INFECTION AND IMMUNITY, Dec. 1995, p. 4928–4932 Vol. 63, No. 12
0019-9567/95/$04.00+0
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Association of Flagellum Expression and Intracellular Growth of *Legionella pneumophila*

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Received 12 May 1995/Returned for modification 4 August 1995/Accepted 14 September 1995

We examined the role of the flagella of *Legionella pneumophila* in the infection of amoebae and human monocyte-like cells. Insertional mutants were constructed with mini-Tn*10*. Ten mutants (F^-) which did not react with polyclonal *L. pneumophila* antiflagellar antisera were identified. Ten randomly selected mutants (F^+) that did react with the polyclonal antiflagellar antiserum were also identified. The infectivity of these 20 mutants in *Hartmannella vermiformis* and human U937 cells was characterized. Seven of the 10 F^- mutants were attenuated in their ability to multiply in the amoebae during the first 3 days of coincubation and failed to multiply in U937 cells. Three of the 10 F^- mutants multiplied as well as the wild-type parent strain did in amoebae and to a limited degree in U937 cells. None of the 10 F^+ mutants were attenuated in either the amoebae or U937 cells. While the flagellar structure is not essential for virulence, the ability of *L. pneumophila* to infect amoebae and human phagocytic cells appears to be linked to flagellar expression. We believe that the attenuated F^- mutants contain insertions in genes critical to both flagellum expression and the infection process.

Legionella pneumophila, the etiologic agent of Legionnaires' disease, is transmitted to humans from aquatic environmental sources. In these environments, the bacteria survive as intracellular parasites of freshwater protozoa (3, 13, 18, 30). In humans, *L. pneumophila* enters and multiplies within phagocytic cells, and this ability to multiply intracellularly is considered a primary virulence factor (19, 27). The infection of human cells has been shown to be related to the bacterium's ability to infect protozoa (14). Attenuated strains of *L. pneumophila* are unable to grow in human macrophages or protozoa and are avirulent in the guinea pig model (8, 17, 25).

This study is part of an ongoing investigation of the process by which *L. pneumophila* infects the amoeba *Hartmannella vermiformis* (1, 17). This species of amoeba is predominate in U.S. potable-water systems (35, 36) and has been associated with a strain of *L. pneumophila* responsible for an outbreak of nosocomial legionellosis (6, 15, 16). Although there are striking ultrastructural similarities between protozoa and human phagocytic cells infected with *L. pneumophila*, we have identified two differences in the early stages of infection of these host cells. First, the uptake by amoebae is accomplished by a microfilament-independent mechanism and is sensitive to methylamine, an inhibitor of receptor-mediated endocytosis (21). The uptake of *L. pneumophila* by macrophages is mediated by microfilament-dependent phagocytosis and has been associated with binding to the CR1 and CR3 complement receptors (26). Second, host cell protein synthesis is required for *L. pneumophila* to infect amoebae but not human macrophages (1). Exposure of amoebae to *L. pneumophila* induces the synthesis of a number of amoebal proteins. These proteins are not induced by the presence of avirulent *L. pneumophila* or other bacteria. The mechanism of initial attachment and the role of the induced proteins are unknown.

This study was initiated to determine if the expression of flagella by *L. pneumophila* plays a role in the early events associated with host cell invasion. Flagellation has been shown to enhance pathogenicity and the colonizing ability of *Pseudomonas* spp. and *Vibrio cholerae* (2, 9, 37). The flagella from *L. pneumophila* have been characterized as single polar or subpolar flagella with a 47-kDa filament subunit (7, 10, 29). Previous studies have shown that *L. pneumophila* strains that are negative for flagella by electron microscopy are virulent in guinea pigs (11). Motility has been associated with the growth phase of *L. pneumophila* (30). These reports have described two phases of growth and motility for legionellae. "Multiplicative-phase" bacteria are actively multiplying bacteria, such as are found in the early stages of host cell infection and agar-grown bacteria. These bacteria are nonmotile and possess "rumpled" cell walls. Multiplicative-phase cells obtained from agar media are frequently long and filamentous. Bacteria in the second phase, or "active infective phase," are highly motile short rods associated with the later stages of infection and lysis of the host cell. These bacterial cells possess smooth walls and may contain β-hydroxybutyrate inclusions. Bacterial motility would allow the organisms to find a new host quickly since the bacteria lose motility approximately 24 h after release from host amoebae (30). Our results provide evidence that the expression of the flagellum in *L. pneumophila* cells is related to the bacterium's ability to infect and multiply in both amoebae and human monocyte-like (U937) cells.

