

Flagellum of *Legionella pneumophila* Positively Affects the Early Phase of Infection of Eukaryotic Host Cells

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***Legionella pneumophila*, the etiologic agent of Legionnaires' disease, contains a single, monopolar flagellum which is composed of one major subunit, the FlaA protein. To evaluate the role of the flagellum in the pathogenesis and ecology of *Legionella*, the *flaA* gene of *L. pneumophila* Corby was mutagenized by introduction of a kanamycin resistance cassette. Immunoblots with anti-flagellin-specific polyclonal antiserum, electron microscopy, and motility assays confirmed that the specific flagellar mutant *L. pneumophila* Corby KH3 was nonflagellated. The redelivery of the intact *flaA* gene into the chromosome (*L. pneumophila* Corby CD10) completely restored flagellation and motility. Coculture studies showed that the invasion efficiency of the *flaA* mutant was moderately reduced in amoebae and severely reduced in HL-60 cells. In contrast, adhesion and the intracellular rate of replication remained unaffected. Taking these results together, we have demonstrated that the flagellum of *L. pneumophila* positively affects the establishment of infection by facilitating the encounter of the host cell as well as by enhancing the invasion capacity.**

Legionella pneumophila, the etiologic agent of Legionnaires' disease, is a ubiquitous microorganism inhabiting natural and man-made freshwater biotopes (5). In these environments, the gram-negative, rod-shaped bacteria survive as intracellular pathogens of protozoan organisms such as *Acanthamoeba castellanii*, *Hartmannella vermiformis*, and *Naegleria* spp. (15). Upon transmission to individuals via *L. pneumophila*-containing aerosols generated by showerheads and air-conditioning systems, the bacteria invade and multiply within alveolar macrophages (1, 2, 7) and nonphagocytic cells (17). The infection which mainly affects immunocompromised patients results in a life-threatening atypical pneumonia (7).

Detailed ultrastructural and molecular studies of the intracellular fate of the bacterium revealed that human macrophages and protozoan cells infected with *L. pneumophila* exhibit remarkable similarities concerning the establishment of a replicative phagosome (3, 16, 22, 42, 45). However, significant differences were observed during early stages of infection (21). Uptake by *Hartmannella* is accomplished by a microfilament-independent mechanism that is sensitive to methylamine, which is an inhibitor of receptor-mediated endocytosis (28). So far, one receptor of *Hartmannella vermiformis*, a Gal/GalNAc lectin, could be identified (46). Attachment of *L. pneumophila* to this lectin results in tyrosine dephosphorylation of multiple host cell proteins. However, depending on the type of amoeba, different receptors might be involved (22). In contrast, the uptake by human macrophages occurs following binding of complement receptors CR1 and CR3 via microfilament-dependent phagocytosis (26). In addition to this cytochalasin D-sensitive mechanism, complement-independent mecha-

nisms for uptake by nonphagocytic cells have been described (39).

The influence of bacterial motility on infection processes or on survival of legionellae in aquatic habitats is not well understood, but motility has been associated with the growth phase of *L. pneumophila* (40). Bacteria which actively multiply within the host cell vacuole are nonmotile, whereas bacteria in the later stages of infection and cell lysis are flagellated and highly motile (8, 38). This trait during the postexponential phase suggests that motility enables *Legionella* to escape from a spent host and facilitates its attempt to find a new host by dispersion into the environment.

The single, monopolar flagellum of *L. pneumophila* is composed of one major subunit, the flagellin, encoded by the *flaA* gene (23). Previous reports suggest that the expression of flagella is temperature regulated, since it is repressed at temperatures higher than 37°C (36). Recently, we demonstrated that the expression of the *flaA* gene is regulated at the transcriptional level (24) by the alternative σ^{28} factor (FliA) and probably by FlaR, a regulator of the LysR family (25). Moreover, the complex flagellum expression and assembly seems to be coordinatively regulated with other virulence-associated traits (6, 16, 38, 40). These traits include thickening of the cell envelope, sensitivity to NaCl, contact-dependent cytotoxicity, osmotic resistance, and evasion of macrophage lysosomes (8). However, experiments with an insertion mutation in the *fliI* gene of *L. pneumophila* indicated that intracellular growth in macrophage-like U937 cells does not require flagellar assembly (32). Therefore, it has been proposed that the flagellum might be a virulence-associated factor in the infection process of *L. pneumophila*. In addition, it has been proposed that proteins involved in the assembly of flagella may be required for export of factors involved in intracellular growth (32).

