech), using fluorescein-labeled primers.

Construction of the chromosomal deletion mutants. Deletion mutants with mutations in the *flaA*, *cheY*, and *cheA* genes were generated by PCR with specific primers with incorporated restriction sites (underlined) to introduce in-frame deletions. (i) To create the Δ*flaA* mutant, the oligonucleotide pair A (GCGGAATTCGCGCAGGTGTTGGAGCCGATG) and B (GCGGGATCCCAAACGTTCTTGCGCTTGAG) with a BamHI restriction site were used to amplify a 428-bp DNA fragment (AB) of the 5′ region of *flaA* (15), encoding the first 32 N-terminal amino acid residues. The oligonucleotide pair C (GCGGGATCCCAAACACCGCAAATGTTAACTC), with a BamHI restriction site, and D (TCGTCGGGTCTTTCTCAAGC) served to amplify a 798-bp DNA fragment (CD) at the 3′ region encoding the last 14 C-terminal amino acids of FlaA. The two PCR products were digested with two restriction enzymes: AB with EcoRI and BamHI and CD with HindIII and BamHI. The resulting PCR products were cloned into the temperature-sensitive vector pAUL-A (7). (ii) To create the Δ*cheY* mutant, the oligonucleotide pair E (GCGAAGCTTTCTCAAGAAGCAACGGAAGC) and F (GCGGGATCCATGAACATTGCATCGTCGAC), with a BamHI restriction site, were used to amplify a 609-bp DNA fragment (EF) of the 5′ region of *cheY* (16) encoding the first 14 N-terminal amino acid residues. The oligonucleotide pair G (GCGGGATCCGGTTTTAGAGGCGTTAGAAAAAGC), with a BamHI restriction site, and H (GCGGAATTCAATCGCTTCTCTGTCTGGCG) served to amplify a 602-bp DNA fragment (GH) at the 3′ region encoding the last 12 C-terminal amino acids of CheY. The two PCR products were digested: EF with HindIII and BamHI and GH with EcoRI and BamHI. The resulting PCR products were cloned into the vector pAUL-A. (iii) To create the Δ*cheA* mutant, the oligonucleotide pair I (GCGGAATTCCAAACACCGCAAATGTTAACTC) and J (GCGGGATCCTTCTCAAGCTGTAACAGATTATC), with a BamHI restriction site, were used to amplify a 796-bp DNA fragment (IJ) of the 5′ region of *cheA* (16) encoding the first 36 N-terminal amino acid residues. The oligonucleotide pair K (GCGGGATCCTCAGATTGCCTTTTCTGGAGC), with a BamHI restriction site, and L (GCGAAGCTTTTAGCATGCGTTTTCCTCCC) served to amplify a 857-bp DNA fragment (KL) at the 3′ region encoding the last 26 C-terminal amino acids of CheA. The two PCR products were digested: IJ with EcoRI and BamHI and KL with HindIII and BamHI. The resulting PCR products were cloned into the vector pAUL-A. (iv) To create the Δ*cheYA* mutant, the oligonucleotide pair M (GCGGAATTCTCTCAAGAAGCAACGGAAGC) and F (GCGGGATCCATGAACATTGCATCGTCGAC), with a BamHI restriction site, were used to amplify a 609-bp DNA fragment (MF) of the 5′ region of *cheY* encoding the first 14 N-terminal amino acid residues. Fragment MF and fragment KL described above were digested: MF with EcoRI and BamHI and fragment KL with HindIII and BamHI. The resulting PCR products were cloned into the vector pAUL-A. Plasmids pAUL-Δ*flaA*, pAUL-Δ*cheY*, pAUL-Δ*cheA*, and pAUL-Δ*cheYA* were transformed into *L. monocytogenes* 12067 by electroporation. To obtain the chromosomal in-frame deletions, recombinant clones were processed as previously described (35). The appropriate gene deletions were confirmed by PCR sequencing of chromosomal DNA from mutants (data not shown).

Complementation. For complementation of the *cheY* mutant, a 796-bp DNA fragment containing the *cheY* gene and its promoter was amplified by oligonucleotides I and J (described above). The PCR product was digested with EcoRI and BamHI and cloned into the shuttle vector pAT19 (56), creating p*cheY*. Plasmid p*cheY* was transformed into the *L. monocytogenes* Δ*cheY* mutant by electroporation. For complementation of the *cheA* and *cheYA* mutants, a 2,685-bp DNA fragment containing the *cheY* and *cheA* genes and their promoter was amplified with oligonucleotides G (GGCGAATTCGGACGAGGGGCTTTTCTTTT) and H (GCGGGATCCCCAGGTTTCTTTTCCACTTCG). The PCR product was digested with EcoRI and BamHI and cloned into the vector pAT19, creating p*cheYA*. Plasmid p*cheYA* was transformed into *L. monocytogenes* Δ*cheA* and Δ*cheYA* mutants.

