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Legionella pneumophila Contains a Type II General Secretion Pathway Required for Growth in Amoebae as Well as for Secretion of the Msp Protease

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We report the identification of a set of *Legionella pneumophila* genes that encode products with homology to proteins of the type II general secretion pathway of gram-negative bacteria. A strain containing a deletion-substitution mutation of two of these genes was unable to secrete the Msp protease. This strain was unable to multiply within the free-living amoeba *Acanthamoeba castellanii* yet was able to kill HL-60-derived macrophages. Because Msp is not required for growth in amoebae, other proteins which are important for growth in amoebae are likely secreted by this pathway.

Legionella pneumophila is the gram-negative facultative intracellular pathogen responsible for Legionnaires' disease. *L. pneumophila* is able to infect and multiply within a variety of eukaryotic hosts, including human mononuclear phagocytes, and a wide variety of protozoa including the free-living amoeba *Acanthamoeba castellanii*. The bacteria are phagocytosed via a unique coiling mechanism and reside in a specialized phagosome that does not acidify or fuse with lysosomes. Following replication, the host cell lyses and the bacteria are released and are able to initiate a new infection cycle (for reviews, see references 1, 18, 46, and 47).

Identification of the *lspFGHIJK* genes. As part of an effort to identify regulatory proteins of *L. pneumophila*, we attempted to complement a mutant gene product from *Escherichia coli* (20, 21). Maintenance and growth of *E. coli* and *L. pneumophila* and all DNA manipulations were carried out as described previously (39). A library of *Eco*RI-digested genomic DNA of *L. pneumophila* Philadelphia-1 cloned into the vector pMMB207 (31, 39) was used in a complementation screen. DNA sequencing of the vector-*L. pneumophila* genomic DNA junctions of a particular clone (plasmid pLM511) revealed homology to the DNA sequence encoding *xcpS* (*gspF*).

The *xcp* genes encode proteins whose products function in the main terminal branch (MTB) of the general secretion pathway (GSP) of *Pseudomonas aeruginosa* (3). The GSP is a type II protein secretion pathway that is highly conserved among gram-negative bacteria and was first described by Pugsley et al. 14, 15; for reviews, see references 34, 37, 38, and 42). Proteins secreted by the GSP have an initial *sec*-dependent step for export across the inner membrane. The proteins are then transported across the outer membrane via an apparatus consisting of the protein products of 12 to 15 genes. A well-studied paradigm for this pathway is the pullulanase secretion system of *Klebsiella oxytoca* (37).

Plasmid pLM511 contains a single *Eco*RI fragment of *L. pneumophila* DNA, which is 4,279 bp in length. Sequence from both strands of DNA was generated (DNA Synthesis and Sequencing Facility of the Comprehensive Cancer Center, College of Physicians and Surgeons of Columbia University). There are two partial open reading frames and five complete open reading frames contained on the fragment (Fig. 1). Because of the homology to the GSP family of proteins, we named the open reading frames *lsp* for *Legionella* secretion pathway. The open reading frames containing homology to the GSP family of proteins were named *lspFGHIJK*, corresponding to the nomenclature of the *pul* operon homologs (37, 38, 42).

Although some of the GSP family of proteins contain an additional two to four genes downstream of *lspK* (homologs of *pulLMNO*), the *L. pneumophila lsp* operon appears to end with *lspK*. No open reading frames encoding homologs of other proteins in this family were found on the contiguous 1 kb of DNA 3′ to *lspK*. A partial open reading frame (*orf1*) is encoded by the downstream region, but *orf1* contains no homology to sequences in the databases. At this time, we cannot rule out the existence of an additional unlinked region encoding proteins with homology to *pulLMNO* located elsewhere on the *L. pneumophila* chromosome. However, in all other examples of genes encoding proteins in the GSP family, the genes are adjacent and in essentially the same order on the chromosome.

The *lspFGHIJK* genes encode proteins with significant homology to the gene products of the respective *xcp* family members (Table 1). Consistent with the cognate GSP homologs, each of the LspGHIJK proteins contains a putative signal sequence, and the predicted localization for all the protein products is in the inner membrane or periplasmic space. The signal sequence consensus site contained in the XcpTUVWX proteins for cleavage by XcpA/PilD (GFXXXE [11, 32]; also see reference [35]) is present in LspGHIJ (Table 1). Additionally, the LspGHIK proteins are devoid of cysteines, a trait common among GSP family members (2).

