

Identification and Temperature Regulation of *Legionella pneumophila* Genes Involved in Type IV Pilus Biogenesis and Type II Protein Secretion

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Previously, we had isolated by transposon mutagenesis a *Legionella pneumophila* mutant that appeared defective for intracellular iron acquisition. While sequencing in the proximity of the mini-Tn10 insertion, we found a locus that had a predicted protein product with strong similarity to PilB from *Pseudomonas aeruginosa*. PilB is a component of the type II secretory pathway, which is required for the assembly of type IV pili. Consequently, the locus was cloned and sequenced. Within this 4-kb region were three genes that appeared to be organized in an operon and encoded homologs of *P. aeruginosa* PilB, PilC, and PilD, proteins essential for pilus production and type II protein secretion. Northern blot analysis identified a transcript large enough to include all three genes and showed a substantial increase in expression of this operon when *L. pneumophila* was grown at 30°C as opposed to 37°C. The latter observation was then correlated with an increase in piliation when bacteria were grown at the lower temperature. Southern hybridization analysis indicated that the *pilB* locus was conserved within *L. pneumophila* serogroups and other *Legionella* species. These data represent the first isolation of type II secretory genes from an intracellular parasite and indicate that the legionellae express temperature-regulated type IV pili.

The gram-negative bacterium *Legionella pneumophila* causes a potentially fatal pneumonia known as Legionnaires' disease (7, 20). This organism normally exists in freshwater ecosystems, either free living within biofilms or as an intracellular parasite of protozoa (23). In order for *L. pneumophila* to cause disease within humans, contaminated aerosols must be inhaled into the lung, where alveolar macrophages serve as the primary sites of bacterial replication (7). Interestingly, alveolar type I and type II epithelial cells are infected in vitro by this bacterium, suggesting a secondary mechanism for survival and spread of the pathogen within its human host (10, 28). Unfortunately, our understanding of *L. pneumophila* pathogenesis is still rather minimal. However, a number of known or candidate virulence factors have been identified. For example, *L. pneumophila* possesses flagella and pili which may aid in adherence of the bacteria to host cells (41). Furthermore, several loci, including *mip*, *dot*, and *icm*, potentiate intracellular survival and replication (3, 5, 9). Finally, a variety of excreted toxins and enzymes, such as proteases and phospholipases, may promote tissue destruction and bacterial spread (7).

The focus of our recent efforts has been to identify bacterial systems which facilitate the intracellular acquisition of nutrients such as iron (17, 18, 24, 33, 36). As one approach toward identifying these virulence factors, we randomly mutagenized *L. pneumophila* 130b (serogroup 1) with mini-Tn10 and screened for mutants with deficiencies in both iron uptake (e.g., resistance to streptonigrin) and growth within U937 cells, a human macrophage-like cell line (36). Seventeen mutants appeared defective for iron uptake, and six of these had infectivity defects. While the genetic basis of the defect in one of these mutants (i.e., NU218) was being determined, an operon

(*pilBCD*) containing genes involved in pilin biosynthesis and type II protein secretion was discovered and characterized.

To determine the genetic loci involved in *L. pneumophila* iron acquisition, we employed inverse PCR to identify sequences near each of the mini-Tn10 insertions in our iron uptake mutants (31). More specifically, 5 µg of genomic DNA was digested with *Hind*III, an enzyme which cuts once within the mini-Tn10, and then the restricted DNA was circularized with T4 DNA ligase overnight at 15°C. After ethanol precipitation of the ligated molecules, PCR products were generated with primers (5'-TGATTTTGATGACGAGCG and 5'-GTGACGACTGAATCCGGT) that recognize sequences on either side of the transposon's *Hind*III site as well as a primer (5'-CCTTAACCTAATGATTTTAC) specific for a sequence in the transposon's inverted repeats. For each mutant, there was the potential to obtain two PCR products, enabling sequencing of the regions immediately surrounding the transposon as well as the DNA flanking the distal *Hind*III sites. The conditions utilized for PCR were 1.5 min at 95°C and 1 min at 47°C, followed by 3 min at 72°C, with 30 cycles and 1.25 U of *Taq* polymerase added in a total reaction volume of 50 µl. To prepare the PCR products for sequencing, approximately 100 ng of PCR product was incubated with 2 U of alkaline phosphatase and 1 U of exonuclease I for 15 min at 37°C, followed by enzyme inactivation at 80°C for 15 min. PCR products and plasmids were sequenced with the Perkin-Elmer sequencing kit according to the manufacturer's specifications (Foster City, Calif.).