Motility assays. Swarming on soft agar was analyzed as described by Kathariou et al. (30), with some modifications. Individual colonies were transferred to a petri plate containing tryptic soy broth (Difco) with 0.25% agar, and the motility plates were incubated for 24 h at 24 or 37°C. The low density of the agar allowed the bacteria to move within the agar, forming a halo of growth around the point of inoculation. Motility was also examined by phase-contrast microscopy at a magnification of ×1,000, using an Axiolab microscope (Carl Zeiss, Oberkochen, Germany) and a hanging-drop preparation from a liquid culture of *L. monocytogenes* grown at 24°C (optical density at 600 nm [OD_{600}], 0.8). The behavior of the bacteria was assessed qualitatively. Linear advances were regarded as swimming, and stationary rotations were regarded as tumbling.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. To analyze total cell lysates of *L. monocytogenes* strains, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed as previously described (15). Bacteria were grown to late logarithmic growth phase (OD_{600}, 0.8), and cell lysates of *L. monocytogenes* were made by sonication of cells. The samples were run on a 12.5% polyacrylamide gel and incubated with a monoclonal antibody against *L. monocytogenes* 4b flagellin, kindly provided by W. Donachie, Moredun Research Institute, Edinburgh, Scotland.

Electron microscopy. Bacteria from liquid cultures incubated at 24°C (OD_{600}, 0.8) were washed twice in physiological saline buffer. A drop of bacterial suspension was placed on a carbon-coated grid. After 90 s, the excess was carefully removed and the preparations were negatively stained in 2% uranyl acetate for 45 s. Air-dried grids were examined in a Philips CM100 transmission electron microscope at 80 kV.

Infection of cell cultures. Determination of cell association and invasion of *L. monocytogenes* was performed as described by Larsen et al. (32), with some modifications. Enterocyte-like Caco-2 cells obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) were cultured in Eagle's minimal essential medium (MEM) enriched with Glutamax and HEPES (Invitrogen, Tåstrup, Denmark) and supplemented with 20% heat-inactivated (30 min at 56°C) fetal calf serum (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and 0.5 ml of gentamicin (50 mg/ml) (Invitrogen) at 37°C under 5% CO_2. The cells were used at passages 10 to 15. They were trypsinized, and the cell concentration was adjusted to 5×10^5 cells per ml. They were grown without gentamicin to a monolayer (68 to 72 h at 37°C) in Eagle's MEM supplemented with 10% heat-inactivated fetal calf serum and 0.1 mM

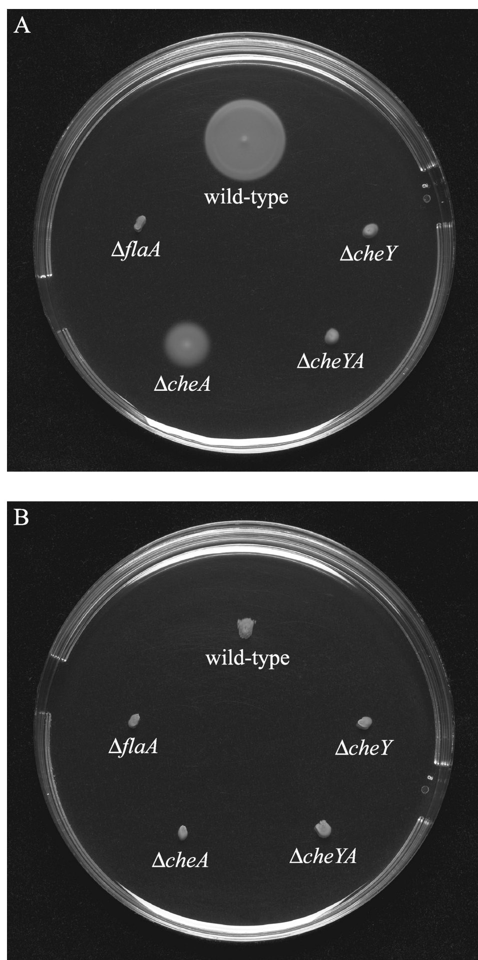

FIG. 1. Swarming of *L. monocytogenes* mutants in semisolid agar. The *L. monocytogenes* WT strain 12067 and the Δ*flaA*, Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants were stabbed into semisolid agar plates (tryptic soy broth plus 0.25% agar). The plates were incubated at 24°C (A) or 37°C (B) for 24 h.