To determine the exact role of the flagellum for the pathogenicity, we constructed and phenotypically characterized a specific *flaA* mutant of *L. pneumophila* Corby. The results of

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this study provide evidence for the importance of the flagellum during the early stage of infection of eukaryotic host cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. pneumophila* Corby (serogroup 1) (27) was used for the construction of the *flaA* mutant strain KH3 carrying the chromosomal *flaA::kam* gene fusion (47). *Escherichia coli* DH5 α was used for propagation of recombinant plasmid DNA, and *E. coli* K-12 (SM10 λ pir) (43) was used for propagation of pCVD442 and pMSS704. Plasmid pUC18 (Pharmacia LKB, Freiburg, Germany) was used for the construction of pKH106, and plasmid pKS (Stratagene, Heidelberg, Germany) was used for the generation of pKH2 and pKH3. The suicide vectors pCVD442 and pMSS704 (11) were used to generate plasmids pKH4 and pCD10, respectively.

Media and chemicals. *E. coli* was cultivated in Luria-Bertani broth. Solid medium for growth of *L. pneumophila* was ACES [N-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal yeast extract medium (ABCYE) (pH 6.9), essentially as described previously (13). Strains were incubated at 37°C for 48 h before being harvested. For broth culture, *L. pneumophila* was grown in ACES-buffered yeast extract broth (YEB; 1% yeast), supplemented with 0.025% ferric pyrophosphate and 0.04% L-cysteine, to stationary growth phase at 37°C unless indicated otherwise. Where indicated, drugs were included in bacteriological media at the following concentrations: ampicillin, 100 mg ml⁻¹; kanamycin, 12.5 to 25 mg ml⁻¹; and chloramphenicol, 5 to 25 mg ml⁻¹. Enzymes were purchased from Pharmacia LKB, Boehringer GmbH (Mannheim, Germany), and GIBCO BRL (Eggenstein, Germany). All other chemicals were supplied by Merck (Darmstadt, Germany), Oxoid (Wesel, Germany), Roth (Karlsruhe, Germany), and Sigma (Deisenhofen, Germany).

DNA techniques. Preparation of genomic DNA and plasmid DNA, as well as DNA-cloning procedures and Southern blot analysis, were performed by standard methods (41). Several oligonucleotide primer sets (MWG, Ebersberg, Germany) were designed and synthesized for amplification of DNA fragments encompassing the chromosomal *flaA* region or the *flaA* region with the inserted antibiotic resistance marker. The nucleotide sequences of the primers were as follows: KHACCD, 5'-TCCAACCGCTCGCTCCCATGGAGCCACCCA-3'; FLA1, 5'-GTAATCAACACTAATGTGGC-3'; and FLA5, 5'-GTTGCAGAATTTGGTTTTGGTC-3'. PCR was carried out using a Thermocycler 60 apparatus from Biomed (Theres, Germany) and GoldStar DNA polymerase (Eurogentec, Seraing, Belgium). Amplification was performed at 95°C for 3 min followed by 30 cycles of denaturation (95°C for 1 min), annealing (55°C for 1.5 min), and extension (72°C for 5 min).

Construction of plasmids. To construct a disrupted *flaA* allele replacement vehicle, plasmid pKH106 was isolated from an expression library of *L. pneumophila* Corby (23). The 2,580-bp *Sau3AI* chromosomal fragment of this plasmid comprises the *flaA* gene, the putative σ^{28} promoter region, and the beginning of an open reading frame which shows significant homology to the *accD* gene of *E. coli*. Subsequent cloning of a 2,170-bp *XbaI-KpnI* fragment containing the *flaA* gene into pKS resulted in pKH2. The cloned *flaA* gene was disrupted by replacement of an internal 109-bp *EcoRI-HindIII* fragment with a Km^r cassette, generating plasmid pKH3. To construct the suicide delivery vector, the 4,000-bp *XbaI-KpnI* fragment of pKH3 was ligated into pCVD442, resulting in pKH4. For complementation, the *SacI-XbaI* fragment (2,170 bp) from pKH106 was cloned into suicide plasmid pMSS704, resulting in pCD10.

