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# Analysis of the *Legionella pneumophila fliI* Gene: Intracellular Growth of a Defined Mutant Defective for Flagellum Biosynthesis

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**Using a PCR-based strategy and degenerate oligonucleotides, we isolated a *Legionella pneumophila* gene that showed high sequence similarity to members of the *fliI* gene family. An insertion mutation that disrupted the *fliI* open reading frame was recombined onto the *L. pneumophila* chromosome and analyzed for its effects on production of flagella and intracellular growth. The mutation resulted in loss of surface-localized flagellin protein but had no effect on the ability of the bacteria to grow within cultured cells. Therefore, in spite of the fact that some aflagellar mutations render *L. pneumophila* unable to grow within macrophages, the isolation of this defined mutant confirms that production of flagella is not required for intracellular growth.**

*Legionella pneumophila* is a fastidious gram-negative bacterium able to replicate within a variety of eukaryotic cells. The microorganism grows within membrane-bound vacuoles within target cells, which include macrophages and amoebae, the latter being its presumed natural reservoir (1, 15, 31). The organism was originally identified as the cause of Legionnaires' disease and related pneumonic diseases, which result from growth of the bacterium within alveolar macrophages. Mutants defective for growth within phagocytic cells are uniformly avirulent in a guinea pig model of pneumonia after aerosol inoculation (4, 14, 23).

Detailed studies of the intracellular growth of the microorganism by electron and immunofluorescence microscopy indicate that phagosomes harboring *L. pneumophila* differ in a number of unusual ways from compartments found in the endocytic pathway (17). Very few plasma membrane proteins are internalized with the newly formed phagosome, and maturation of this compartment involves avoidance or inhibition of fusion with late endosomal or lysosomal compartments (8, 9). Initially, mitochondria appear to be recruited to the phagosome, but within 3 h after uptake the most striking structure associated with the phagosome is the rough endoplasmic reticulum, which appears tightly associated with the phagosomal surface (17, 37). The structure containing the bacteria, called a replicative phagosome, expands during microbial growth, and the bacteria are eventually released from the phagosome as the macrophages die. In transformed cells, this last step resembles apoptosis (26). Most of the characterized bacterial mutants that are unable to grow intracellularly also appear to be defective for proper targeting of the bacteria to a replicative phagosome, with mutant organisms localized in a late-endosomal compartment shortly after uptake (2, 3, 18, 23, 38).

The distinct lack of association of endosomal marker proteins with the developing phagosomes of wild-type organisms suggests that during internalization of the bacterium by host cells, *L. pneumophila* deposits factors that modify the phagosomal membrane. Presumably, a specialized bacterial secretion machinery promotes the transfer of these proteins. Precedents for this model exist, as a variety of gram-negative bacteria have highly conserved factors that allow the export of proteins across the outer membrane as well as the deposition of the secreted proteins directly into mammalian cells (27, 29, 33, 35, 45).

Most of the microorganisms that directly transfer proteins from adherent bacteria to host cells do so in a signal sequence-independent fashion, as exemplified by *Salmonella typhimurium*, *Shigella flexneri*, and *Yersinia* species (24, 27, 35, 45). For each of these microorganisms, there are approximately 20 sequence-conserved components necessary to promote protein translocation. This apparatus is often referred to as a type III secretion system (33). The functions of some of these proteins are conserved across species, as components from one species have been shown to function properly in the secretion of heterologous proteins in other species (16, 30). Several of these components also have homologs found in the biosynthetic apparatus of flagella of both gram-positive and gram-negative bacteria, such as the products of the *fliI* and *flhA* genes (7, 11).

To attempt to isolate genes that express components of an *L. pneumophila* apparatus that function in a fashion analogous to type III secretion, a PCR-based approach was taken. Each of the characterized type III secretion systems has a component containing a consensus nucleotide binding sequence proposed to couple nucleotide hydrolysis to the translocation process (12, 40, 43). By aligning the sequences of the appropriate genes, i.e., *yscN* of *Yersinia enterocolitica* (43), *spa47* of *S. flexneri* (40), and *invC* of *S. typhimurium* (12), several degenerate oligonucleotides that corresponded to highly conserved regions of these genes were synthesized. Pairs of primers, containing synthetic restriction sites for either *Hin*dIII or *Sal*I, were then used to attempt to amplify short regions internal to this gene by using *L. pneumophila* chromosomal DNA, isolated as described previously (25), as a template. The degenerate primer pair that gave a PCR product of the appropriate size, GGA AGCTTGATTC(T/C/A)TT(G/A)ACN(C/A)G(G/A)TATGC (primer A) and GGGTGACAC(A/G)GTATAAAA(T/A/G)G CNGT(A/T)AT (primer B), terminated at nucleotides corresponding to the codons for amino acids 259 and 298 of *spa47* (40) (Fig. 1).