nonessential amino acids. For infection experiments, overnight bacterial cultures were diluted 25-fold and grown at 24 or 37°C with agitation until the OD_{600} reached 0.8. Bacteria were harvested, adjusted to approximately 3×10^6 bacteria per ml, and added to wells of 12-well plates, resulting in a multiplicity of infection of about 30 bacteria per cell. Following 1 h of incubation at 37°C, the cells were washed three times with phosphate-buffered saline (PBS). To kill extracellular bacteria, 2 ml of MEM with gentamicin (10 μg/ml) was added to the wells, and the mixture was then incubated for 1 h at 37°C under 5% CO_2. The cells were washed three times and lysed by adding 1 ml of 0.1% Triton X-100. The number of viable bacteria released from the cells was assessed on agar plates. In some experiments, bacteria were centrifuged on the cells at $1,000 \times g$ for 1.5 min. The CFU after the first 1 h of incubation reflects the bacteria associated with the monolayer (attached on the surface or at some stage of internalization). The CFU 1 h after the addition of gentamicin in the extracellular environment represents the viable bacteria that are internalized by the mammalian cells. All parameters were assayed in duplicate. Each experiment was repeated twice. The data were analyzed statistically by Student's *t* test.

Mouse infections. Overnight cultures of bacteria were diluted 25-fold and incubated at 24 or 37°C with agitation to an OD_{600} of 0.8. Bacteria were diluted in PBS prior to infection. Eight-week-old female BALB/C mice (Taconic M & B, Ry, Denmark) were infected intragastrically with 2×10^9 bacteria (five mice per group). Seven-week-old female C57BL/6 mice (WT) and TNFR-p55$^{-/-}$ mice (43), bred in our animal facilities, were infected intragastrically with 4×10^8 bacteria (six per group). The mice were sacrified at different times after infection, and the spleens and livers were removed and homogenized in PBS containing 0.1% Triton X-100. Tenfold serial dilutions of the lysates in PBS were plated on BHI agar plates. Colonies were counted after overnight incubation at 37°C. The data were analyzed statistically by Student's *t* test.

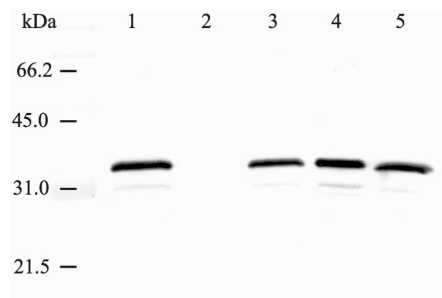

FIG. 2. Western blot analysis of whole-cell lysates of the Δ*flaA*, Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants grown at 24°C (5×10^7 CFU). Samples were run on a 12.5% polyacrylamide gel and incubated with a monoclonal antibody specific for the *L. monocytogenes* 4b flagellin. Lanes: 1, *L. monocytogenes* 12067 (WT); 2, Δ*flaA* mutant; 3, Δ*cheY* mutant; 4, Δ*cheA* mutant; 5, Δ*cheYA* mutant. Molecular mass standards are shown on the left.

RESULTS

Construction and characterization of defined *L. monocytogenes flaA*, *cheY*, *cheA*, and *cheYA* mutants. To analyze the involvement of the *flaA*, *cheY*, and *cheA* gene products in the virulence of *L. monocytogenes*, we constructed mutant strains carrying in-frame deletions in each of the *flaA*, *cheY*, and *cheA* genes (Δ*flaA*, Δ*cheY*, and Δ*cheA*) as well as an in-frame deletion in both the *cheY* and *cheA* genes (Δ*cheYA*). These chromosomal mutants were obtained by allelic exchange in *L. monocytogenes* strain 12067. This WT strain was chosen for construction of mutants since the only *flaA*, *cheY*, and *cheA* sequences available at the time this project was initiated were those from *L. monocytogenes* strain 12067 (15, 16). The phenotype of the mutants were analyzed by semisolid swarm plate assays (Fig. 1A). After incubation at 24°C, the WT strain showed good swarming, with concentric rings that increased with the period of incubation. The Δ*flaA* mutant did not show swarming and produced small, compact colonies with sharp boundaries. The Δ*cheY* and the Δ*cheYA* mutants formed a small fuzzy-looking swarm ring, while the colonies of the Δ*cheA* mutant showed swarming, but the swarm ring was much smaller than that of the WT, indicating that *cheY* is required for swarming and that *cheA* is contributing to a lesser degree. At 37°C, none of the strains showed swarming (Fig. 1B). None of the four mutants were affected for growth rate in BHI medium (data not shown).