Electrotransformation of *L. pneumophila*. For transformation of *L. pneumophila*, bacteria were grown on ABCYE agar plates at 37°C for 24 h. They were then suspended in 200 ml of chilled (4°C) water containing 10% glycerol, and pelleted by centrifugation. This procedure was repeated three times, and the final pellet was suspended in 1 ml of 10% glycerol. Aliquots of 80 μ l were stored at -80°C. Samples were prepared for electroporation by mixing competent cells and plasmid DNA on ice. The samples were placed into prechilled 0.1-cm electroporation cuvettes (Bio-Rad) and pulsed with the pulse controller set at 2.3 kV, 25 mF, and 100 Ω of resistance. Immediately following electric discharge, 1 ml of prewarmed YEB was added to each cuvette. Phenotypic expression was allowed to occur overnight in 4 ml of YEB. For selection of recombinants, cultures were plated onto ABCYE agar plates containing the appropriate antibiotic, with or without 5% sucrose. After 4 to 5 days of incubation at 37°C, colonies were isolated and streaked again on selective ABCYE medium. All strains were stored at -80°C to avoid serial-passage effects.

SDS-PAGE and immunoblotting. Total-cell extracts of *L. pneumophila* strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was performed by use of the discontinuous buffer system of Laemmli (30). *Legionella* cells were grown in overnight cultures at 37°C to the stationary phase unless indicated otherwise. A

1-ml volume was pelleted by centrifugation, the cells were suspended in 100 μ l of SDS sample lysis buffer, and a 5- μ l volume was then loaded onto an SDS-13% polyacrylamide gel. Purified flagellar extracts were prepared from cultures grown on agar plates for 5 days at 30°C. Flagella were isolated by differential centrifugation as described elsewhere (34). Western blot analyses were carried out as described elsewhere by using a polyclonal monospecific antibody against *L. pneumophila* Corby flagellin (23).

Cell culture and growth of *L. pneumophila* in HL-60 cells. The human leukemia cell line HL-60 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was maintained at 37°C under 5% CO₂ in RPMI 1640 medium (PAA, Cölbe, Germany) supplemented with 2 mM L-glutamine (Gln) and 10% fetal calf serum (FCS) (Sigma). HL-60 cells were differentiated into macrophages by incubation for 2 days with 10 ng of phorbol 12-myristate 13-acetate per ml in RPMI-2 mM Gln-10% FCS in 24-well plates (Falcon, Schwandorf, Germany). Adherent cells were washed three times and then incubated with RPMI-2 mM Gln-10% FCS prior to infection.

The ability of *L. pneumophila* strains to grow in macrophage-like cells was determined in coculture assays. Bacterial strains were cultivated overnight at 37°C in YEB to the beginning of the stationary phase. They were adjusted in RPMI-2 mM Gln-10% FCS medium to a concentration of 2×10^3 cells per ml prior to infection. Differentiated HL-60 cells (2×10^5 cells per well) in 24-well plates were infected with 1 ml of bacterial suspension (multiplicity of infection (MOI) 0.01) and incubated for 4 days. Macrophages were lysed daily with 1 ml of cold H₂O and combined with the culture supernatant, and serial dilutions were spread on ABCYE plates to determine the number of bacterial CFU.

To analyze the intracellular growth rates during the first 24 h, a gentamicin assay was used. HL-60 cells were infected at an MOI of 10 for 2 h. After extracellular bacteria were killed by addition of gentamicin (80 μ g/ml) for 1 h, CFU were determined immediately and after 24 h.

In situ hybridization of infected host cells was performed as described elsewhere (19). Briefly, HL-60 cells were infected with bacteria at a MOI of 0.01 in Permax chamber slides (Nunc, Wiesbaden, Germany). After 72 h, the bacteria were labeled with the *Legionella*-specific probe LEG705. Fluorescence was detected using a Zeiss (Oberkochen, Germany) AxioLab microscope and the Zeiss filter set 00 and 10.

To determine the number of intracellular bacteria at the beginning of infection, HL-60 cells were infected with 2×10^6 bacteria, (MOI, 10), incubated for different time intervals (15, 30, 60, and 120 min), treated with gentamicin (80 μ g/ml) for 1 h, washed twice, lysed as described above, and plated on agar plates in serial dilutions. To minimize uptake during the attachment assay, HL-60 cells were pretreated with 1 μ g of cytochalasin D per ml for 1 h (44). The number of adherent bacteria was determined after sedimentation of bacteria by centrifugation (1,000 \times g for 5 min) and a 20-min incubation in the presence of cytochalasin D, followed by vigorous washing. All assays were performed independently in triplicate.