While sequencing a region more than 1 kb away from the mini-Tn10 insertion in the mutant NU218, we found sequences encoding a predicted protein with strong similarity to PilB of *Pseudomonas aeruginosa*. PilB and its homologs in other bacteria are components of type II protein secretion systems that are required for the assembly of type IV pili (22, 29, 35). The importance of type IV pili for mediating the attachment of *P. aeruginosa* and other pathogens to epithelial cells has been well documented (14, 15, 26, 46). Early studies by Rodgers and

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colleagues had detected pili in *L. pneumophila*, but the nature of these structures and the genes and proteins involved in their biosynthesis have remained elusive (40, 41). In many species, the gene encoding the PilB homolog is adjacent to the gene for the type IV pilus subunit as well as other genes involved in pilin biogenesis (29, 35). One of these nearby genes, encoding the prepilin peptidase PilD in *P. aeruginosa*, is also involved, albeit indirectly, in the export of toxins and enzymes (25). We therefore sought to confirm the existence of a *Legionella pilB*-like gene and to identify other genes in its vicinity that might contribute to the biosynthesis of pili and/or type II protein secretion.

To isolate clones containing the putative *pilB* homolog, the 1.8-kb PCR product generated from NU218 was labeled with digoxigenin (Boehringer Mannheim, Indianapolis, Ind.) and used to probe genomic libraries of strain 130b (1, 17). Southern blots confirmed the presence of the gene within four different cosmids and one plasmid (data not shown). To facilitate sequencing of this locus, the 5-kb segment of *Legionella* DNA from the recombinant plasmid was subcloned into pSU2719 (4, 27), and the resulting plasmid, pML218, was subjected to unidirectional deletion with exonuclease III (13). To help determine sequences downstream of the putative *pilB* analog, a 6-kb *Bgl*II fragment from one of the cosmids (i.e., C5) was subcloned into pSU2719, yielding pML219.

The 4,259-bp region that was sequenced had three open reading frames (ORFs) which were predicted to encode products with significant similarity to proteins involved in type II protein secretion and pilin biogenesis (Fig. 1). The first ORF was 1,723 bp in length, and the deduced amino acid sequence predicted a 62-kDa protein with 52% identity and 72% similarity to *P. aeruginosa* PilB. This predicted product was also similar in terms of sequence and size to PilB analogs in *Aeromonas hydrophila*, *Dichelobacter nodosus*, and *Neisseria gonorrhoeae*, among others, and possessed the highly conserved Walker sequence, an ATP-binding motif found in PilB-like proteins (Fig. 2) (50). Although the exact cellular location and function of PilB are unknown, the protein is believed to be present at the cytoplasmic face of the inner membrane, where its nucleotide-binding domain may provide energy for the introduction of prepilin into the inner membrane (46). Due to the considerable similarity of the predicted protein to *P. aeruginosa* PilB, we designated the first ORF as the *L. pneumophila pilB* gene. Immediately downstream of *L. pneumophila pilB* was an ORF predicted to encode a 45-kDa protein which had 50% identity and 72% similarity to *P. aeruginosa* PilC, as well as comparable similarity to PilC homologs in other species (data not shown). As was the case for PilB, mutational analysis had determined that PilC is required for pilus expression (37, 49). More specifically, PilC-like proteins, because of their putative transmembrane domains, are believed to be anchored within the inner membrane where they may facilitate pilin translocation (46). Our designation for the second *L. pneumophila* ORF was *pilC*. The region downstream of *pilC* revealed a third ORF predicted to encode a 33-kDa protein with significant similarity to *P. aeruginosa* PilD and PilD homologs in other bacteria (Fig. 3). PilD is a bifunctional enzyme which cleaves prepilin and N methylates the first residue of the resultant mature pilin (25). Furthermore, this peptidase also processes the secreted prepilin-like proteins (XcpT, XcpU, XcpV, and XcpW) that are required for the terminal branch of type II protein secretion in *P. aeruginosa* (30). Thus, PilD, unlike PilB and PilC, has the additional function of contributing to the export of important toxins and enzymes, such as exotoxin A, phospholipase C, and elastase (30, 47). Although clearly significant, the sequence homology between *L. pneu-*