To determine whether the deletions in *cheY* and *cheA* had effects on flagellin production, we performed Western blot analysis with a monoclonal antibody directed against the flagellin protein of *L. monocytogenes*. The Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants and WT bacteria showed similar flagellin levels (Fig. 2). Microscopic examination of cells grown in liquid culture to late-logarithmic phase showed that the Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants all had flagella (Fig. 3). As expected, no flagella and flagellin were detected in the Δ*flaA* mutant as analyzed by electron microscopy or Western blotting, respectively (Fig. 2 and 3). Phase-contrast microscopy

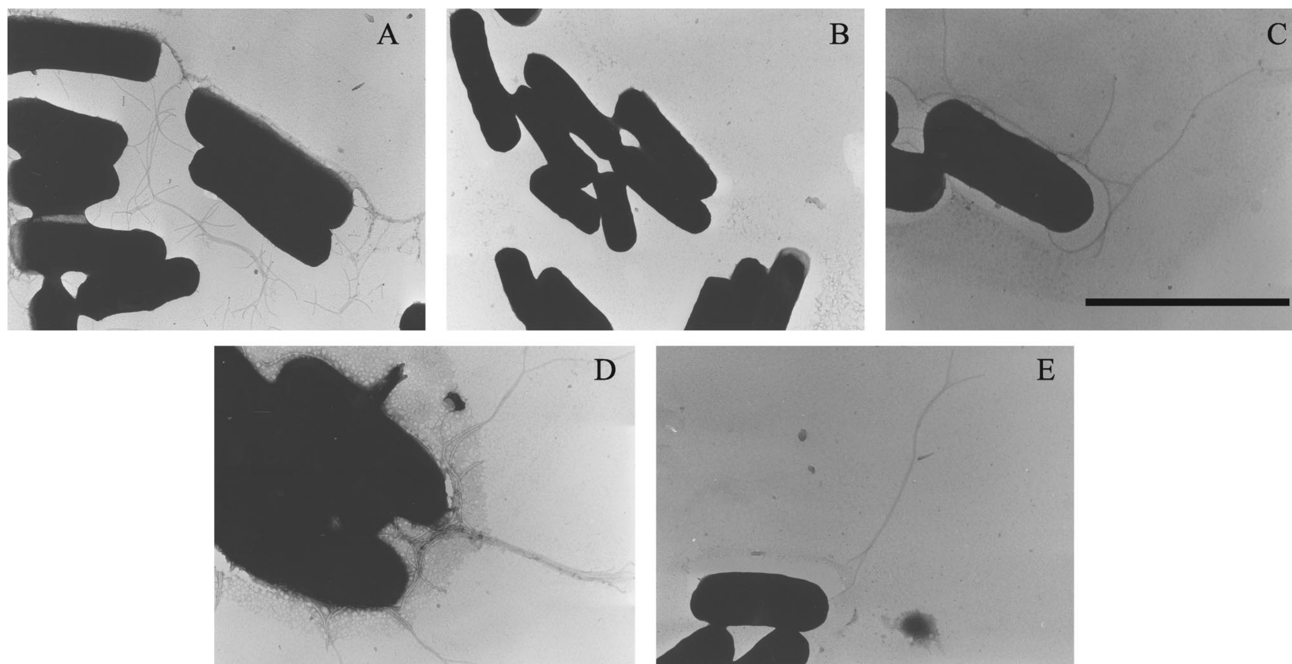

FIG. 3. Electron micrographs of the flagellated *L. monocytogenes* WT strain (A), the nonflagellated Δ*flaA* mutant (B), the Δ*cheY* mutant (C), the Δ*cheA* mutant (D), and the Δ*cheYA* mutant (E). Bacteria were grown at 24°C to late logarithmic growth phase, applied to carbon-coated grids, shadowed with 2% uranyl acetate, and examined under a transmission electron microscope. The scale bar, shown in panel C and valid for all panels, represents 1.5 μm (B), 1 μm (A, C, and E), and 0.5 μm (D).

revealed that the Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants were all motile and showed only tumbling behavior whereas the WT showed tumbling and smooth swimming.

For complementation of the Δ*cheY* mutant, the vector pAT19 containing the *cheY* gene and its promoter region (p*cheY*) was introduced into the Δ*cheY* mutant. For complementation of the Δ*cheA* and the Δ*cheYA* mutants, pAT19 containing the *cheY* and *cheA* genes and their promoter (p*cheYA*) was introduced into the Δ*cheA* and Δ*cheYA* mutants. As controls, the WT and the Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants carrying the vector without inserts were used. On semisolid agar, swarming of the complemented mutants was restored (Fig. 4). Phase-contrast microscopy demonstrated that the three complemented strains showed both tumbling and smooth swimming like the WT. None of the phenotypes were restored by the plasmid vector alone.