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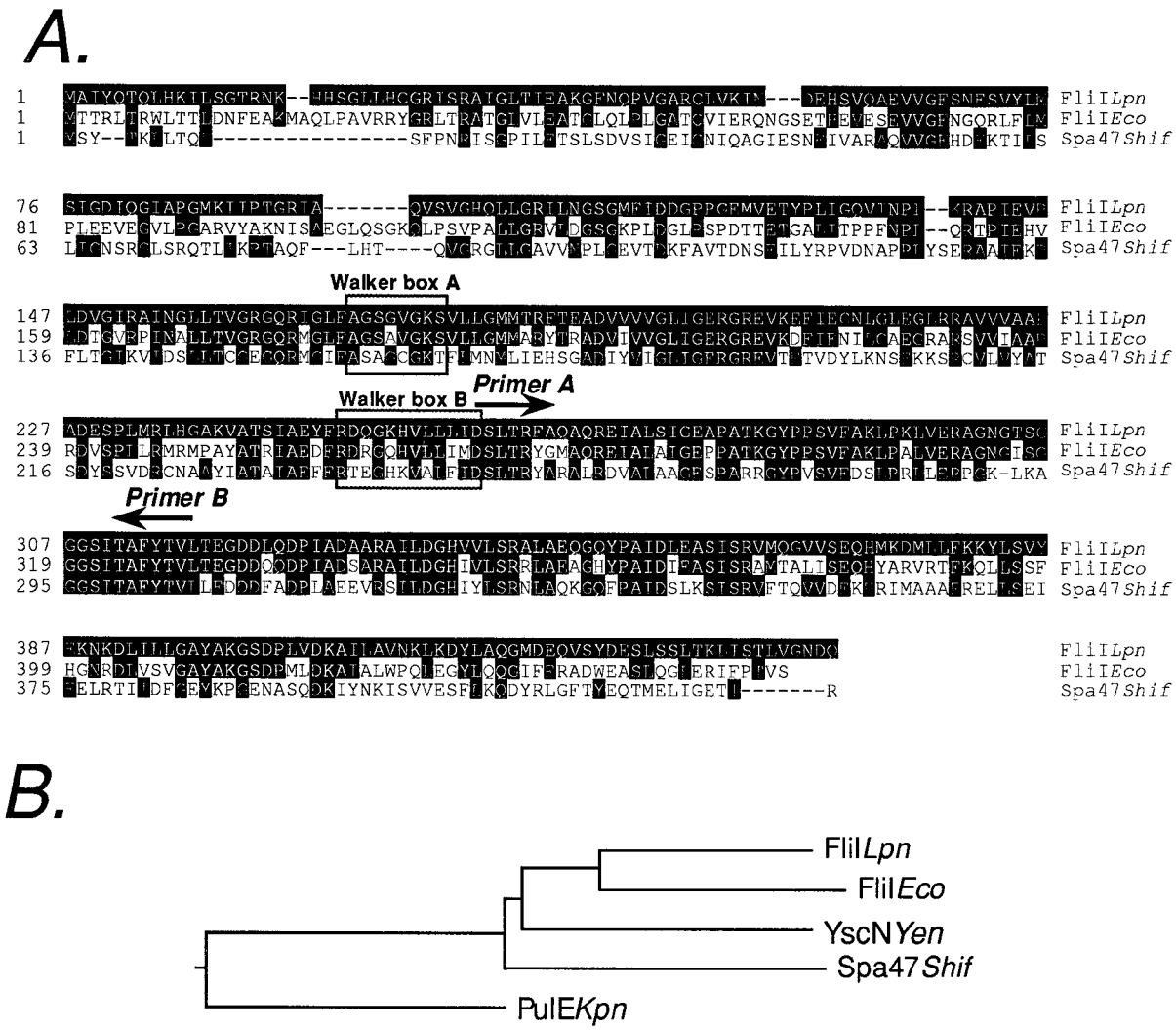