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1  AAAACCTTAATACCTTCAAAATTTTATTTATTTCCAAAGAGGTTGGTGTATAGCCGCTTTTGCAGAAAATTTGATATGCAAAATGACCAAGAAATAGA
-35 -10
101  TTTGGGGGAGCTCCAAAACCGGGTTGGTTCAAAAGCATGCAATTTAAAATAATTTTACATATTTAACTAGATACATTTTGAATATAATGAA
pilB
201  ATGCTCTAGCTACAGAAATATAGATTAACGGGAATCGGGACGCTTCTGCTCGGAAAGCTCTAGATAAACAAGAAAGCAATGACATGACAAAGC
M A L A T E E Y R L Q G I G Q L L V L E K L L D K T K A I E L H K
301  TGGCAGCGGAAAGATGCTTCTGCAATACATTTGAAAATAAATAATATTTCTGCTGAAACAAATGCTGACGACGCTGCAATATTTGGCT
L A A E K M S L L Q Y I V K N L I L S A E Q I A L T A A Q N F G V
401  ACCCATGTGGATTAACCTGATGATGAGGACCACTTCTGCAATCTGTTATGAGGAAATTAATTAAGCTGACGCAATGGTCTCTTTTAC
F N L D I N C I D V G T I P A N L V N E K L I K R H A M V P L I F S
501  CGCGGTACCAATTTATCTCCAGACAGATGATCTAGTAAACAGCTCTATTAAGSAAATACATTTCCACCGGATTAATTAATCTGATGCGATGTTG
V E T D K L S A L I D N L L T A K F S S Q G L S E F V E D S G D L E G
601  TAGAACAGATAAATCTAGTCCCTGATGATAACCTGTTAAACCAAGAAAGCTCAGCGCTTACAGATGTTGTAAGACTTGGAGCTTGGAGG
V E T D K L S A L I D N L L T A K F S S Q G L S E F V E D S G D L E G
701  TTTAGAAATAGCTGATGATGAGGATCAAGATAGTACTGCAACATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
L E I S A D D E D Q D S D T A T S V T D D A P I V I C V N K I L L
801  GATCGATAAGCAGCGGCTTCTGATACAGCTTGAACCTTATGAAAGGAAATCCGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATG
D A I R Q G A S D I H F E F E Y E R E Y R I R Y R Q D G I L H E V A
901  CCGCTCCCGAAGCTGATCTGATCTGATACAGCAGATCAAGCTTAACTGATTTGGATATACAGACCGGCTATCCCGGAGGCTGGCTTCAA
T F P A S L S S R I T A R I K V M S N L D I S E R I R I P Q D D G G F
1001  AATGAAATTCAGCTCAGCAATGATTTACAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
M K I S K S R A I D F R V S T C T P S A G E K V V M R V L D S G A
1101  GCTAATAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
A K L G I E A L G F N P V Q R T N F L K A I Q R P Q G M I L V T
1201  CTATGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
P T G S G K T V T L Y T A L N L I N T I E V N I S T A E D P V E I K
1301  AGTCCGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
V P G I N Q V N I N P X A G L T F S G A L R S F L R Q D C A P G C C T A T T A T A T A T
1401  GTGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
V G E I R D L E T A E I A V K A A Q T G H L V L S T L H T N S A A
1501  AAATCTTAATGTTTATGAAATCTGGATACCACTTTTAAATCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
E T L N R L V K H G D T Q L L N I A S S V T L I I A Q R L A R K L C
1601  CAATCAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
N Q Y F A V R D D E F T N Q G L I E L G G A S D L L K Y K A
1701  GTTGGTCCGCAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
V G S G T S G Y R G R V L G F E V L P F K A E L G Q L I M T
1801  GTATTCGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
G N S L D I L K L A Q S E G H L T I F S G I E K V E G I T T A E
pilC
1901  GGAGGCTCAATGGTAAACCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
E V N R V T V D * M D K N S P T L L E T F H Y Q G I N K A Q K
2001  TGGAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