Effects of mutations in flagellar and chemotaxis genes on bacterial association with cells. The ability of the Δ*flaA*, Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants to associate with and invade the human enterocyte-like cell line Caco-2 was analyzed. Caco-2 cell association was significantly reduced for the mutants grown at 24°C compared to the WT strain (10.6 to 12.7% of control values; $P < 0.001$) (Table 1). Likewise, invasion by the mutant bacteria was significantly reduced in comparison to that of the WT strain ($P < 0.001$), with levels from 1.4 to 7.0% of the wild-type values. To further determine if the reduced invasion capacity of the Δ*flaA* and Δ*cheYA* mutants was due to impaired locomotion, the bacteria were centrifuged onto the monolayers prior to the invasion assay. Centrifugation increased cell association to 78.8 and 75.8% of the WT values, respectively, indicating that flagella and chemotaxis are important to ensure that bacteria contact host cells. However, cell association was still lower than that of the WT strain ($P < 0.005$) (Table 1).

Cell association of the Δ*cheYA* mutant grown at 37°C was only slightly reduced as compared to that of the WT strain. No differences in cell association between the WT strain and the Δ*flaA* mutant grown at 37°C were observed. The mutant and

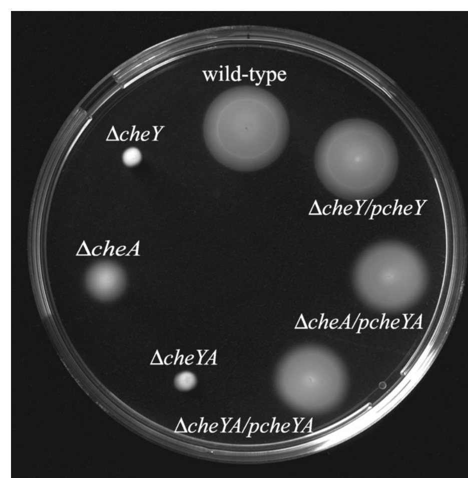

FIG. 4. Swarming of complemented Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants in semisolid agar. The Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants with plasmids carrying the *cheY* (p*cheY*) or *cheY*/*cheA* (p*cheYA*) genes were stabbed into a semisolid agar plate. The WT strain and the Δ*cheY*, Δ*cheA* and Δ*cheYA* mutants carrying the parent vector pAT19 served as controls. The plate was incubated at 24°C for 30 h.

TABLE 1. Association and invasion of Caco-2 cells by wild-type *L. monocytogenes* 12067 and the isogenic flagellum and chemotaxis mutants

Growth temp (°C) and strain	Without centrifugation		With centrifugation	
	No.[a] (%)[b] of cell-associated bacteria (CFU/ml)	No.[a] (%) of invading bacteria (CFU/ml)	No. (%) of cell-associated bacteria (CFU/ml)	No. (%) of invading bacteria (CFU/ml)
24				
WT	$(3.3 \pm 0.1) \times 10^{6}$ (100)	$(2.7 \pm 0.3) \times 10^{5}$ (100)	$(6.6 \pm 0.5) \times 10^{6}$ (100)	$(1.4 \pm 0.4) \times 10^{6}$ (100)
Δ*flaA* mutant	$(3.5 \pm 0.6) \times 10^{5c}$ (10.6)	$(3.8 \pm 0.5) \times 10^{3c}$ (1.4)	$(5.2 \pm 0.4) \times 10^{6d}$ (78.8)	$(3.6 \pm 0.9) \times 10^{5d}$ (25.7)
Δ*cheY* mutant	$(4.2 \pm 0.2) \times 10^{5c}$ (12.7)	$(1.2 \pm 0.2) \times 10^{4c}$ (4.4)	ND	ND
Δ*cheA* mutant	$(4.0 \pm 0.3) \times 10^{5c}$ (12.1)	$(1.9 \pm 0.1) \times 10^{4c}$ (7.0)	ND	ND
Δ*cheYA* mutant	$(4.1 \pm 0.1) \times 10^{5c}$ (12.4)	$(1.6 \pm 0.2) \times 10^{4c}$ (5.9)	$(5.0 \pm 0.4) \times 10^{6d}$ (75.8)	$(8.4 \pm 0.6) \times 10^{5d}$ (60.0)
37				
WT	$(2.6 \pm 0.2) \times 10^{5}$ (100)	$(1.2 \pm 0.3) \times 10^{5}$ (100)	ND	ND
Δ*flaA* mutant	$(2.4 \pm 0.2) \times 10^{5}$ (NS)[e] (92.3)	$(1.1 \pm 0.1) \times 10^{5}$ (NS) (91.7)	ND	ND
Δ*cheYA* mutant	$(2.2 \pm 0.1) \times 10^{5d}$ (84.6)	$(9.3 \pm 0.7) \times 10^{4}$ (NS) (77.5)	ND	ND

[a] Association and invasion assays were performed without or with centrifugation of the bacteria onto the monolayer. Bacteria were grown in BHI broth at either 24 or 37°C, and invasion assays carried out as described in Materials and Methods. Each experiment was repeated twice, and the mean number of associated (both attached and intracellular) and invading bacteria were recorded. Results are given as mean ± standard deviation.