FIG. 1. Sequence similarity of the predicted *L. pneumophila fliI* product with putative nucleotide binding proteins associated with type III secretion systems. (A) Shown is the predicted amino acid sequence encoded by each ORF based on nucleotide sequence analysis (22, 40). Blackened areas denote amino acids that match the *L. pneumophila fliI* gene product rather than consensus amino acids. Boxed areas are regions containing amino acids corresponding to the two predicted nucleotide binding motifs as noted by Walker (42) (Walker boxes) from analysis of $F_1F_0$ ATPases. Primers indicate regions of the predicted Spa47 protein sequence that were used to determine the appropriate oligonucleotide sequences for PCR amplification of the *L. pneumophila* chromosome. FliI*Lpn*, predicted *L. pneumophila* FliI sequence; FliI*Eco*, predicted *E. coli* FliI sequence; Spa47*Shif*, predicted *S. flexneri* Spa47 sequence. (B) Phylogenetic tree of selected nucleotide binding proteins. The *L. pneumophila fliI* gene product is more closely related to a component of the flagellum apparatus than to *S. flexneri spa47*. PulE*Kpn* (10) (the product of the *pulE* gene from *Klebsiella pneumoniae*), which is involved in signal sequence-dependent secretion, is included as a comparison to a distantly related nucleotide binding protein that is not a member of this family. YscN*Yen*, predicted *Y. enterocolitica yscN* gene product (43).

The single species generated by the procedure described above was digested with *Sal*I and *Hin*dIII and inserted into pSK1(+), and the nucleotide sequence was determined. The cloned region was subjected to BLASTX alignment and found to be highly similar to the genes used to design the primers, as well as to *fliI* (41). To isolate the complete gene(s) that encompasses the cloned fragment, MC1061 harboring a gene bank previously constructed in the vector pKB5 was probed (36) with the potentially heterogeneous PCR product derived from the chromosomal template. Seven individual positive molecular clones that hybridized and that had four different restriction digestion patterns were isolated. The site of hybridization of two of these clones indicated that only a portion of the desired gene could be present on the isolated molecular clones, whereas the other two showed hybridization signals that were sufficiently internal to their molecular clones to contain complete genes.

The predicted protein sequence, derived from the nucleotide sequence of the complete open reading frame (ORF) that hybridized to the PCR product, contains sequences found in a variety of nucleotide binding proteins (42) (Walker boxes [Fig. 1A]). These sequences are found in all examples of this component of type III secretion systems (12, 40, 43). The predicted protein product, however, had a higher degree of similarity to FliI from *Bacillus subtilis* (44) and *Escherichia coli* (22) than to Spa47 or other virulence-associated components (40). The percentage of identical amino acids found in the *L. pneumophila* ORF and *E. coli fliI* products was 59%, whereas the percent identity to Spa47 was only 40%. This difference was particularly striking in the predicted protein sequence C terminal to