Amoeba culture and growth of *L. pneumophila* in *A. castellanii*. *Acanthamoeba castellanii* was obtained from the American Type Culture Collection (ATCC 30234). Axenic cultures of *A. castellanii* were prepared in 20 ml of *Acanthamoeba* medium PYG 712 (4) at room temperature. Subculture of the amoebae was performed at intervals of 4 days. The axenic culture was adjusted to a titer of 2×10^5 cells per ml, and 1 ml of culture was pipetted into each well of 24-well plates. Following overnight incubation, the medium was replaced with *Acanthamoeba* buffer (i.e., PYG 712 medium without proteose peptone and yeast extract), and the next day the amoeba cultures were infected with bacteria as described for HL-60 cells (see above). To determine the number of adherent bacteria, amoebae were incubated with cycloheximide (100 μ g/ml) and cytochalasin D (5 μ g/ml) for 2 h prior to and throughout the infection (29).

Electron microscopy. Bacteria were grown to stationary phase in supplemented YEB at 37°C. They were then carefully resuspended in distilled water, and a drop of the suspension was directly applied to Formvar-coated copper grids. After sedimentation of the bacteria and removal of the remaining fluid, the samples were stained with 2% uranyl acetate and examined with a transmission electron microscope (EM10; Zeiss) at 60 kV.

RESULTS

Construction of an *L. pneumophila flaA* mutant and a *flaA*-positive complementant. Plasmid pKH4 was used to inactivate the targeted *flaA* locus of *L. pneumophila* strain Corby (Fig. 1). We obtained three putative mutants (KH1, KH2, and KH3) on ABCYE plates plus kanamycin and sucrose, where the allelic

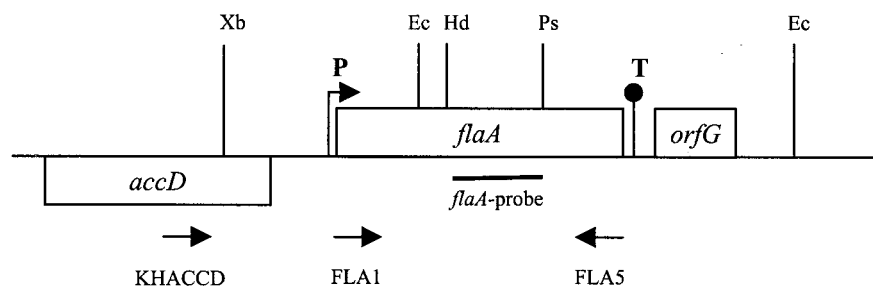


FIG. 1. *flaA* region of *L. pneumophila*. Bars represent open reading frames; *flaA* is the flagellin-encoding gene, *orfG* is the homolog to *flaG* of *P. aeruginosa*, and *accD* is the homolog to *accD* of *E. coli*. Deduced promoters (P), terminators (T), binding sites for primers (arrows), and the *flaA*-specific probe are indicated. Restriction sites: Ec, *EcoRI*; Hd, *HindIII*; Ps, *PstI*; Xb, *XbaI*.

exchange was possibly due to a double crossover. An additional three recombinants (KH4, KH5, and KH6) were isolated from ABCYE plates plus kanamycin as a result of single crossover.

Candidate flagellar mutants were screened by PCR with a primer pair specific for the 5' (FLA1) and 3' (FLA5) region of the *flaA* gene. Amplification products of the predicted length of 1,300 bp were observed for the wild type and the single-crossover mutants. In contrast, strains KH1, KH2, and KH3 revealed amplification products with a predicted length of 3,000 bp, indicating the integration of the *neo* gene (1,800 bp) and the deletion of 109 bp within the *flaA* locus. During a second screen using primer pair KHACCD and FLA5, we excluded the possibility of extrachromosomal copies or a single-crossover event for these mutants, since the binding site of KHACCD is lacking in construct pKH4. The desired recombination event was confirmed by Southern blot analysis using a *flaA* specific probe as well as a *Km^r* gene probe (data not shown). Strain KH3 was chosen for complementation and further characterization.

Successful reintegration of the intact *flaA* gene into the chromosome was achieved after cloning the 2.2-kb *SacI-XbaI* fragment of pKH106H4 into the suicide vector pMSS704. After delivery of the complete vector into the chromosome by a single crossover, the presence of the intact *flaA* gene was proven by PCR and Southern blotting with *flaA*-specific, *Km^r*-specific, and pMSS704-specific probes (data not shown). The insertion was shown to be upstream of the disrupted *flaA* gene, and the wild-type phenotype was completely restored.