Bacterial strains and culture media. *L. pneumophila* serogroup 1 strain RI-243 is a virulent strain that was initially isolated from a cooling tower implicated in an outbreak of legionellosis (21). The strain had been passaged less than four times on buffered charcoal-yeast extract (BCYE) agar (12). Strain RI-243A is an avirulent, nonflagellated variant of strain RI-243, isolated after passage on Mueller-Hinton agar plates as described previously (17). Both RI-243 and RI-243A were

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maintained on BCYE agar. The RI-243 mini-Tn*10* mutants were maintained on BCYE supplemented with 25 μg of kanamycin per ml (22, 28). All cultures were grown in 2.5% CO_2 at 35°C.

Purification of flagella. Flagella were purified from bacterial cells as previously described (5, 9). Confluent growth of *L. pneumophila* was harvested from five BCYE agar plates by flooding of each plate with 5 ml of sterile distilled H_2O and incubation at ambient temperature for 30 min. The plates were then swirled to dislodge the bacteria from the agar. The plates were washed twice; the washes were pooled with the original suspension. The bacterial suspensions were sheared by passage through a 27-gauge needle. The sheared suspension was centrifuged at 8,000 rpm in an SS-34 rotor (DuPont, Wilmington, Del.), and the supernatant was harvested. The supernatant was centrifuged at 42,000 rpm for 25 min in an OTD75B ultracentrifuge (DuPont). The pellet was resuspended in 0.5 ml of distilled H_2O, and the protein concentration was measured with Coomassie Plus protein concentration reagent (Pierce Co., Rockford, Ill.) by following the manufacturer's recommendations. The flagellum suspensions were stored at −20°C.

An aliquot of the flagellar preparation from RI-243 was further purified by gel filtration chromatography with a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column equilibrated with phosphate-buffered saline (pH 7.2). The sample was eluted with the same buffer. Fractions (1 ml) were collected. Two peaks were detected and concentrated with an Amicon stirred cell containing a YM10 membrane (Amicon Corp., Beverly, Mass.). The protein concentrations of the peaks were measured as previously described. The concentrated peaks were electrophoresed through a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel by using the Laemmli buffer system (23) and analyzed by Western blotting (immunoblotting).

Production of antiserum to flagellar proteins. Polyclonal antiserum to *L. pneumophila* RI-243 flagellum was produced by immunizing a rabbit with a 100-μl intramuscular injection in each hindquarter. The purified flagella (200 μg) were mixed with an equal volume of TiterMax adjuvant (Vaxcel, Inc., Norcross, Ga.). The rabbit was bled after 3 and 6 weeks. The antiserum was absorbed with *L. pneumophila* RI-243A and centrifuged at 10,000 × *g* for 10 min to pellet the bacterial cells and any bound antibody. *L. pneumophila* RI-243A is an avirulent subclone of RI-243 and does not possess flagella. The resulting antiserum was shown to be specific for the flagellin by a rapid immunoblot procedure (32) and immunofluorescent-antibody staining of RI-243 and RI-243A cells.

Construction of nonflagellar mutants by using mini-Tn*10*. *L. pneumophila* RI-243 was rendered electroporation competent as follows. *L. pneumophila* organisms were scraped from 3-day-old BCYE agar plates. The bacteria were resuspended in 100 ml of sterile ice-cold 10% glycerol and pelleted by centrifugation at 7,000 rpm for 10 min in a GSA rotor (Dupont). The bacteria were again resuspended in 100 ml of sterile ice-cold 10% glycerol and pelleted as before. After the second wash, the pelleted bacteria were resuspended in 2.0 ml of sterile ice-cold 10% glycerol and then stored at −70°C.

Two micrograms of the purified plasmid pCDPO5, containing mini-Tn*10* (22, 28), was electroporated into *L. pneumophila* RI-243 by using cuvettes with a 0.1-cm electrode gap distance in a Gene Pulser (Bio-Rad, Hercules, Calif.) set at 2,000 V, 25 μF, and 200 Ω. Recombinants were selected by growth on BCYE agar containing 25 μg of kanamycin per ml. The recombinants were harvested and replated on BCYE agar containing 25 μg of kanamycin per ml and 5% sucrose to ensure genomic insertion of Tn*10* and loss of the plasmid (28).

Screening of recombinants. The mini-Tn*10* mutants were screened for failure to react with the absorbed polyclonal rabbit antiserum by a rapid dot blot procedure (32). All potential mutants were rescreened three times to confirm that the mutants did not react with the antiserum. Strains that failed to react with this antiserum in the dot blot procedure were designated F^-; those that did react were designated F^+.