Construction of a strain containing a mutation in the *lspGH* genes. To help identify protein products secreted by the *L. pneumophila* GSP, we constructed a mutation in the *lspGH* genes. The 4,279-bp *Eco*RI fragment from pLM511 was subcloned into pBR322 to generate pLM569. Plasmid pLM569 was digested with *Nco*I to remove an internal 587-bp fragment (Fig. 1). The larger 3,692-bp *Nco*I fragment containing the vector sequences was treated with Klenow enzyme, and a ligation was performed between the Klenow-treated fragment and a 2,118-bp *Hin*cII DNA fragment encoding gentamicin resistance (a gift from David Figurski). This resulted in plasmid

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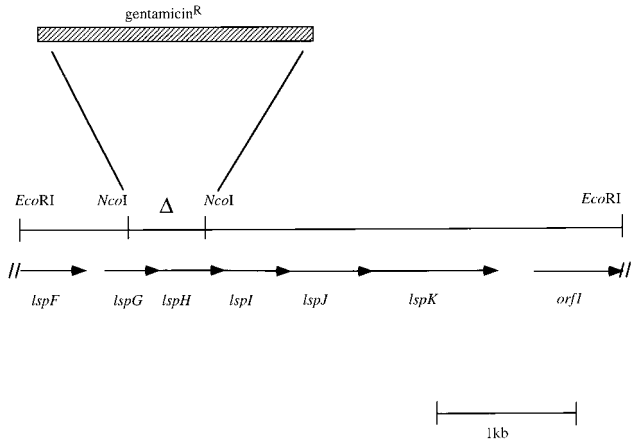

FIG. 1. Schematic diagram of open reading frames (indicated by arrows) encoded on pLM511 and construction of a deletion-substitution that inactivates the *lspGH* genes. The orientation of the arrowheads denotes the direction of transcription. The *Eco*RI fragment contained in pLM511 is 4,279 bp in length and contains the complete DNA sequence encoding the *lspGHIJK* genes and the partial DNA sequence encoding the *lspF* and *orf1* genes. For construction of a mutation in the *lspGH* genes, an internal 587-bp *Nco*I fragment was deleted (indicated by △) and a 2,118-bp gentamicin resistance cassette (hatched box) was inserted in its place.

pLM808 containing an gentamicin resistance cassette inserted within the *lspGH*-coding region (Fig. 1). Because the coding regions for the *lspGHIJK* genes overlap, such an insertion would likely be polar on the *lspIJK* genes and would therefore represent a null phenotype of the *L. pneumophila* GSP.

The 5,232-bp *Eco*RV fragment containing *lspFGH*::Gent^r *IJKorf1* from plasmid pLM808 was subcloned into the *Eco*RV site of the vector pLAW344 for allelic exchange (50). The resultant plasmid, pLM826, was electroporated into the wild-type strain *L. pneumophila* JR32. Allelic exchange of the *lspGH*::Gent^r mutation onto the chromosome of JR32 was performed as described previously (50) and generated strain LM1520. Southern blot analysis confirmed the construction (data not shown).

A complementing plasmid, pLM828, was constructed by cloning the original 4,279-bp *Eco*RI fragment containing the *lspFGHIJKorf1* genes from pLM511 into the vector pMMB207αc (a *mobA* Kan^s derivative of pMMB207αb-Km-14 [45]). Plasmid pLM828 was electroporated into strain LM1520, resulting in strain LM1559. Strain LM1558 is strain LM1520 containing the vector pMMB207αc.

Identification of a protein secreted by the *L. pneumophila* GSP. We were next interested in identifying a protein secreted by this system. A prime candidate is the major secretory protein (Msp) of *L. pneumophila*. Msp is a 38-kDa Zn^{2+} metalloprotease with caseinolytic and hemolytic activities and is the most abundant protein found in culture supernatants (17, 24). Msp contains homology to elastase, a Zn^{2+} metalloprotease which is secreted by the *xcp*-encoded GSP of *P. aeruginosa* (5, 25).