Flagellar expression and motility. The effect of targeted mutagenesis on the expression of the *L. pneumophila* FlaA protein was assessed by SDS-PAGE and Western blot analysis by probing whole-cell lysates with a polyclonal monospecific antibody against *L. pneumophila* Corby flagellin (reference 23 and data not shown). The *flaA* mutant strain KH3 was shown to be devoid of the 48-kDa FlaA protein, while wild-type strain Corby and the complemented *flaA* mutant CD10 clearly expressed the protein. The formation of intact flagella correlated with the flagellin expression, as demonstrated by electron microscopy (Fig. 2). Light microscopy was used to monitor the motility of the wild-type strain and the complemented mutant. Consistent with the results obtained by Western blotting and electron microscopy, this motile phenotype was absent in mutant KH3.

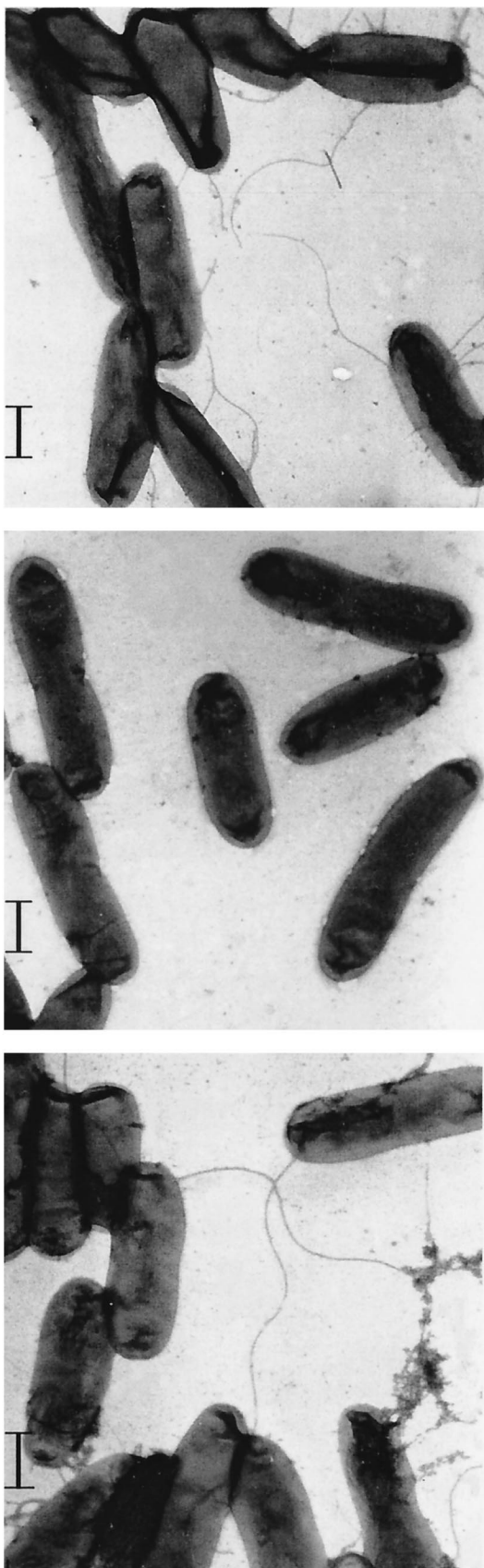
Adherence to host cells. To investigate the effects of the disruption of the *flaA* gene on the life cycle of *Legionella*, we

evaluated the ability of the *L. pneumophila* wild-type strain, the mutant strain KH3, and the complemented strain CD10 to adhere to host cells. In the presence of phagocytosis inhibitors, the bacteria were centrifuged onto *A. castellanii* and differentiated HL-60 cells, and the number of adherent bacteria was determined after 20 min of coinoculation. No significant differences in adherence among the three strains and their host cells were observed (data not shown).

Early stages of invasion. Gentamicin infection assays revealed a clear difference in the number of intracellular bacteria at the onset of multiplication (Fig. 3). After 30 min of coinoculation with *A. castellanii*, 10-fold fewer bacteria of strain KH3 than of the wild-type strain were found inside the host cells. In HL-60 cells, the rate of invasion of strain KH3 was 150-fold lower, while the complemented strain CD10 exhibited the wild-type phenotype in both host cell systems. After 120 min of coinoculation, the flagellated and nonflagellated bacteria still showed a 5-fold difference for amoebae and a 130-fold difference for HL-60 cells.