Western blot analysis. Western blot analysis was performed on both whole-cell suspensions and purified flagellum extracts. For whole-cell suspensions, bacteria were resuspended in sterile distilled H_2O and adjusted to a final concentration of 10^8 cells per ml. An aliquot containing 10^6 cells was mixed with 2× SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, 0.001% bromophenol blue). For the purified flagellar extracts from both purification methods, flagellum preparations (as described above) were mixed with 2× SDS sample buffer to yield 20 or 200 ng of protein for F^+ or F^- strains, respectively. The samples were electrophoresed through an SDS–10% polyacrylamide gel by using the Laemmli buffer system (23). The proteins were transferred to a Magnagraph nylon membrane (MSI, Westboro, Mass.) by established protocols (34). The flagellin subunit was detected by probing with a 1:1,000 dilution of absorbed rabbit polyclonal antiserum to the flagella with the sequential addition of a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) and 3,3′-diaminobenzidine and hydrogen peroxide.

DNA manipulation. Genomic DNA was isolated from *L. pneumophila* RI-243 and the mutant strains by resuspending the bacteria in 500 μl of TE buffer (10 mM Tris-HCl–1.0 mM EDTA [pH 8.0]). Lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 100 μg/ml, and the bacterial suspension was incubated at 37°C for 30 min. The suspension was adjusted to 0.5% SDS and 500 μg of proteinase K (Sigma) per ml and incubated at 37°C for 2 h. Genomic DNA was recovered after phenol-chloroform extraction and ethanol precipitation. Plasmid DNA was isolated by alkaline lysis (4).

Digestion of DNA with *Eco*RI, *Bam*HI, and *Pst*I was performed according to the manufacturer's recommendations (Gibco-BRL, Gaithersburg, Md.). Plasmid DNA for electroporation was purified by electrophoresis in low-melting-point agarose, followed by digestion of the agarose with β-agarase according to the manufacturer's recommendations (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The 1.8-kb *Bam*HI fragment from pCDP05 containing the kanamycin resistance gene was isolated by electrophoresis in low-melting-point agarose. Nick translation of the probe with [α-^{32}P]dCTP was performed by using the nick translation system (Gibco-BRL) by following the manufacturer's recommendations.

Southern blot analysis. Approximately 10 μg of genomic DNA was digested to completion with the restriction enzymes *Pst*I and *Eco*RI. Following electrophoresis in a 1× Tris acetate-EDTA buffer–0.7% agarose gel, the restricted DNA was transferred by capillary blot to a Magnagraph nylon membrane (MSI) (31). The blot was hybridized with the nick-translated probe as previously described (31).

Amoeba culture and coincubation with *L. pneumophila*. *H. vermiformis* CDC19 (ATCC 50237) has been established as a model for studying the pathogenesis of *L. pneumophila* (15, 17). The amoebae were maintained in ATCC culture medium 1034, and coincubation with *Legionella* organisms was performed in assay medium as previously described (21). Triplicate aliquots from each flask were cultured on BCYE agar plates to determine the numbers of *L. pneumophila* CFU at 0,

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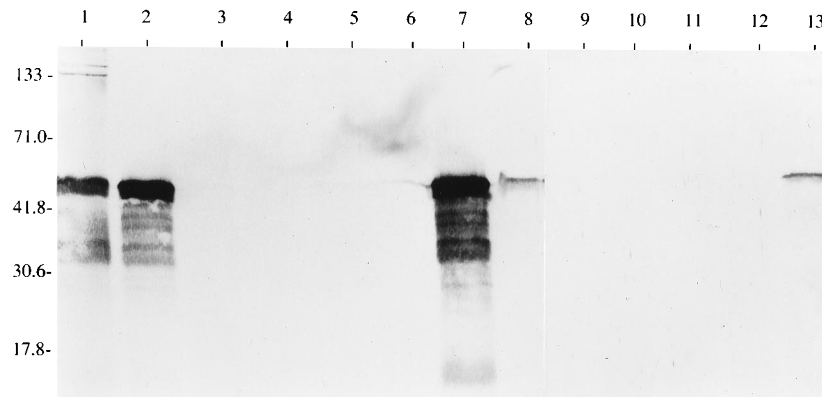

FIG. 1. Western blot analysis of purified flagellum preparations from F^+ and F^- *L. pneumophila* Tn*10* mutants by using polyclonal rabbit anti-*L. pneumophila* flagellum serum. For RI-243 and mutant 1 (F^+), 20 ng of purified protein was loaded per well. For all other mutants (F^-), 200 ng of purified protein was loaded per well. Lane 1, RI-243; lane 2, mutant 1; lane 3, RI-243A; lane 4, mutant 50; lane 5, mutant 123; lane 6, mutant 406; lane 7, mutant 78; lane 8, mutant 426; lane 9, mutant 507; lane 10, mutant 317; lane 11, mutant 198; lane 12, mutant 187; lane 13, mutant 182. Numbers on the left are molecular weight standards in thousands.