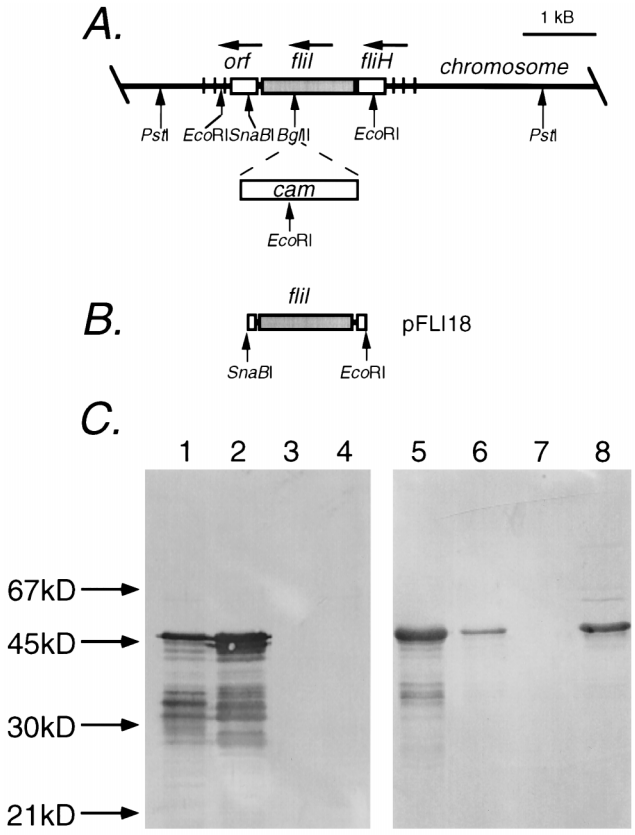


FIG. 2. Absence of exported flagellin protein in *L. pneumophila fliI* mutants. (A) Structure of the *L. pneumophila* chromosome having an insertion mutation in the *fliI* ORF that confers resistance to chloramphenicol. The mutant was constructed as described in the text. Strain LP02 has an intact *fliI* locus, whereas strains LP2016 and LP2328 contain the noted *cam* insertion in *fliI*. Arrows refer to the direction of translation of the noted genes. Vertical lines indicate regions that are not sequenced and are presumed extensions of the noted genes. (B) Limits of the region of the *L. pneumophila* chromosome present in pFLI18. Shown is the region on the plasmid spanning *fliI*, containing 80 nucleotides of the upstream ORF that is similar to *fliH* of *S. typhimurium* (41) and 189 nucleotides of the downstream ORF. The resulting fragment was inserted into the plasmid pKB5 (2), a derivative of RSF1010, as described in the text. (C) Immunoblot analysis of flagellin isolated from *fliI* insertion mutants and demonstration of complementation in *trans*. Bacterial strains were grown for 3 days on a single 100-mm-diameter dish containing charcoal-yeast extract medium at 37°C and harvested, and flagella were isolated from bacterial cell surfaces by shearing through a small-bored needle (13). The resulting preparations were suspended in 15 μl, and aliquots were loaded onto a sodium dodecyl sulfate–11% polyacrylamide gel (20) and prepared for immunoblotting (39). The blots were probed with a rabbit polyclonal antibody directed against flagellin isolated from *L. pneumophila* serogroup 2 (13) and revealed as described previously (21). Flagella were prepared from strains as follows: LP03 *fliI*$^+$*dotA* (1.5 μl of preparation) (lane 1), LP02 *fliI*$^+$*dot*$^+$ (1.5 μl of preparation) (lane 2), LP2016 *fliI*::*cam* (1.5 μl of preparation) (lane 3), LP2328 *fliI*::*cam* (1.5 μl of preparation) (lane 4), LP02 *fliI*$^+$ (1.5 μl of preparation) (lane 5), LP02 *fliI*$^+$ (0.15 μl of preparation) (lane 6), LP2328 *fliI*::*cam*/pKB5 (1.5 μl of preparation) (lane 7), and LP2328 *fliI*::*cam*/pFLI18*fliI*$^+$ (1.5 μl of preparation) (lane 8). Preparations displayed in lanes 1 to 4 were isolated on different days from those displayed in lanes 5 to 8.

primer A (Fig. 1A) (67% percent identity with *E. coli* FliI compared to 45% identity with Spa47). Phylogenetic tree analysis also predicted higher identity of the ORF to *fliI* than to *spa47* (Fig. 1B). Continued sequencing of the region upstream of this ORF revealed a sequence predicted to be highly similar to *fliH*, which is often found in the same operon with *fliI* (Fig. 2A). The organization of these two genes is identical to that seen in flagellar assembly systems in other organisms such as *S. typhimurium* and *B. subtilis*. This lends support to the hypothesis that the product of the *L. pneumophila* gene isolated by PCR probably has a function identical to that of FliI found in other bacteria (41, 44).

A previous study suggested that there was a physiological link between the presence of flagella and growth of *L. pneumophila* in either cultured cells or amoebae (28). In that study, among 10 aflagellar mutants analyzed, 7 had defects in intracellular growth. At least some of the mutants lacking flagella were able to grow intracellularly, indicating no direct requirement for the presence of flagella. Although the genes affected by these mutations have not been identified, this previous work raises the possibility that proteins involved in the assembly of flagella may be required for export of factors involved in intracellular growth. For this reason, an insertion mutation in the putative chromosomal *fliI* gene was constructed to determine if the protein product is specifically involved in the biosynthesis of flagella, as well as to determine if a defined mutation in a gene known to be involved in flagellum biosynthesis could prevent intracellular growth.