A simple test for the extracellular proteolytic activity of a *msp*$^+$ strain is a ring of casein hydrolysis around a patch of wild-type *L. pneumophila* organisms grown on a agar plate containing casein (48). We tested the ability of the strain containing the mutation in *lspGH* to hydrolyze casein. Strains LM1558 and LM1559 were patched onto buffered yeast starch extract (BYSE) medium containing 10 g of casein per liter as described previously (48). As a control, we patched the wild-type strain JR32 onto the same plate. We also patched strains LS2102 (*L. pneumophila mspA1*::Tn*9*) and its cognate wild-type parent LS2029 (48) as Msp$^-$ and Msp$^+$ controls, respectively.

A ring of hydrolysis was observed around the patch of JR32 growth but not around the patch of LM1558 growth (Fig. 2). This result indicates that the strain LM1558 cannot hydrolyze casein. Because the caseinolytic activity of Msp accounts for virtually all of the proteolytic activity of *L. pneumophila* (13, 48), we conclude that strain LM1558 is defective in the secretion of Msp. Plasmid pLM828 containing the wild-type *lspFGHIJK* genes (strain LM1559) is able to complement the inability to hydrolyze casein (Fig. 2). This result provides evidence that the Msp$^-$ phenotype of strain LM1558 is due to the loss of the *L. pneumophila* GSP and not to an extraneous mutation elsewhere in the genome.

To confirm the results obtained in the casein hydrolysis experiment, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the presence of Msp in *L. pneumophila* culture supernatants. Cell culture supernatants of the wild-type strain LS2029 and the Msp$^-$ strain LS2102 were analyzed for the presence of Msp, and the results compared to cell culture supernatants of strains JR32, LM1558, and LM1559. No Msp was observed in the culture supernatant of strain LS2102, as predicted (Fig. 3A). The culture supernatant from strain LM1558 contains low levels of Msp activity compared to that in the supernatant of the wild-type strain JR32 (Fig. 3A). Plasmid pLM828 containing the wild-type *lspFGHIJK* genes (strain LM1559) is able to complement the inability to secrete Msp into the culture supernatant (Fig. 3A). Taken together with the results from the casein

TABLE 1. Characteristics of proteins encoded on pLM511

Gene	No. of amino acids in open reading frame	Predicted molecular mass (kDa)	Presence of signal sequence (PSORT)[a]	Signal peptide consensus sequence (GFXXXE)[b]	Predicted location (PSORT)[c]	% Amino acid sequence identity to Xcp proteins (LALIGN)[d]
lspF	>126	>13.7	?	?	IM (1)	40.8
lspG	140	15.4	Yes (29)	GFSLIE	IM (1)	59.4
lspH	161	17.8	Yes (31)	GFSLIE	OM/PP	28.8
lspI	125	14.1	Yes (28)	GFSLIE	PP	30
lspJ	205	23.7	Yes (20)	GFSLIE	PP/OM	28.9
lspK	322[e]	36.1	Yes (37)	NF	PP/OM	25.6
orf1	>213	>24.3	No		IM (4)	

[a] Amino acid position at predicted cleavage site is indicated in parentheses. ? indicates unknown.
[b] Consensus sequence for the prepilin peptidase XcpA/PilD cleavage site (see references 11 and 32). NF, not found.
[c] IM, inner membrane (number of predicted transmembrane domains is given in parentheses); PP, periplasmic space; OM, outer membrane.
[d] Values for *lspF* and *orf1* were calculated by using the partial sequence information.
[e] Contains five possible Met start codons.

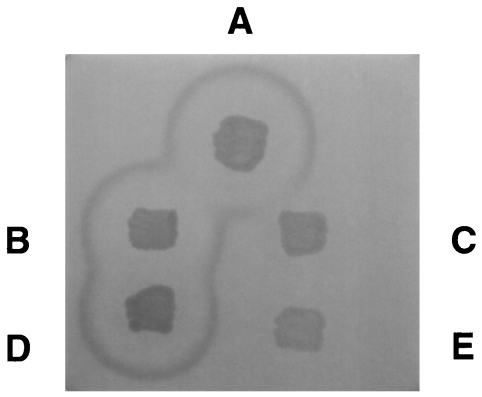

FIG. 2. Casein hydrolysis of the strain containing a mutation in the *lspGH* genes and the complemented strain. Various strains were patched onto a BYSE agar plate containing casein and incubated for 3 days at 37°C as described previously (48). Patches: A, Wild-type strain JR32; B, wild-type strain LS2029 (isogenic to LS2102); C, *msp* mutant strain LS2102 (LS2029 *mspA1*::Tn*9*); D, LM1558 (JR32 *lspGH*::Gent^r pMMB207αc::*lspFGHIJKorf1*); E, LM1559 (JR32 *lspGH*::Gent^r pMMB207αc).

hydrolysis experiment, these results confirm that Msp is a substrate for the *L. pneumophila* GSP. The fact that Msp requires secretion by the GSP confirms the functionality of the system in *L. pneumophila*. It has been shown that wild-type *E. coli* possesses a complete GSP operon, but this operon is not expressed during growth under laboratory conditions (19, 36).