Intracellular multiplication in amoebae and macrophage-like HL-60 cells. To determine whether the disruption of the *flaA* gene influences the intracellular multiplication of the bacteria in host cells, *A. castellanii* and HL-60 cells were infected with the same set of flagellated and nonflagellated strains. The results of the intracellular growth assays are shown in Fig. 4. In *A. castellanii*, no difference between flagellated and nonflagellated bacteria was observed. All strains showed a 50,000 to 70,000-fold increase in numbers by 4 days postinfection (Fig. 4A). However, a significant difference in infection by flagellated bacteria and the nonflagellated mutant KH3 was observed in HL-60 cells 24 to 96 h postinfection (Fig. 4B). While the wild-type strain Corby had multiplied 600-fold by 4 days postinfection, the *flaA* mutant had only multiplied 14-fold. The defect of growth of mutant KH3 was completely restored in the complemented strain CD10. To exclude variable growth kinetics of the wild-type and mutant strains, growth curves for the growth of both strains in YEB were established. No difference in growth could be observed. To distinguish between invasion and intracellular growth, an intracellular growth assay (MOI, 10) with gentamicin treatment (2 h postinfection) to kill extracellular bacteria was performed. With this method, the intracellular growth rate of the wild-type strain can be calculated with respect to that of the mutant strain. The number of intracellular bacteria was determined directly after the gentamicin treatment and again after 24 h. The two strains showed

FIG. 2. Electron micrographs of the flagellated wild-type strain (A), the nonflagellated mutant KH3 (B), and the complemented mutant strain CD10 (C). Bacteria were grown to stationary phase, suspended in H₂O, applied to Formvar-coated copper grids, shadowed with 2% uranyl acetate, and examined with a Zeiss EM10 transmission electron microscope. Bars, 0.5 μm.



A.

B.

C.

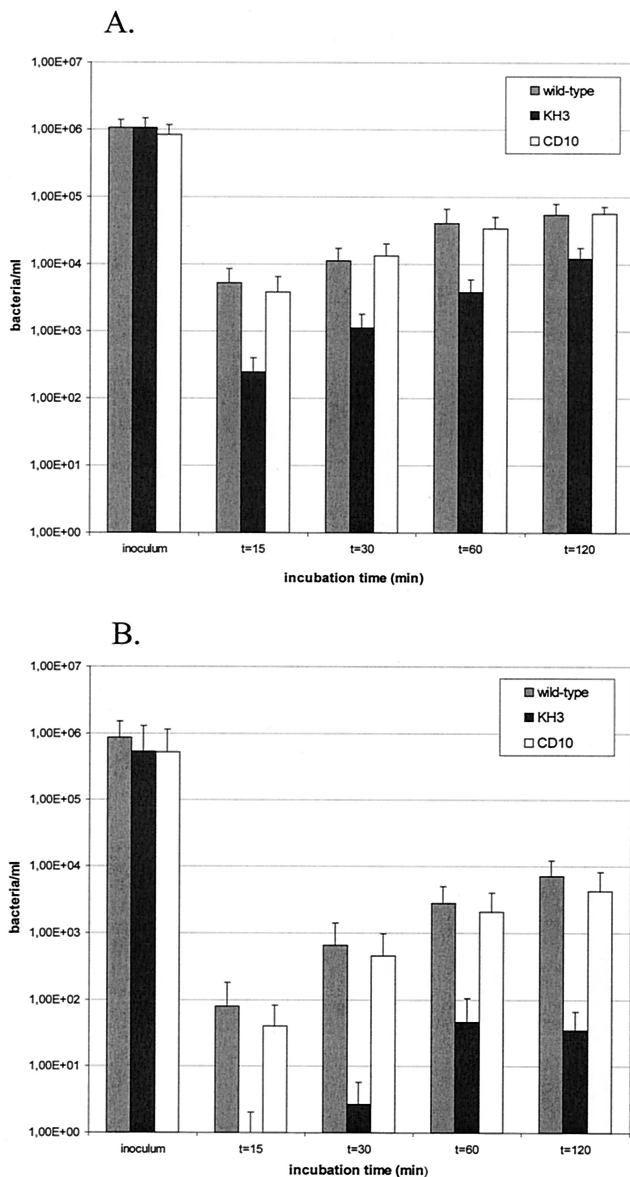


FIG. 3. Invasion efficiency of *L. pneumophila* Corby wild type, the *flaA* mutant KH3, and the complemented mutant CD10 into *A. castellanii* cells (A) and HL-60 cells (B). Adherent host cells were incubated with legionellae at an MOI of 10 for different time intervals and treated with gentamicin (80 μg/ml) for 1 h, and the CFU were determined by plating on ABCYE agar plates. Error bars indicate the standard deviations obtained from three independent experiments.

