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Influence of the Alternative σ^{28} Factor on Virulence and Flagellum Expression of *Legionella pneumophila*

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The *fliA* gene of *Legionella pneumophila* encoding the alternative σ^{28} factor was inactivated by introducing a kanamycin resistance cassette. Electron microscopy and Western blot analysis revealed that the *fliA* mutant strain is aflagellate and expresses no flagellin. Reporter gene assays indicated that the *flaA* promoter is not active in the *fliA* mutant strain. The *fliA* mutant strain multiplied less effectively in coculture with amoebae than the wild-type strain and was not able to replicate in coculture with *Dictyostelium discoideum*.

Legionella pneumophila, a pathogen of humans that causes a severe pneumonia termed Legionnaires' disease, is a ubiquitous microorganism that inhabits freshwater biotopes and man-made water systems. In the environment, this bacterium replicates intracellularly in amoebae and other protozoan host cells (5, 26, 28). *Legionella* infection occurs after inhalation of aerosolized bacteria. *Legionella* invades and proliferates in alveolar macrophages of the human lung (25, 26).

In order to evaluate the role of the flagellum in the pathogenesis and ecology of *Legionella*, we recently mutagenized the *flaA* gene of *L. pneumophila* Corby. We demonstrated by coculture analysis that the flagellum of *L. pneumophila* positively affects establishment of infection by enhancing the capacity to invade (4). In contrast, the intracellular rate of replication seems to be unaffected (4, 17).

The complex flagellum expression and assembly system seems to be coordinately regulated with other virulence-associated traits (2, 3, 6, 24, 26). Therefore, it has been proposed that the flagellum might be a virulence-associated factor in the *L. pneumophila* infection process. Flagellin is the major subunit of the flagella of *L. pneumophila*, and it has been shown previously that various *L. pneumophila* strains and isolates of members of the family *Legionellaceae* other than *L. pneumophila* are flagellated (11, 23). Furthermore, we demonstrated that expression of the *flaA* gene seems to be regulated at the transcriptional level by the alternative σ^{28} factor FliA (11, 12) and probably by a regulator of the LysR family (14). The *L. pneumophila fliA* gene was able to restore flagellation and motility of an *Escherichia coli fliA* mutant, suggesting that the FliA protein of *L. pneumophila* can bind to the *E. coli* core RNA polymerase and direct transcription initiation from flagellum-specific promoters (12). Furthermore, we demonstrated that *flaA* expression is regulated by temperature and is influenced by the growth phase, by amino acids, and by the viscosity and the osmolarity of the medium (13).

Genes belonging to the σ^{28} family (designated *sigD*, *fliA*, and *rpoF*) are required for expression of motility and chemotaxis genes in several organisms (1, 10, 19, 22, 27). These genes are expressed in a complex transcriptional cascade (8, 9). In this study we generated and characterized an *fliA* mutant strain of

TABLE 1. Plasmids and oligodeoxyribonucleotides

Plasmid or oligonucleotide	Characteristics or sequence	Reference
Plasmids		
pKH23	pMMB207 3,560-bp *Hin*dIII-*Kpn*I fragment containing the p *flaA-luxAB* fusion	12
pKH12	pMMB207 containing the p *flaA-lacZ* fusion from pKH20	13
pKH27	pUC18 1,028-bp PCR product containing *fliA* and part of *flhF* (*ylxH*)	12
pCD33	pBC KS containing the complete *fliA* gene disrupted by a Km^r cassette	This study
pKHfli10	2,400-bp *Sal*I-*Sph*I fragment of pCD33 containing the disrupted *fliA* gene cloned in pBOC20	This study
pfli12	pBC KS containing the complete *fliA* gene from pKH27	This study
Oligonucleotides		
ylxSalI	5′-AGCCATAGCGTTATGTCGACT-3′	This study
fli3SpeI	5′-CACTAGTGATTGATAGTCTCCCTC-3′	This study
fli5SpeI	5′-CAAGTCAACTAGTTGTACACTC-3′	This study
motSphI	5′-CAGGCATGCAACGACCATTTCATC-3′	This study
fliU5	5′-GGATGTGCAGTTAGATTAC-3′	12
fliR5	5′-TTTATTCCGGTAATCTTGATC-3′	12
Ka5-EcoR5	5′-CGTGATATCATCCTTCAACTC-3′	This study
Ka3-Spe1	5′-GTCTGACTAGTCGGGAAGATG-3′	This study