3, and 7 days after inoculation. Each coculture was performed in duplicate.

Tissue culture and infection with *L. pneumophila*. The human monocyte-like cell line U937 was maintained in RPMI 1640 (pH 8.0) with L-glutamine (Gibco-BRL) supplemented with 5% heat-inactivated fetal calf serum at 37°C in a 5% CO_2 humidified atmosphere (33). The *L. pneumophila*-monocyte cocultures were prepared as previously described (21). U937 cells were inoculated to a final density of 10^5 cells per ml into 25-cm^2 tissue culture flasks containing fresh RPMI 1640–heat-inactivated fetal calf serum. *L. pneumophila* was suspended in sterile distilled H_2O and inoculated to a final bacterial concentration of 10^3 CFU/ml. Triplicate aliquots from each flask were cultured on BCYE agar plates to determine the numbers of *L. pneumophila* CFU at 0, 3, and 7 days after inoculation. Each coculture was performed in duplicate.

The first 20 amino acids of the 47-kDa protein were determined with a protein sequencer (Porton Instruments, Tarzana, Calif.). This amino acid sequence was used to search protein databases and was shown to be homologous to flagellin subunits from *Escherichia coli* and *Salmonella typhimurium.*

The specificity of the antiflagellum antiserum was determined by immunofluorescent-antibody assay and rapid immunodot blotting with the flagellated strain RI-243 and its nonflagellated subclone, RI-243A. In the immunofluorescent-antibody assay, the antiserum reacted only with the flagella on RI-243 cells. Flagella clearly stained with the antiserum, and no reaction was observed with somatic antigens. The antiserum did not react with strain RI-243A. In the rapid dot blot, the antiserum reacted only with RI-243 cells or flagellar preparations from RI-243 but not with RI-243A cells or flagellar preparations from RI-243A. The specificity of the antiserum is also supported by the identical banding profiles seen in the Western blot analysis of flagellum preparations purified by both methods.

Approximately 550 mutants carrying the Tn*10* insert were screened. Ten F^- mutants were identified by the rapid blot screen. These 10 mutants were screened three times to ensure that they consistently failed to react with the antiserum. Ten F^+ mutants were chosen at random to serve as controls. All F^- mutants grew as well as the wild-type parent strain on microbiological medium. Flagella were purified from the F^- and F^+ Tn*10* mutants as well as the wild-type parent strain (RI-243) and its attenuated variant (RI-243A).

Western blot analysis of whole-cell suspensions showed that the antiflagellar immune serum reacted with a 47-kDa protein of the parent strain and a representative F^+ mutant but none of the 10 F^- mutants (data not shown). Western blot analysis of purified flagella showed that the antiflagellar immune serum reacted strongly with a 47-kDa protein and weakly with a collection of variously sized proteins from the RI-243 parent strain (Fig. 1) and all F^+ mutants (data not shown). The antiserum did not react with any proteins from extracts of the avirulent strain RI-243A and seven F^- mutants. The antiserum reacted weakly with a 47-kDa protein from two F^- mutants and strongly with a 47-kDa protein from one F^- mutant. Western blot analysis of flagella purified from RI-243 by both methods showed identical banding profiles.

Southern blot analysis indicated a single insertion in the bacterial genome of each mutant, as demonstrated by a single band detected upon hybridization with the 1.8-kb *Bam*HI probe from pCDP05 (data not shown). Restriction analysis with *Eco*RI and *Pst*I indicated that each F^- mutant had a unique insertion site for the transposon (data not shown).

All 10 F^+ mutant strains and the wild-type parent strain showed increases of $>10^3$ CFU after 3 days' coincubation with *H. vermiformis*. Seven of the 10 F^- mutants failed to increase in number of CFU by 1 log after 3 days' coincubation with *H. vermiformis* (Table 1). The growth of four of these F^- mutants was significantly less than that of the wild-type parent strain after 7 days' coincubation with the amoebae ($P < 0.01$, t test). Three F^- mutants multiplied as well as the wild-type parent strain. The avirulent strain (RI-243A) showed a decrease in the number of CFU over the 7-day period.