During this analysis, we noticed that the protein profiles of the culture supernatants of the Lsp$^-$ and Lsp$^+$ strains differed significantly (Fig. 3B). The proteins from an SDS-PAGE gel were electroblotted onto a polyvinyl difluoride membrane (Millipore), and three bands (p74, p49, and p36) were excised from the membrane. The samples were subjected to N-terminal sequencing for 8 cycles each (Protein Chemistry Core Facility, Columbia University). In this manner, the N-terminal amino acid sequences were obtained for p74 (AQPTACVN), p49 (YYTSQGSI), and p36 (KDVYEIKH). The sequence databases do not contain any proteins with homology to these three amino acid sequences. The proteins p74, p49, and p36 represent examples of additional proteins that are likely secreted by the *L. pneumophila* GSP (Fig. 3B).

Analysis of the intracellular growth phenotype of the strain containing a mutation in the *lsp* genes. We were then interested in examining the ability of the strain containing a mutation in the *lspGH* genes to replicate within eukaryotic hosts. We first tested the ability of strain LM1520 for cytotoxicity of HL-60-derived macrophages. The assay was performed as described previously (27, 28). The cytotoxicity of strain LM1520 was compared with the results obtained from the *L. pneumophila* wild-type strain, JR32, and the mutant strain 25D (22). Strain LM1520 was able to kill macrophages in a manner identical to that of wild-type strain JR32 (data not shown). This finding indicates that the putative secretion system encoded by the *lsp* operon, or a protein secreted by it, is not required for killing of a macrophage-like cell line.

L. pneumophila also has the ability to multiply intracellularly within the free-living amoeba *A. castellanii* (12, 30, 40, 41). Therefore, strain LM1558 was tested for its ability to replicate within amoebae. Growth and maintenance of *A. castellanii* was carried out as described previously (12, 30). The assay for replication within amoebae was based on previously described methods (12, 30). *L. pneumophila* at a multiplicity of infection (MOI) of 10 was added to an adherent monolayer of 1.2×10^5 amoebae. After incubation for 30 min at 37°C to allow for infection, the wells were washed three times with 0.5 ml of *Acanthamoeba* medium buffer to remove extracellular bacteria. A sample of the infection supernatant was removed once every 24 h for 4 days. Colony forming units (CFUs) of extracellular bacteria were quantitated on ACES-buffered charcoal yeast extract (ABCYE) plates. Wild-type strain JR32 replicated 10^4-fold within 72 h while the mutant strain 25D did not (Fig. 4A). Strain LM1558 containing a mutation in the *lspGH* genes is clearly defective for replication within amoebae. The 4,279-bp *Eco*RI fragment was able to complement the growth defect in *A. castellanii* (Fig. 4A). Therefore, either the secretion apparatus itself or, more likely, another protein that is secreted by this system is required for replication within protozoa. In order to rule out Msp as this protein, we tested the ability of strains LS2029 (Msp$^+$) and LS2102 (Msp$^-$) to replicate within *A. castellanii*. The results show that both strains replicate approximately 10^4-fold in amoebae (Fig. 4B). This result indicates that the growth defect of strain LM1558 in amoebae is