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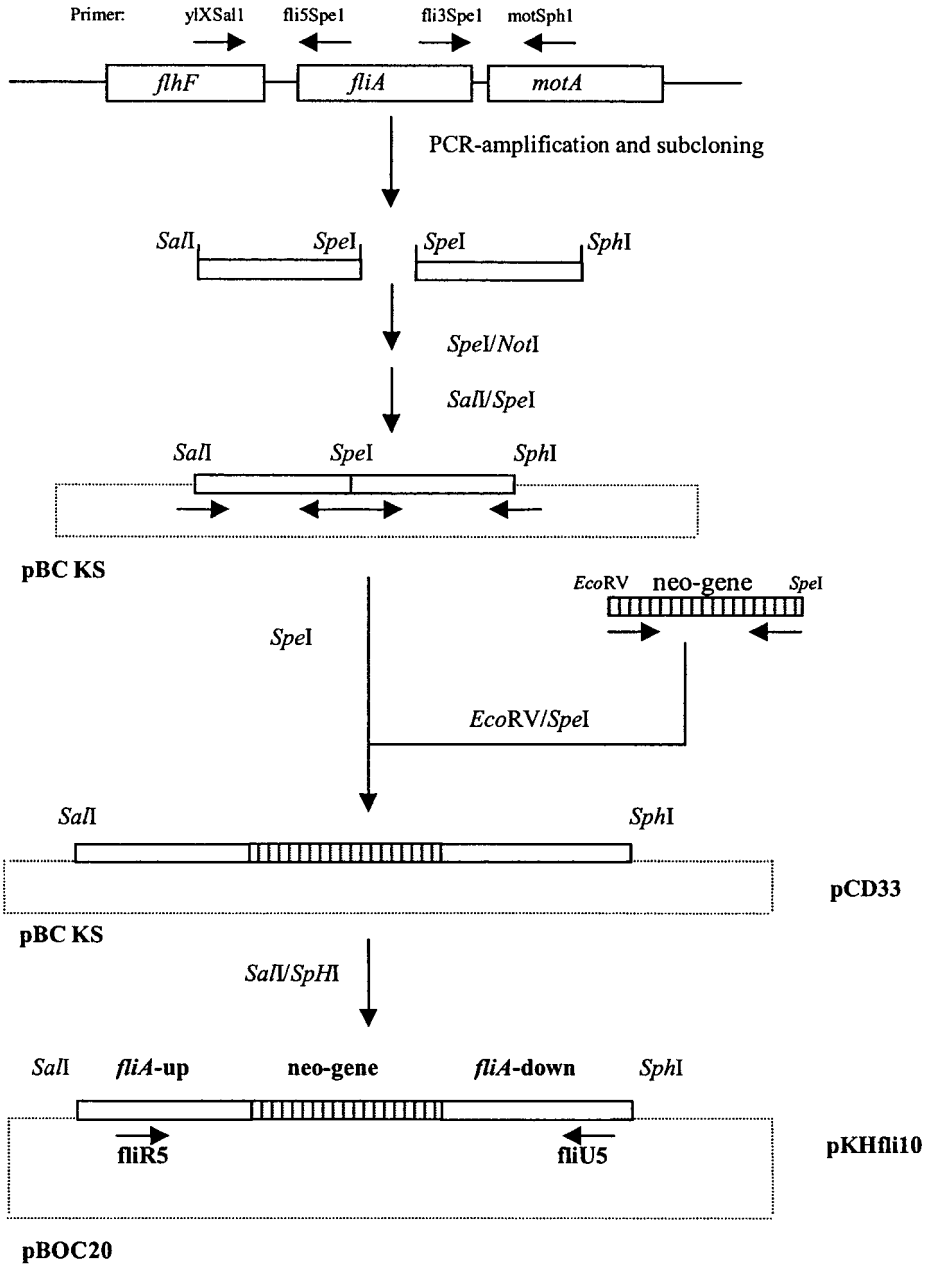

FIG. 1. Cloning scheme used to inactivate the *fliA* gene of *L. pneumophila* Corby. Plasmid designations are indicated on the right, and the vectors used are indicated on the left. The primers used for PCR are indicated by arrows. The restriction endonuclease sites used for cloning are also indicated. neo-gene, kanamycin resistance cassette.