[b] The values for association and invasion were normalized to the values for the WT strain (under identical culture and centrifugation conditions), which were arbitrarily set at 100.

[c] Significantly different from the WT strain under identical culture and centrifugation conditions ($P < 0.001$).

[d] Significantly different from the WT strain under identical culture and centrifugation conditions ($P < 0.005$).

[e] NS, not significantly different from the WT strain under identical culture and centrifugation conditions.

WT strains grown at 37°C showed similar invasion ability (Table 1).

Virulence of *flaA* and *cheYA* mutants in WT and TNFR-p55$^{-/-}$ mice. To analyze the effect of deleting the *flaA* and *cheYA* genes on the virulence of *L. monocytogenes*, bacterial levels in mice intragastrically (i.g.) infected with Δ*flaA* and Δ*cheYA* mutants were compared to those infected with the WT strain. In a first series of experiments, we observed that spleens of BALB/c mice 3 days after i.g. infection with the Δ*flaA* mutant showed 100-fold larger bacterial numbers than did the spleens of mice infected with the WT strain (Fig. 5A). Spleens of mice infected i.g. with the Δ*cheYA* or WT *L. monocytogenes* strain showed similar bacterial levels. No differences in bacterial numbers in spleens were observed when the Δ*flaA*, and Δ*cheYA* mutants and WT *L. monocytogenes* were grown at 37°C (Fig. 5B). These results indicate that abolished expression of the *flaA* gene increases the virulence of *L. monocytogenes* in BALB/c mice compared to the flagellated WT strain and the Δ*cheYA* mutant.

To address whether TNF-mediated protective immune responses are involved in the discrepancy between invasion and virulence of Δ*flaA* and Δ*cheYA* mutants, the bacterial load in

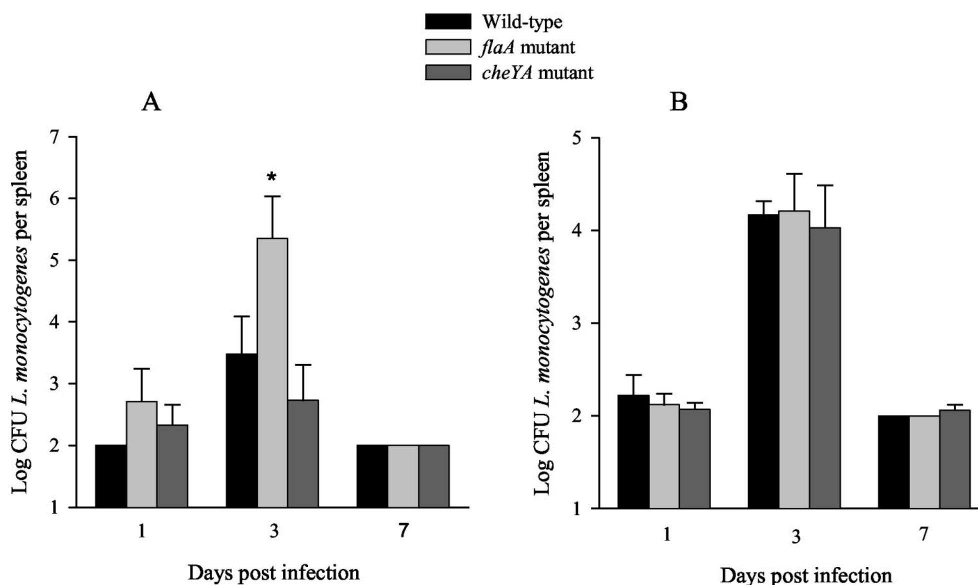

FIG. 5. CFU in the spleens of i.g. infected BALB/c mice. *L. monocytogenes* WT and the Δ*flaA* and Δ*cheYA* mutants were grown at either 24°C (A) or 37°C (B) before being used to infect BALB/c mice (five mice per group). The mice were sacrified at the indicated time points after infection, and their spleens were homogenized and plated. The means and standard errors of the mean are shown. An asterisk indicates a significant difference in the log CFU of the mutant compared to the WT ($P < 0.05$ as determined by Student's t test). A representative of two independent experiments is shown.