M E G D I Q A R S L A I A K A D L R K Q G I V T N K V I K K R K P L
2101  GTTGTAGAAAATAAATAAATACACAGGAGATATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
F D R K N K K I T Q A D I T V F S R Q L D A T M I E S G I F A Q C A
2201  TTTGATTTGTCAGGAGCAAGCAATAAAGATTAAGACTTAAGACTTAAGACTTAAGACTTAAGACTTAAGACTTAAGACTTAAGACTTAAGACTTA
F D I V A K Q S N K R L K D L I E T I K I E T I G L T L A E S
2301  TANTAACTCCACTTATTAATGATTTTGTAAATTTGTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
L I K H P L Y F N E L F C N L V D A G E K S G L D I M L D K V A T
2401  GTATGAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA
Y R E K I E I I K K I K K A L T Y F I A V M V V A L L V T A G L T
2501  TTAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
L Y V F E S L F K G A D L P A M T R A V I T H E
2601  AGGCTTTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
Q A Y M Y I I F G A L G G V V Y S F A T G A K N H S L C E A T T I G C T G T I D R
2701  AATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
V N L K A P F V I G P I L E K A A I A R F A R T L S I T F A G L P
2801  CTGGTGAAGATGAAATCTGATGCTGAGCGACCGGGAATTTATTTGCAAGCGCATGATAAATCAGAGAAGATGCAACAGGACAGCAAA
L V E A L K S V A G A T G H I I Y A K A T D R I K R E E V A T Q
2901  TGTATCTGCTGAAATCTACTTATTTTCCAAATATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
M F T A I E N T H L F P N M V I Q M V A I G E E S G A L E K M L S K
3001  AATGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
V A D F Y E E E V N N A V D A L S L L E P I I M S I L G L I L F S
3101  GGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
G L V V G M Y L H I F S L G E A V *
pilD
3201  TGAATAAGCTAATCAATATCCAGCTTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
M I N A L I N Y P W E M Y L V V G L F S L A V G S L L N V I I Y R
3301  CTTCAGATTTTTCAGGAGAGTGAAGAGCAATGCTGCTGAGCTTCCATTTTGGACCGGAAAGAAATAAATAAATAAATAAATAAATAAATAA
L P I I L G E E W K E Q C C E L L F T F E P G K E K I K L L L F L P
3401  GGTCTTTTTCCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
R S P C F H C K A M V K A W Q N I P L L A I L V L R G R C Y Q C D
3501  GTCATTTTCATGCTGCTTCTGTTGAGACGTTGACACAGTTTATCTCTTACCGCGGATGCGATTTGGTGTGAGGAGAGCTGTACCAATGATG
S P F S I R Y F F V E T L T V L S L Y A S W H F G E T I Q L L F A
3601  TTTATGACTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
L L A I W I L I S L V F I D L D H Q L L P D S L L T L G L L W I G L
3701  ATGCTAAAGCCAGATGATTTGAGCTTGAAGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
T A N T Q H V E V S L D V A V L S C A G Y V L A L W L F I N D F Y
3801  TGAACATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
L M T C T K V M G H G D F K L F A A T F G A L T P G A W H V L L I L L I
3901  ATCTCAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
S I E T G A I I G L I Y L E K I N G A T T A C C A G A T T C C A A T T A I F F V H L C E I S
4001  GATTAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
G L I A H F W G D S I I N W T I G Y W M *
4101  AGCCACAGCCCAAAATACATGAGAAGAGATCCCCCAAGAGATTTCTGCTGTAATACCCCAAAAGAGCTCCGGCGTAARATTAAT
4201  GAGCAAGGTTAACCCCAAGTGAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG

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FIG. 1. Nucleotide sequence of the *L. pneumophila pilBCD* genes. The deduced amino acid sequences of the three ORFs and the termination codons (*) are indicated. The direction of transcription-translation of each ORF is indicated by a horizontal arrow. The possible binding sites for the alternative σ^{28} factor are indicated by the -35 and -10 designations. Although Northern blot analysis indicated otherwise (see below), no transcriptional terminator was evident at the end of the *pilBCD* locus. The locations of the F2 and R7 primers used to prepare a *pilB*-specific probe are also indicated. The sequence between nucleotides 1 and 3140 was obtained from analysis of the pML218 insert, whereas the sequence from nucleotides 689 to 4259 was from the pML219 insert. Double-stranded sequence data were compiled with Gene Runner (Hastings Software, Inc., Hastings, N.Y.).

	1				50
PPilB	...MNDSIQL	SGLSRQLVQA	NLLDEKTAIQ	AQTQAQRNKL	SLVTHLVQNK
TapB	...MTSSPN	SGLALSIAAS	SLLESSESQR	YLSQAQRKR	PFVTFLEINE
FimN	...MSEY	DELIAYMKN	KVATPEQLKE	VQISVQKRRG	NFLQQLSDG
PilF	...MS	VGLLRILVLE	QVTVPEQAEH	YVNESQAGK	EVLPMFLSDG
LPilB	MALATEEYRL	QGIGQLLVLE	KLLDKTKAIE	LHKLAAAEKM	SLLYQYVKNK
Consensus	-----*	-----*	-----*	-----*	-----*
	51				100
PPilB	LVSGLALAEI	SAEQFGIAYC	DLNSLDRESF	PRDAISEKLV	RQHRVPLWR
TapB	ILDSKALADF	CELEYGVPLL	DLAAPDLAEI	PQKYLNQKLI	EKHHVLPYIT
FimN	LLDASKLSKI	NREVYMLPIV	ALRELNIRRE	LTAQFKEAVV	RKHTAMPVYA
PilF	VISPKSLAAL	IARVFSYSIL	DLRHYPRHRV	LMGVLTEEQM	VEFHCVVFR
LPilB	ILSAEQIALT	AAQNFVGPML	DINCIDVGTI	PANLVNEKLI	KRHAMVPLFS
Consensus	-----*	-----*	-----*	-----*	-----*
	101				150
PPilB	RGNKLVFGIS	DAANHQAIND	VQFSTGLTT	EAILVEDDKL	GLAIDKLFEN
TapB	QGHTLYIAMS	DPTNVALED	FGFSFGLHT	EALLVEENKL	TTAIGKLLS
FimN	NGGRLFIATI	DPNNRSMLEE	FKYQKFTSV	EPIIANLDAI	EALIEEHYAG
PilF	RGDKVFVAVS	DPTQMPQIQK	TVSAAGI.AV	ELVIVEDDQL	AGLLDWVCSR
LPilB	RGTNLYLATD	DPSQASLKE	IQFHTGLNT	HAIVVETDKL	SALINDLL.T
Consensus	-G-***-D	-D***-D	-----*	-----*	-----*
	151				200
PPilB	ATD..GLAGL	..DDVDLEGL	DVGVKETSQG	EDTGAE.ADD	APVVRVVKM
TapB	DQDALGMEDI	..DESSISEL	EVSDENSRLD	ESVNTT.DDD	APIVKYINKI