L. pneumophila in order to analyze the effect of the mutation on flagellum expression.

Bacterial strains, plasmids, and oligonucleotides. *L. pneumophila* Corby (serogroup 1) (15), *flaA* mutant strain KH3, and complemented *flaA* mutant strain CD10 (4) were used in this study. *E. coli* DH5α was used for propagation of recombinant plasmid DNA. The following vectors were used: pUC18 (Pharmacia LKB, Freiburg, Germany), pBC KS (Stratagene, Heidelberg, Germany), plasmid pMMB207 (20), and plasmid pBOC20 (21). All of the plasmids and oligonucleotides used in this study are listed in Table 1.

Construction of an *L. pneumophila fliA* mutant and a complemented *fliA* mutant strain. The *fliA* gene was amplified by PCR, and a kanamycin resistance cassette (*neo*) was cloned into the *Spe*I site, which was introduced by PCR (Fig. 1). This construct was then cloned into vector pBOC20, resulting in pKHfli10. This plasmid was used to inactivate the *fliA* locus of *L. pneumophila* Corby. We obtained eight putative mutants that grew on ABCYE plates containing kanamycin and sucrose, suggesting that the allelic exchange was due to a double crossover. FliA mutants were screened by PCR performed with primers binding to the 5′ (fliU5) and 3′ (fliR5) regions of the *fliA* gene (Table 1). Amplification products of the predicted length (1,000 bp) were observed for the wild type, whereas

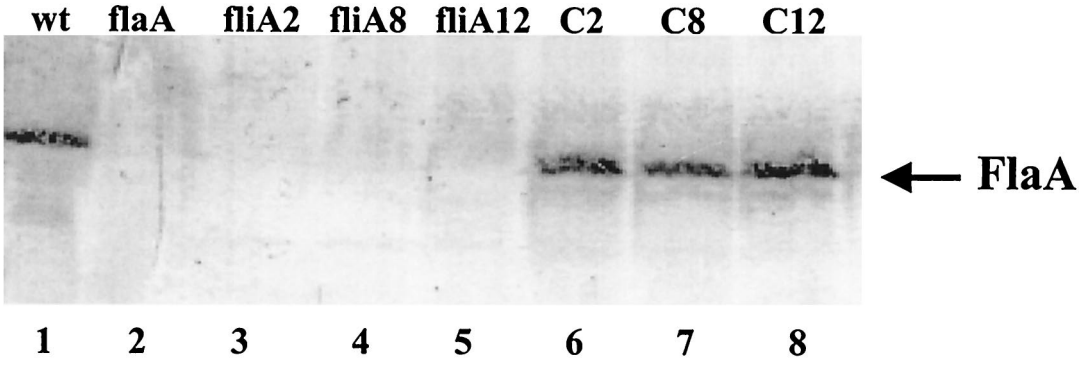

FIG. 2. Western blot analysis with the anti-flagellin antibody. Equal amounts of whole-cell extracts were loaded onto the polyacrylamide gel. The position of the FlaA protein (48 kDa) is indicated on the right. Lane 1, *L. pneumophila* Corby (wild type [wt]); lane 2, flagellin mutant strain KH3 (flaA) of *L. pneumophila* Corby; lanes 3 to 5, σ^{28} factor mutant strains fliA2, fliA8, and fliA12 of *L. pneumophila* Corby; lanes 6 to 8, *fliA* mutant strains harboring plasmid pfli12 (complemented clones 2 [C2], 3 [C3], and 12 [C12]).

2,400-bp amplification products (the predicted length) were obtained for the mutant strains, indicating that integration of the 1,400-bp *neo* gene occurred (data not shown). The recombination event was confirmed by Southern blot analysis using an *fliA*-specific probe and a *neo* gene-specific DNA probe (data not shown). Strain KHfli12 was used for complementation and further characterization. Complementation was done by electroporating (2.3 kV, 100 Ω, 25 μF; Bio-Rad Gene Pulser) plasmid pfli12 (Table 1), which contained the complete *fliA* gene of *L. pneumophila* Corby cloned into vector pBC KS, into *fliA* mutant strain KHfli12. Clones growing on ABCYE agar plates supplemented with chloramphenicol were used in the following experiments.