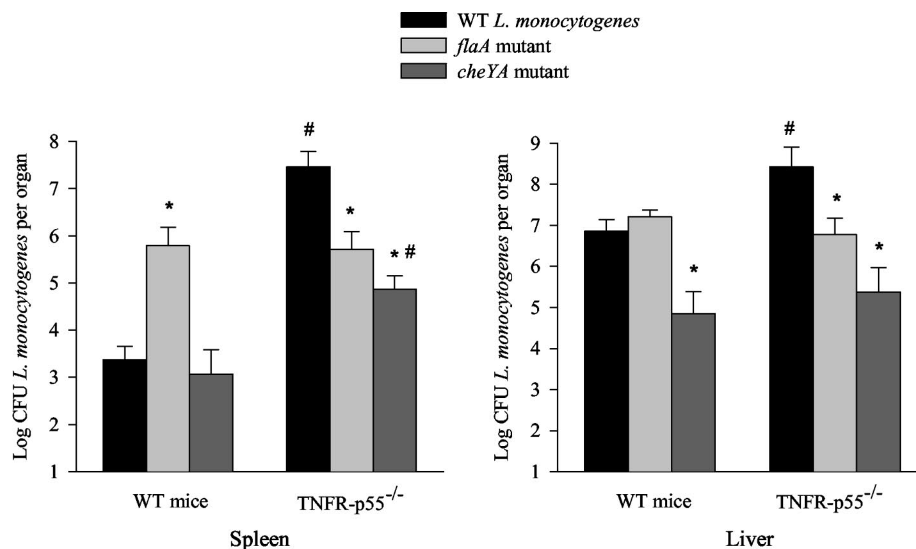

FIG. 6. CFU after i.g. infection of TNFR-p55$^{-/-}$ and WT mice. *L. monocytogenes* WT and mutant strains were inoculated i.g. into WT or TNFR-p55$^{-/-}$ mice (six mice per group). Three days after infection, the mice were sacrified and bacterial loads (CFU) in their spleens and livers were measured. The means and standard errors of the mean are shown. The asterisk indicates a significant difference in the log CFU of a bacterial mutant relative to the WT ($P < 0.05$ as determined by Student's *t* test). The pound sign indicates a significant difference between two groups of mice infected with the same bacterial strain ($P < 0.05$ as determined by Student's *t* test).

mice deficient in TNFR-p55, the main TNF receptor, was measured. TNFR-p55$^{-/-}$ mice have been previously shown to possess a dramatically increased susceptibility to *L. monocytogenes* (43, 47). The susceptibility of TNFR-p55$^{-/-}$ mice to WT *L. monocytogenes* and to the Δ*flaA* and Δ*cheYA* mutants grown at 24°C was compared. The spleens and livers from TNFR-p55$^{-/-}$ mice showed a dramatically increased number of WT *L. monocytogenes* bacteria compared to the same organs of C57BL/6 control mice (Fig. 6). Differences in bacterial numbers in organs from control and TNFR-p55$^{-/-}$ mice were significantly smaller when mice were infected with Δ*flaA* or Δ*cheYA* mutants. Moreover, bacterial numbers of the Δ*flaA* mutant recovered from the spleens of control and TNFR-p55$^{-/-}$ mice were similar (Fig. 6). Also, similar levels of Δ*flaA* and Δ*cheYA* in the livers of TNFR-p55$^{-/-}$ mice and WT mice were found (Fig. 6). This suggests that flagellin and *cheYA*-encoded polypeptides participate in bacterial invasion but the presence of flagella is deleterious to systemic infection, which seems to be dependent on the TNF-p55 receptor.

DISCUSSION

In this work we characterized the flagellin gene, *flaA*, and the two chemotaxis genes, *cheY* and *cheA*, of *L. monocytogenes* through the construction of deletion mutants and compared their virulence with that of WT *L. monocytogenes* in vitro and in vivo.

Phenotypic characterization of the mutants showed that the deletion mutant with a mutation in *flaA* was completely defective in the production of flagella and motility. Analysis of the mutants with deletions in *cheY* and *cheA* showed impaired swarming and tumbling behavior during motility. *Trans*-complementation of the Δ*cheY*, Δ*cheA*, and Δ*cheYA* mutants restored swarming and smooth swimming behavior, indicating that the mutations had no polar effects. In *B. subtilis*, *cheY* and *cheA* null mutants are tumbly, in contrast to *E. coli*, where *cheY* and *cheA* null mutants exhibit a smooth-swimming phenotype (5, 22, 28). These data suggest that the *cheY* and *cheA* gene products of *L. monocytogenes* contribute to chemotactic signal transduction, as suggested from the amino acid sequence similarity (16), and that the mechanism by which they confer their control may be similar to the mechanism in *B. subtilis*.