To isolate a chromosomal insertion, a plasmid containing the 5.5-kb *Pst*I fragment (Fig. 2A) that encompasses the *L. pneumophila fliI* gene was digested at the unique *Bgl*II site in the *fliI* gene, and a fragment encoding the *cam* gene from pBR325 (5) was then ligated into this site to create pSPA3 (Table 1). The chromosomal region containing this disruption was inserted into pSR47, a suicide plasmid encoding kanamycin resistance and containing the replication origin of plasmid R6K and the origin of transfer of RP4 (pSPA4 [Table 1]) (25). The resulting plasmid was then introduced into *L. pneumophila* LP02 (*rpsL fli*$^+$) by mating (2), with selection for integration by plating on chloramphenicol-containing medium. To isolate disruptions in *fliI*, the resulting strain containing the integration event was grown in culture, and individual Cam$^r$ colonies were screened for Kan$^s$ to identify candidate strains that had lost plasmid sequences. Southern hybridization analysis was

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Source or reference |
|---|---|---|
| *L. pneumophila* strains[a] | | |
| philadelphia 1 | Clinical isolate | Centers for Disease Control and Prevention |
| LP02 | *rpsL thyA hsdR* | 2 |
| LP03 | LP02 *dotA03* | 2 |
| LP2016 | LP02 *fliI*::*cam* | This work |
| LP2328 | LP02 *fliI*::*cam* | This work |
| *E. coli* strains | | |
| MC1061 | F$^-$ *hsdR hsdM*$^+$ | M. Casadaban |
| SM10($\lambda_{pir}$) | RP4 *thr leu supF* (λpir) *tet*::Mu *recA* | 34 |
| SY327λpir | F$^-$ *araD* Δ(*pro lac*)*XIII argE*(Am) *rpoB gyrA recA56*(λpir) | 25 |
| Plasmids | | |
| pKB5 | pMMB67EH, *td*Δ*i* (Thy$^+$) | 2 |
| pJJM36 | pKB5 *fliI*$^+$ | This work |
| pSR47 | *ori*T RP4 *ori*R6K *kan* | S. Rankin |
| pSPA3 | pSK(+) *fliI*::*cam* | This work |
| pSPA4 | pSR47 *fliI*::*cam* | This work |
| pFLI18 | pKB5 *fliI*$^+$ | This work |

[a] All *L. pneumophila* strains are derived from *L. pneumophila* philadelphia 1 (Centers for Disease Control and Prevention, Atlanta, Ga.).

then used to confirm that *fliI* was disrupted and that no intact *fliI* gene was present (Fig. 2A; Table 1 [LP2328 and LP2016]).

As predicted for a factor involved in biosynthesis or export of flagella, strains bearing the *fliI*::*cam* mutation were defective for the production of flagella (Fig. 2C). Preparations of flagellin protein (13) from the isogenic *fliI*$^+$ and *fliI*::*cam* strains were fractionated on sodium dodecyl sulfate-polyacrylamide gels and analyzed by immunoprobing with rabbit polyclonal antibody directed against the *L. pneumophila* flagellin protein (13) (kind gift of W. Johnson). The presence of flagellin protein was readily detected as a species with an apparent molecular weight of 47,000, with a series of lower-molecular-weight degradation products, from the strain LP02 *fli*$^+$ (Fig. 2C, lanes 2 and 5). On the other hand, no flagellin antigen could be detected from LP2016 *fliI*::*cam* or LP2328 *fliI*::*cam* (Fig. 2C, lanes 3 and 4). Even when extracts of the *fliI*::*cam* mutants were loaded in 10-fold excess relative to those displayed in Fig. 2C, no flagellin antigen could be detected by immunoprobing (data not shown).

To demonstrate that the defective production of flagella was due to the insertion mutation in the putative *fliI* gene and not due to a second unlinked lesion, a complementation test was performed. A fragment encompassing the wild-type *L. pneumophila fliI* gene and little other chromosomal material was inserted into the broad-host-range plasmid pKB5 (2), creating pFLI18 (Fig. 2B). The presence of the wild-type *fliI* gene was able to restore production of flagella to the insertion mutant, whereas no flagella could be isolated from a *fliI*::*cam* strain harboring the cloning vector pKB5 (Fig. 2C, lane 7). The amount produced in the *fliI* heterozygote, however, was approximately 30% of that found in the wild-type strain (Fig. 2C, compare lane 8 to lanes 5 and 6). Presumably, the absence of a promoter driving the chromosomal fragment in pFLI18 was the cause of lowered efficiency of flagellum biosynthesis relative to the wild-type strain, or the mutation was partially polar on a downstream gene.