FlaA expression of the *fliA* mutant strain. Using an *fliA* mutant strain of *E. coli* (YK4104), it was recently shown that, in the recombinant system, expression of the *fliA* gene depends on the presence of an intact FliA protein (12). To determine whether the *fliA* mutant strain of *L. pneumophila* Corby is able to express the flagellin gene, total cell extracts of *L. pneumophila* strains in the early stationary phase were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting by using a polyclonal monospecific antibody against *L. pneumophila* Corby flagellin. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (16). Three hundred microliters of a *Legionella* cell suspension (optical density at 600 nm, 1) was pelleted by centrifugation, and then the cells were suspended in 100 μl of SDS sample lysis buffer and equal amounts of protein were loaded onto an SDS–13% polyacrylamide gel. Western blot analysis revealed that the *fliA* mutant strain did not express the FliA protein (Fig. 2, lanes 3 to 5), whereas the complemented strain produced a flagellin band (Fig. 2, lanes 6 to 8) comparable to that of the wild-type strain (Fig. 2, lane 1). For electron microscopy, single drops of the *Legionella* suspension were directly applied to Formvar-coated copper grids. After sedimentation of the bacteria and removal of the remaining fluid, the samples were shadowed with platinum-palladium and examined with a Zeiss 10A transmission electron microscope. The *fliA* mutant strain had no flagella, whereas the complemented strain was flagellated (data not shown).

Using plasmid pKH12 (*flaA* promoter fused to a promoterless *lacZ* gene; cloning method described by Heuner et al. [13]), we showed that in contrast to the *L. pneumophila* wild-type strain, the *fliA* mutant strain exhibited no reporter gene activity (Table 2). Previously, we showed that the σ^{28} consensus sequence acts as the promoter for *flaA* expression in *L. pneumophila* (11). These results, together with the results of the present study, demonstrate that expression of the flagellin gene in *L. pneumophila* is directly regulated by the alternative σ^{28} factor FliA. The *fliA* gene product is also used in transcription of flagellar and chemotaxis genes in various other species (9).

Intracellular multiplication in *Acanthamoeba castellanii* and *Dictyostelium discoideum*. In axenic culture the *fliA* mutant strain grew as well as the wild-type strain (data not shown). To determine whether disruption of the *fliA* gene influences intracellular multiplication of the bacteria in host cells, *A. castellanii* and *D. discoideum* were infected with the *fliA* mutant strain or the complemented strain, as described recently (4, 7). The results of the coculture experiments are shown in Fig. 3. The *fliA* mutant strain multiplied less effectively in coculture with *A. castellanii* than the wild-type strain (Fig. 3A) and was not able to replicate in *D. discoideum* (Fig. 3B). The defects were fully complemented by introduction of the wild-type *fliA* gene back into the *fliA* mutant strain. Previously, it was demonstrated that the *flaA* mutant strain had only a moderate effect in coculture with *A. castellanii* (4). Compared with the *fliA* mutant strain, the *flaA* mutant strain had only a minor

TABLE 2. Characteristics of *fliA* mutant, complemented mutant, and wild-type strains of *L. pneumophila* Corby

Strain	Reaction with anti-FlaA antibodies[a]	Flagellation[b]	*flaA* promoter activity (β-galactosidase activity)[c]
L. pneumophila Corby	+	+	434.3 ± 8.1
KHfliA12[d]	−	−	11.7 ± 1.8
KHfliA12/ pfli12[e]	+	+	ND[f]

[a] Determined by Western blot analysis of whole-cell extracts of the bacteria.
[b] Determined by electron microscopy.
[c] Values are expressed in Miller units, measured as described by Miller (18).
[d] *fliA* mutant strain.
[e] Complemented strain.
[f] ND, not determined.