The flagellum (Δ*flaA*) and chemotaxis (Δ*cheY*, Δ*cheA*, Δ*cheYA*) mutants had a reduced capacity to associate with cells when the bacteria were grown at 24°C, and centrifugation of bacteria onto cells could only partly complement this. In accordance with observations that the production of flagella is markedly reduced at 37°C (42), the ability of the aflagellate Δ*flaA* and chemotaxis Δ*cheYA* mutants to associate with cells was only slightly decreased when they were grown at 37°C prior to infection. These findings suggest that flagella and chemotaxis are important to ensure that *L. monocytogenes* contacts host cells, as also suggested by Flanary et al. (21). The fact that centrifugation did not completely revert the phenotype suggests a role of flagella in adhesion or invasion other than locomotion. On centrifugation, the Δ*cheYA* mutant was more invasive than the Δ*flaA* mutant, probably due to the presence of *flaA*. It has been suggested that components of bacterial flagella can act as adhesins and mediate the binding to host cells and mucosal surfaces (2, 59). Similar effects of mutations in motility-related genes on cell association have been shown for other bacteria. For example, mutations in the *Yersinia enterocolitica flhDC* or *fliA* genes, which are transcriptional regulators of the flagellar regulon and are required for the expression of motility (29, 61), affect the effective migration of bacteria to the host cells (60), and in *S. enterica* serovar Enteritidis the flagella assist in colonization of epithelial cells by enabling motility rather than providing an adhesin (12). The importance of chemotaxis of *L. monocytogenes* in the interac-

tions with cells has been previously studied by using a chemotaxis mutant of *L. monocytogenes* strain NCTC 10527 with a transposon insertion in the promoter region of the *cheYA* operon. This strain was impaired in cell association but not in invasion of fibroblasts (21).

Spleens from i.g. infected BALB/c and C57BL/6 mice contained larger numbers of the Δ*flaA* mutant than of the WT strain, whereas spleens from mice infected with Δ*cheYA* and WT *L. monocytogenes* strains contained similar bacterial numbers, indicating that the presence of flagella is deleterious to systemic infection. In fact, *L. monocytogenes* is able to repress flagellar expression at 37°C (42). This temperature regulation might serve as a mechanism to evade recognition by the innate immune system in the host. Interestingly, when comparing *L. monocytogenes* with the nonpathogenic but closely related species *L. innocua*, Kathariou et al. (30) found that the repression of flagellin in *L. innocua* was not as strong as the repression in *L. monocytogenes*. The discrepancy of the in vivo virulence test of the Δ*flaA* mutant with the in vitro invasion experiments with this mutant can be reconciled through the observation that the Δ*flaA* mutant were present at lower levels than WT bacteria in TNFR-p55$^{-/-}$ mice. The data suggest that the flagella of *L. monocytogenes* are important for triggering TNF-mediated growth control of *L. monocytogenes*. These data correspond to previous reports that flagella from gram-negative bacteria such as *S. enterica* serovar Enteritidis, *P. aeruginosa*, and *Y. enterocolitica* induce the production of cytokines, e.g., TNF-α and interleukin-1 (8, 9, 58). Interestingly, it has been shown that two regions in the conserved N- and C-terminal parts of the flagellin of *E. coli* are required for interleukin-8 release and TLR5 activation in a human epithelial cell line (14). Likewise, it was demonstrated that the N- and C-terminal regions of *S. enterica* serovar Dublin flagellin induce TNF-α production in monocyte cell lines (19). The N- and C-terminal regions are conserved across bacteria, and a considerable degree of homology was also found to these regions of the flagellin of *L. monocytogenes* (15), suggesting a potential role of those conserved domains of flagellin of *L. monocytogenes* in activation of the innate immune response. Indeed, the TLR5 recognition site of flagellin has been mapped to highly conserved N- and C-terminal amino acids which are present in flagellin of *L. monocytogenes* (52). Moreover, a recent study, based on conformation analysis, has predicted that a region in the N-terminal domain of flagellin of *L. monocytogenes* is involved in binding to TLR5. This region overlaps with the N-terminal binding site (27). However, mice deficient in the adaptor protein MyD88, which is required for TLR signaling (1, 38), contained only slightly augmented bacterial numbers of WT *L. monocytogenes* in their spleens (unpublished results), indicating that TNF-mediated control of *L. monocytogenes* is at least partially MyD88 independent.

Factors other than temperature can stimulate the expression of *L. monocytogenes* flagellin, which is present in trace amounts at 37°C (42). Indeed, flagellin expression is regulated by osmolarity (49). Thus, we propose that the observed effect of flagella and chemotaxis on cell invasion and the suspected role of flagella in TNF induction may be relevant to the biology of infection with *L. monocytogenes*.

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