If flagellum biosynthesis is required for intracellular growth, then the *fliI*::*cam* mutant should be unable to replicate efficiently within cultured cells. To test this proposition, the histiocytic cell line U937 was phorbol ester transformed and the phagocytic cells were challenged with a variety of *L. pneumophila* derivatives, including the two independent *fliI*::*cam* recombinants (Fig. 3). The low-passage *L. pneumophila* philadelphia 1 isolate and LP02, its derivative that served as parent for all genetic manipulations, grew efficiently within these cells. In contrast, the targeting-defective mutant LP03 *dotA* was unable to replicate intracellularly, as previously demonstrated (2) (Fig. 3). The two *fliI*::*cam* mutants, however, showed no such growth defects, giving yields in triplicate assays that were indistinguishable from those of the wild type (Fig. 3). This indicates that the assembly of flagella is not required for intracellular growth within mammalian cells.

In concert with previous work (28), elimination of an essential component in flagellum biosynthesis failed to uncover a direct connection between the presence of flagella and intracellular growth. Neither the *fliI* product nor assembled flagella are required for intracellular growth. On the other hand, the results described here do not eliminate the possibility that some specific components of the flagellar assembly apparatus are important for proper intracellular targeting of the bacteria. Arguing against the latter model is the fact that, among 16 *L. pneumophila* loci associated with replicative phagosome formation, none are similar to components of type III secretion systems (2, 6, 32, 40a).

Consistent with the previous report of decreased flagellum assembly (28), we have found that the *dotA3* mutation used in this study (Fig. 2C, compare lane 1, LP03 *dotA3*, to lane 2, LP02 *dot*$^+$) and several other recently characterized mutations caused decreased amounts of exported flagellin antigen (data not shown). There must be some physiological link between an ability to grow in macrophages and the biosynthesis of flagella. Perhaps these mutations cause pleiotropic defects that interfere with the biosynthesis of flagella, or perhaps the absence of some component in these mutants interferes with the expression of a factor necessary for flagellum biosynthesis. Potentially pleiotropic effects that result from *L. pneumophila* intracellular growth-defective mutants have been documented in the literature. For example, such mutants are more resistant than wild-type strains to the presence of NaCl in the culture medium (32). Based on current information, there is no direct molecular explanation for how a single lesion could cause simultaneous defects in flagellum formation, intracellular growth, and NaCl resistance.

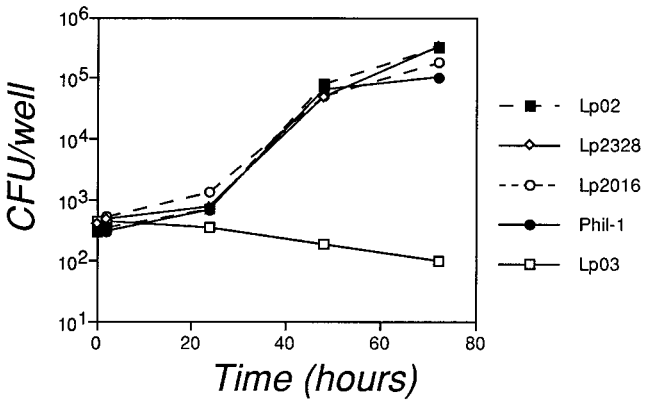


FIG. 3. The insertion mutation in *fliI* causing a defect in flagellum production is competent for intracellular replication. The U937 cell line, treated with tetradecanoyl phorbol acetate as described previously, was seeded onto microtiter wells and exposed to *L. pneumophila* derivatives introduced at a multiplicity of infection of 0.1 (2). Growth was monitored for 3 days by determining the titers for viable counts on charcoal-yeast extract medium at 24-h intervals, as described previously (2). The value at each time point represents the mean of three determinations. Phil-1, *L. pneumophila* philadelphia 1 low-passage isolate; Lp02, LP02 *dotA*$^+$, replication competent derivative; Lp03, LP03 *dotA*, defective for intracellular replication; and Lp2328 and Lp2016, two independently derived *fliI*::*cam* derivatives.

In summary, although many mutants defective for intracellular growth are deficient in flagellum assembly, the absence of a growth defect in the *fliI*::*cam* mutants as well as the previously mentioned genetic studies indicates that intracellular growth does not require flagellar assembly. Further studies, using animal infection models, will be required to determine if flagellum assembly plays some role in disease other than intracellular growth.

**Nucleotide sequence accession number.** The predicted sequence coding for the protein described in this work has been submitted to the GenBank database under accession no. U85783.

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