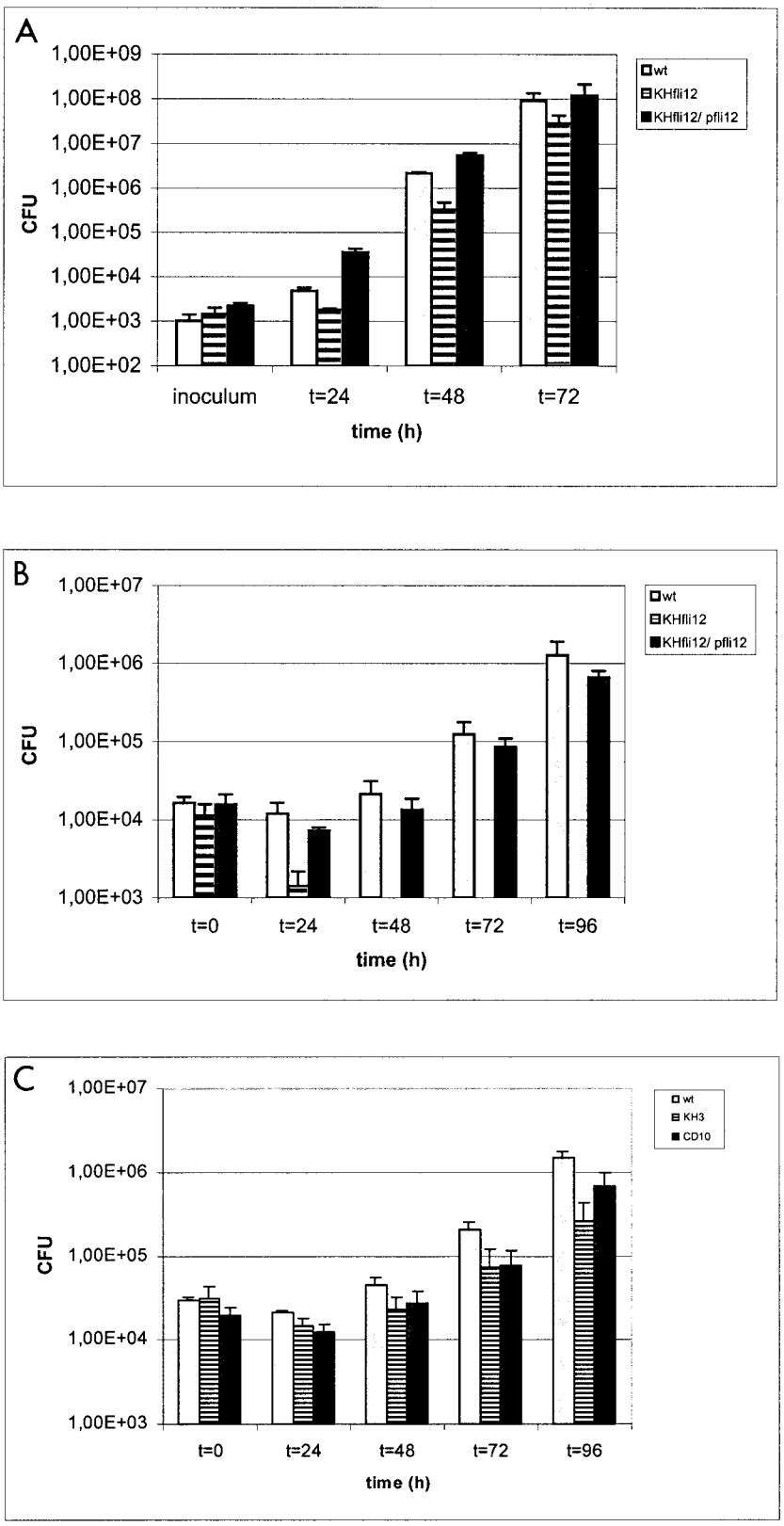

FIG. 3. Analyses of *L. pneumophila* Corby (wt), *fliA* mutant strain fliA12, a complemented *fliA* mutant strain harboring plasmid pfli12 (KHfli12/pfli12), *flaA* mutant strain KH3, and complemented *flaA* mutant strain CD10 in cocultures with *A. castellanii* (A) or *D. discoideum* (B and C). *A. castellanii* cultures (2×10^5 cells/ml) and *D. discoideum* cultures (5×10^5 cells/ml) were infected with 1×10^3 and 1×10^4 bacteria, respectively. The number of CFU per well was determined in duplicate by plating on ABCYE plates. The error bars indicate the standard deviations based on at least three independent experiments.

effect on replication in *D. discoideum* cultures (Fig. 3C). These results suggest that in addition to the *flaA* gene, the FliA protein regulates other putative virulence factors of *L. pneumophila*.

Genes involved in flagellum expression are tightly regulated and are organized in a complex hierarchy. In *E. coli* and *Salmonella enterica* serovar Typhimurium, there are three levels. The first level of the cascade includes the *flhDC* operon coding for master regulators, which control class II genes. The class II gene product FliA is required for transcription of class III genes. The last level of the hierarchy includes flagellin genes, as well as genes involved in motility, such as *motA* and chemotaxis genes (for reviews, see references 8 and 9). To our knowledge, the genomic sequence of *L. pneumophila* contains no *flhDC* homologues. Experiments are under way to further analyze this cascade in *L. pneumophila* in order to identify the proposed master regulator of flagellum expression and to identify additional putative virulence genes regulated by FliA to obtain more information about the link between motility and expression of the virulent phenotype.

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