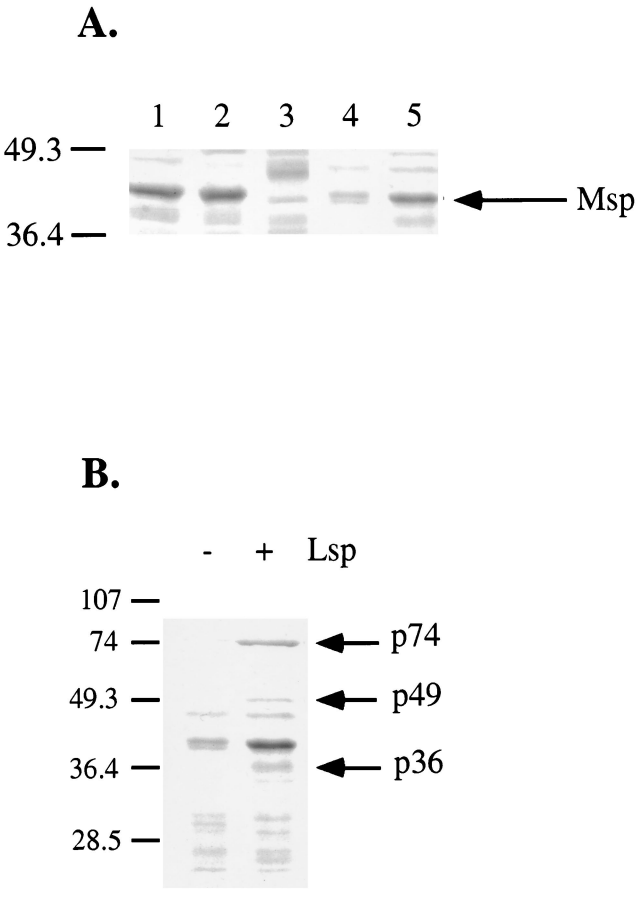

FIG. 3. SDS-PAGE analysis of *L. pneumophila* culture supernatants. Supernatants of *L. pneumophila* overnight cultures were filtered through a 0.45-μm filter (Millipore), and the proteins from the supernatant were precipitated in 10% trichloroacetic acid. SDS loading buffer was added to the protein pellet, and an aliquot was electrophoresed on a 12% polyacrylamide gel. The proteins were visualized by Commassie blue staining. Molecular mass markers (in kilodaltons) are indicated on the left. (A) Lanes: 1, wild-type strain JR32; 2, wild-type strain LS2029 (isogenic to LS2102); 3, *msp* mutant strain LS2102 (LS2029 *mspA1*::Tn*9*); 4, LM1558 (JR32 *lspGH*::Gent^r pMMB207αc); 5, LM1559 (JR32 *lspGH*::Gent^r pMMB207αc::*lspFGHIJKorf1*). (B) Culture supernatants of Lsp$^-$ (LM1558) and Lsp$^+$ (LM1559) strains. The three proteins which are present in Lsp$^+$ but not in Lsp$^-$ culture supernatants, with approximate molecular masses of 74, 49, and 36 kDa, are indicated on the right.

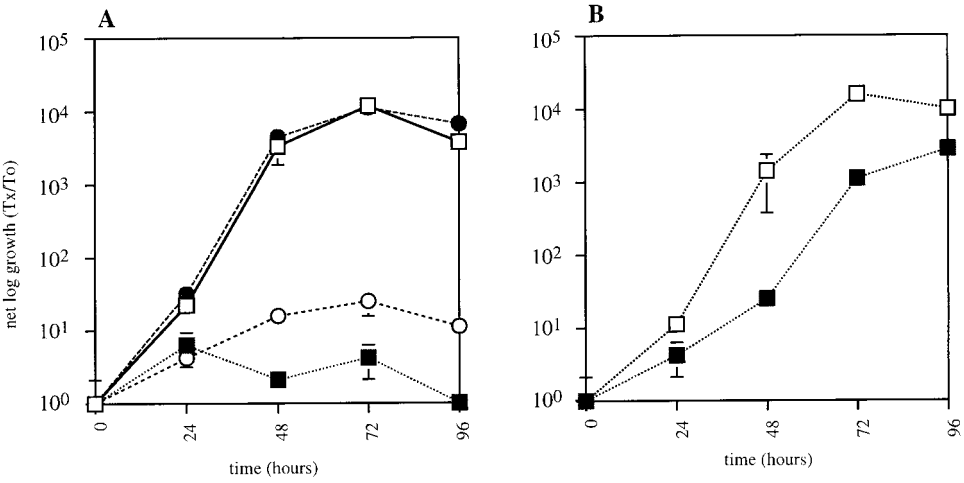

FIG. 4. Intracellular growth of strains in *A. castellanii*. The net log growth of the various strains is plotted as a function of time. Error bars represent standard deviation and may not be visible. (A) Wild-type *L. pneumophila* strain JR32 (open squares) and the mutant 25D (closed squares) are the controls. Strain LM1558 (open circles) is strain LM1520 (JR32 *lspGH*::Gent^r) containing the vector pMMB207αc. Strain LM1559 (closed circles) is strain LM1520 containing the plasmid pMMB207αc::*lspFGHIJKorf1*. (B) Wild-type *L. pneumophila* strain LS2029 (open squares) and the isogenic *msp* mutant strain LS2102 (filled squares).