All F^- mutants showed decreases in the number of CFU after 3 days' coincubation with human U937 cells (Table 1). The three F^- mutants which multiplied as well as the parent strain in the amoebae showed increases of approximately ≤ 1.0 log in the number of CFU after 7 days' coincubation with the U937 cells. The wild-type parent strain, RI-243, and all F^+ mutants multiplied intracellularly in U937 cells.

All 10 mutants failed to react in a rapid colony blot screening procedure with antiserum against purified *L. pneumophila* flagella. Western blot analysis indicated that seven of the F^- mutants produced no detectable flagellin, two produced traces of a 47-kDa flagellin, and one (strain 78) produced flagellin similar to that of the wild-type parent strain (Fig. 1). Flagellar extracts of strain 78 repeatedly showed flagellin by Western

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TABLE 1. Characteristics of F^- mutants[a]

Strain	Flagellar protein[b]	Growth in[c]:			
		Amoebae		U937 cells	
		Day 3	Day 7	Day 3	Day 7
50	−	+	+	−	+
78	+	−	+	−	−
123	−	+	+	−	+
182	+	−	±	−	−
187	−	−	±	−	−
198	−	−	±	−	−
317	−	−	+	−	−
406	−	+	+	−	+
426	+	−	+	−	−
507	−	−	±	−	−

[a] F^- mutants are defined as strains containing the Tn*10* transposon which failed to react with antiflagellum serum in the rapid colony blot.

[b] Absence or presence of proteins that react with the antiflagellum serum in the Western blot analysis of extracted flagellar proteins.

[c] +, growth; −, no growth; ±, difference in CFU of >1.5 log from the growth in CFU of the F^+ strains and RI-243.

blotting, but no protein could be detected by colony blotting or Western blotting on whole-cell suspensions. We believe that this strain produced flagellin but either was unable to assemble or attach these proteins or produced them in a soluble form. This is supported by the fact that bacterial cells are washed in the colony blot (which was negative) but not in the Western blot procedure (which was positive).

Seven of the 10 F^- mutants were attenuated in the *H. vermiformis* amoeba model (Table 1). These same strains failed to multiply in human monocyte-like (U937) cells. Attenuated strains exhibited little or no growth after 3 days' coincubation with the amoebae. Four of these seven F^- strains showed significantly less multiplication than the parent strain after 7 days' coincubation ($P < 0.01$, t test). None of the 10 randomly selected F^+ mutants were attenuated or significantly different from the wild-type parent strain in the ability to infect amoebae or U937 cells. The attenuation of the F^- strains is not due to lack of motility (i.e., ability to contact a host cell), since all *L. pneumophila* organisms, F^+ or F^-, are nonmotile when harvested from BCYE agar. These findings suggest that flagellar expression is related to the ability of *L. pneumophila* to infect amoebae and U937 cells.

Three of the F^- mutants multiplied as efficiently as the wild-type parent strain. None of these three mutants produced any detectable flagellin. This suggests that while flagellar expression may be related to the ability to infect host cells, the flagellin itself is not critical to this process. This supports a previous study showing that nonflagellated strains, obtained by passage on certain media, retained their virulence for guinea pigs (11).

The sequence of events associated with the infection of amoebae by *L. pneumophila* has been previously described (13, 30). A striking feature of this infection process is that *L. pneumophila* becomes motile at a well-defined point while inside the host cell. By light microscopy, highly motile bacteria can be observed within the host cell and swimming away from ruptured host cells. Later, when most of the host cells have been lysed, large numbers of *L. pneumophila* organisms can be observed swarming throughout the tissue culture flask. Approximately 24 h later, no motile bacteria can be detected. Such events suggest a complex and precise growth cycle in which motility is closely associated with release from the host cell. The synthesis and assembly of bacterial flagella and associated motility systems are also quite complex. These systems are under elaborate regulation and are controlled by approximately 40 genes in *E. coli* and *S. typhimurium* (20, 24). These genes have been grouped into three transcriptional levels, each with its own regulatory system. We believe that the attenuated F^- mutants have insertions in the regulatory genes associated with the bacterial flagellum systems. Such a mutation could easily affect other genes critical to the infection process. Observations associating motility with the infection cycle would support this assumption about coordinate regulation.

In this study, we have shown an association between flagellar expression and the infection process. Further studies are needed to identify the mutated genes, either regulatory or structural, in the attenuated strains and the role of these genes in the flagellar regulon. If the mutations are in regulatory genes, the genes controlled by these mutated regulatory genes should be identified and characterized.

We thank N. Cianciotto and C. Pope for the generous gift of the plasmid containing mini-Tn*10*. We thank H. Lipman for statistical analysis. We also thank T. Hine for her assistance in amino acid analysis.

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Editor: B. I. Eisenstein

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