comparable intracellular growth rates. The wild-type and CD10 strains replicated 75- to 125-fold, and the KH3 strain replicated 150-fold. To determine the number of infected host cells and the number of bacteria per host cell, in situ hybridization was performed at the later stages of infection (72 and 96 h). These experiments showed that the percentage of infected HL-60 cells was higher for the wild-type strain than for the *flaA* mutant. Taken together, these results demonstrate that the expression of the FlaA protein is especially relevant for the invasion efficiency of macrophage-like HL-60 cells but not for intracellular growth.

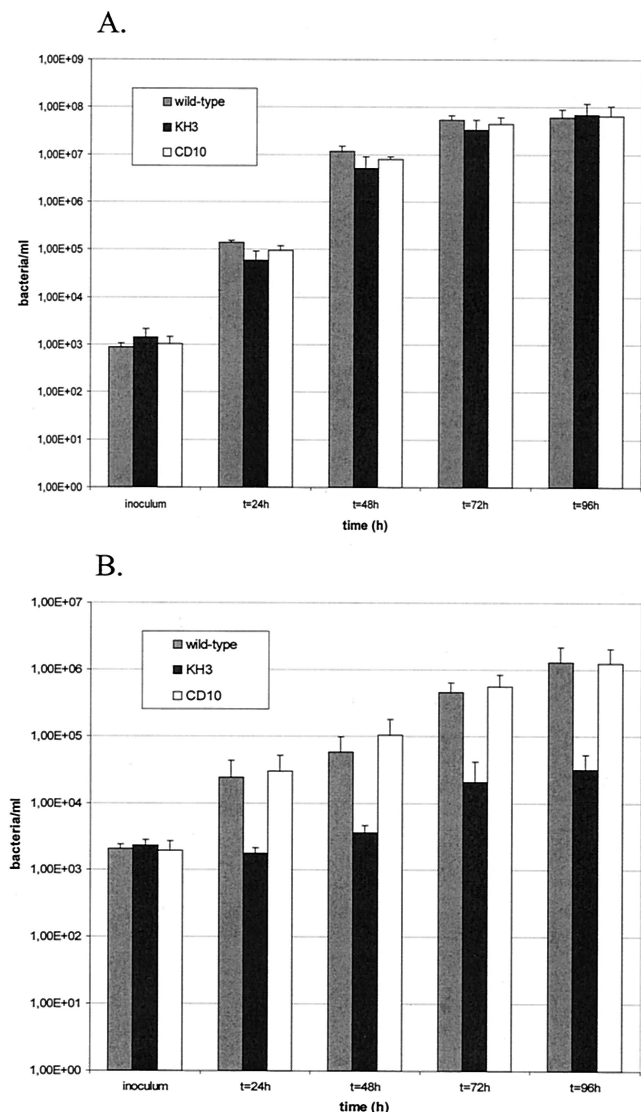


FIG. 4. Multiplication of *L. pneumophila* Corby wild type, the *flaA* mutant KH3, and the complemented mutant CD10 in *A. castellanii* and HL-60 cells. (A) *A. castellanii* cells (2×10^5 /ml) were infected with 2×10^3 bacteria, and the CFU per well were determined daily in duplicate by plating on ABCYE plates. (B) Differentiated HL-60 cells (2×10^5 cells/ml) were infected and treated as described for *A. castellanii*. Error bars indicate the standard deviations obtained from three independent experiments.