not due to the inability of this strain to secrete Msp and provides evidence for the existence of other secreted proteins that are important for growth in amoebae. An alternative explanation for our results is that the deletion-substitution mutation is polar on expression of *orf1*, and it is the *orf1* gene product that is required for growth within amoebae. However, we do not believe that *orf1* contributes to this phenotype because the open reading frames of the *lspGHIJK* genes overlap and there is considerable distance between the end of *lspK* and the beginning of *orf1* (314 bp). Additionally, *orf1* does not have homology to genes in the GSP family, further suggesting that it is not a member of this operon. Therefore, we believe that it is unlikely that a mutation in *lspGH* would affect the function, if any, of *orf1*.

In summary, our analysis shows that the *L. pneumophila* GSP or, a protein secreted by it, is required for growth within amoebae. Many of the proteins secreted by the GSP of gram-negative bacteria play a role in pathogenesis, as many of the organisms from which these components originate are plant or human pathogens. Our evidence indicates that Msp is secreted by the *L. pneumophila* GSP. Indeed, it was postulated that Msp might be a virulence factor (4, 6–10, 13, 29, 48). However, Msp is not required for *L. pneumophila* to either kill macrophages (29, 48) or multiply within *A. castellanii* (reference 29 and this work). Therefore, it is likely that one or more proteins other than Msp secreted by the *L. pneumophila* GSP are required for replication within *A. castellanii*. Several organisms that have a GSP secrete more than one protein, and *L. pneumophila* secretes many other exoenzymes, including acid and alkaline phosphatases and lipases (16). Further work is needed to identify other proteins that are secreted by the GSP and to determine the precise requirements for replication within *A. castellanii*. The observation that a set of genes (encoding the *L. pneumophila* GSP) is absolutely required for growth within *A. castellanii* but not in a macrophage-like cell line supports the protozoan host as a more restrictive model of *L. pneumophila* intracellular growth.

It has been shown previously that *L. pneumophila* possesses another type II secretion system involved in type IV pilus biogenesis. Liles et al. (26) reported the identification of the *L. pneumophila* homologs of the *P. aeruginosa pilBCD* genes. We note that the *lsp* operon reported herein does not encode the homolog of the prepilin peptidase, PulO, that is required for the processing of the signal sequences present on several of the Pul proteins in *K. oxytoca* (33). The *xcp* operon of *P. aeruginosa* also lacks the O homolog (3; also see references 23 and 43). In the *xcp* system, the prepilin peptidase PilD (of the pilus biogenesis system) substitutes for the function of the missing O protein in the *xcp* system, in effect performing the signal peptide cleavage and modification processes for both the *pil* and *xcp* gene products (3, 32). Unless *L. pneumophila* encodes an as-yet-undiscovered unlinked O homolog, the *L. pneumophila pilD* gene product (26) may function for both pilus biogenesis (*pil*) and the GSP (*lsp*) in the same manner as that observed for *P. aeruginosa*. It remains to be determined if the *pilBCD* system is required for the pathogenesis of *L. pneumophila*. The *dot-icm* gene products are proposed to function as a novel secretion system that is required for inhibition of phagosome-lysosome fusion and for intracellular multiplication (44, 49). *L. pneumophila* is not unique in this aspect as multiple secretion systems have also been found in other prokaryotes, most notably bacterial pathogens. Therefore, it seems likely that *L. pneumophila* is typical in its acquisition of some common types of secretion systems for use in pathogenesis in eukaryotic hosts.

Nucleotide sequence accession number. The *L. pneumophila lspFGHIJK* sequence has been deposited in the GenBank database under accession no. AF111940.

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ADDENDUM IN PROOF

Liles et al. (M. R. Liles, P. H. Edelstein, and N. P. Cianciott, Mol. Microbiol. **31:**959–970, 1999) recently showed that a *pilD* mutant strain is defective in the secretion of Msp, which further supports our hypothesis that PilD functions as the prepilin peptidase in the *Legionella pneumophila* GSP.

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