DISCUSSION

Surface structures, such as capsules, lipopolysaccharides, pili, and flagella, play an important role in bacterial pathogenicity and in the ability of bacteria to survive in the environment. Motility-defective bacteria of some species have been reported to be affected in virulence because of aberrations in host cell adherence, invasion mechanisms, or other unknown factors (10, 14, 35, 36). Previous studies utilizing insertional mutagenesis have linked the expression of flagella by *L. pneumophila* and the ability of this pathogen to infect *A. castellanii*, *H. vermiformis*, and human U937 cells (6, 38). Moreover, flagellated legionellae have been found in lung alveolar spaces of

legionellosis patients (9). In contrast, other reports do not support a direct link between flagella and virulence in *Legionella*. The insertion mutation in the *flaI* open reading frame, an essential component of flagellum assembly, had no effect on intracellular growth in cultured cells (32). Also, the finding that flagellar expression is not required for the intraperitoneal infection of guinea pigs argues against the involvement of flagella in virulence (12). Due to this conflicting results, other authors have suggested that *Legionella* virulence factors may be co-regulated with flagellar expression (6).

To elucidate the exact role of the flagellum of *L. pneumophila* during the infection process of amoebae and human macrophages, we constructed a specific flagellum-negative mutant of *L. pneumophila* Corby. Construction of this flagellar mutant, KH3, was accomplished by using a kanamycin gene cassette for targeted disruption of *flaA*. The allelic exchange completely abolished the assembly of the flagellar filament and, accordingly, revealed that only one copy of *flaA* exists in the *L. pneumophila* genome. The redelivery of the intact *flaA* gene into the chromosome completely restored the flagellated motile wild-type phenotype.

Earlier reports have shown that pili improve the adherence of *L. pneumophila* to mammalian and protozoan cells (44). By using the nonflagellated mutant KH3, we found that the *flaA* mutation does not additionally affect the adhesion of *Legionella* organisms to their respective host cells but clearly reduces the capacity to invade macrophage-like HL-60 cells and *A. castellanii*. After 30 min, the motile wild type shows a 150-fold-higher invasion rate in HL-60 cells and a 10-fold higher invasion rate in *A. castellanii* cells than the nonflagellated mutant does. Therefore, we conclude that the flagella of *Legionella* facilitate the initial encounter of the host cell and somehow enhance the process of uptake whereas intracellular replication is not affected. Similar effects have also been shown for other organisms. For example, the flagella of *Agrobacterium tumefaciens* are required for efficient encounter of the root surface and possibly aid in orientating the bacterial cells at various sites for infection (10). The construction of a nonmotile, nonflagellated *Campylobacter jejuni* mutant resulted in a decrease of internalization by a factor of 30 to 40 compared to the parent strain, while attachment appeared to be unaffected (18). *Pseudomonas aeruginosa* nonflagellated mutants exhibited a lower rate of uptake by murine macrophages, whereas the attachment via flagella did not play a major role (14). Similar results were also obtained for *Proteus mirabilis* (33). Adherence studies with concanavalin-pretreated *A. castellanii* demonstrated that the flagella of *Pseudomonas fluorescens* and *Proteus mirabilis* interact with carbohydrates on the host cell surface (37). Moreover, in *P. aeruginosa* the presence of the bacterial flagellum is required for nonopsonic phagocytosis by macrophages (31). Accordingly, similar opsonin-independent adherence and uptake mechanisms have been described for *L. pneumophila* (39). However, further studies are needed to determine whether the flagellum of *Legionella* is involved in this specific mode of uptake.

The negative effect of the *flaA* mutation decreases during the course of infection in *A. castellanii* but not in HL-60 cells. This indicates that the severely reduced invasion efficiency results in smaller intracellular numbers of bacteria in HL-60 cells even at later stages of infection. In amoebae, this effect of

the *flaA* mutant is not observed due to a higher invasion efficiency compared to that in HL-60 cells. This may also be the explanation for the observed replication of the nonflagellated *flil* mutant in U937 cells (32). Differences between human macrophages and amoebae have also been observed on infection with mutants with mutations in the macrophage-specific infectivity loci (*mil*) of *Legionella* (20).

Earlier reports that flagella are not required for the intracellular growth of legionellae correlate with our results and the finding that flagellin is not expressed during the replicative phase of infection (8, 24, 32). Our current view holds that the expression of the flagellum is especially relevant for the initial encounter of the host. This may be important in the environment, where the availability of host cells may be limited. Additionally, our results show that flagellation improves the invasion capacity of *Legionella* organisms into their respective host cells. The observation that intracellular legionellae are highly motile after the multiplicative phase of the bacteria raises the question whether flagellation also contributes to the lysis of the host cell. Further studies with our nonflagellated mutant KH3 and with motility-defective flagellated strains will help to elucidate whether chemotaxis, host cell lysis, or anchorage to biofilm components in the environment are responsible for the widespread dissemination of *L. pneumophila*.

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