FimN	LGGMDDLFD	ETEEKDLDAI	SNALGGLDAN	E.....EE	APVVRVVTGM
PilF	STSLQLQELGE	QEEESHTL	YIDNEE....AED	GPVPRFIHKT
LPilB	AKESQGLSEY	VEDSGDLEGL	EISADDEDQD	SDTATSVTDD	APIVIVCNKI
Consensus	-----*	-----*	-----*	-----*	-----*
	201				250
PPilB	LLDAIKGGSS	DLHFPEYEKI	YRVRFRTDGM	LHEVAKPPIQ	LASRISARLK
TapB	MMDAIRKRGAS	DLHFPEYETK	YRIRFRIDGI	LHEIATPPVN	LANRFSARLK
FimN	LLDAIRITGAS	DLHFPEYETG	YRIRFRRDVG	LQEVAAAPPS	IATRIARLTK
PilF	LSDAIRSGAS	DIHFPEYEHN	ARIRFRVDQG	LREVVQPIIA	VRGQLASRIK
LPilB	LLDAIRIQGAS	DIHFPEYERE	YRIRYRQDGI	LHEVATPPAS	LSSRITARIK
Consensus	*-DA**G-S	D*HFE-YE-	-R*R-R-DG-	L-E*-PP--	*-***-R*K
	251				300
PPilB	VMAGLDISER	RKPQDGRIM	RVSK.TKSID	FRVNTLPTLW	GEKIVMRILD
TapB	VMARLDIAER	RLPQDGRIKL	KLSR.NKSM	MRVNTLPTMW	GEKIVIRLLD
FimN	VMADLDIAEK	RVQDGRIM	YVSD.TKAI	FRVNSLPTLW	GEKIVIRLLD
PilF	VMSRLDISEK	RIPQDGRIM	TFQGGKPEVD	FRVSTLPTLF	GEKIVMRILD
LPilB	VMSNLDISER	RIPQDGGFIM	KISK.SRAID	FRVSTCPTSA	GEKIVMRILD
Consensus	VM*-LDI*E-	R-PQDG-*	-----D	-RV---PT-GEK*V-R*L-	
	301				350
PPilB	SSSAQMGIDA	LGYEEDQKEL	YLAALKQPPG	MILVTGPTGS	GKTVSLYTLG
TapB	SSAARLNIEQ	LGFPDRQKQ	YLRALSFKPG	MILVTGPTGS	GKTVSLYTLG
FimN	SSAAKLNIIEI	LGFEPPQKQ	YLDALSKPQG	LVLVTGPTGS	GKTVSLYTLG
PilF	SDAASLNIDQ	LGFEPPQKRL	LLEAIRHPYG	MVLVTGPTGS	GKTVSLYTLG
LPilB	SAAAKLGIEA	LGFNVPQRTH	FLKAIQRQGG	MILVTGPTGS	GKTVLYTLAL
Consensus	S--A--I*-	LG-E-Q---	-L-A*-P-G	**LVTGPTGS	GKTV-LYT-L
	351				400
PPilB	NILNNTDINI	STAEDPVEIN	LEGINQVNVN	PRQGMDFSA	LRAFLRQDPP
TapB	NILNNTTEVNI	STAEDPVEIN	LPGVNQVQVN	PKAGLTFASA	LRSFLRQDPP
FimN	NILNKPVTNI	STAEDPVEIN	LPGINQVNVN	PKTGLDFSA	LKAFRLRQDPP
PilF	NILNTEVNI	ATAEDPAEIN	LPGINQVNVN	DKQGLTFASA	LKSFRLRQDPP
LPilB	NILNTEVNI	STAEDPVEIK	VPGINQVNVN	PKAGLTFESA	LRSFLRQDPP
Consensus	NILN---NI	-TAEDP-EI-	*-G*NQV-*N	---G*-F-A	L*FRLRQDPP
	401				450
PPilB	VIMVGEIRD	ETAETAIKAA	QTGHVMVSTL	HTNSAAETLT	RLLNMG.VFA
TapB	VIMVGEIRD	ETAETAIKAA	QTGHVVLSTL	HTNSAAETLT	RMMNMG.VFA
FimN	IIMVGEIRDI	ETAETAIKAA	QTGHVVLSTL	HTNDVPQTIA	RLVNI.G.IEP
PilF	IIMVGEIRD	ETAETAIKAA	QTGHVMVSTL	HTNNAPATLS	RMLNMG.VAP
LPilB	IIMVGEIRD	ETAETAIKAA	QTGHVVLSTL	HTNSAAETLN	RILVKGDTQL
Consensus	**MVGEIRD*	-TA*IA*KAA	QTGH*V-STL	HTN----T*-	R*---G----
	451				500
PPilB	FNLATSVNLI	IAQRLARKLC	SHCKK.EHDV	PKETLLHEGF	.PEELIGTFK
TapB	FNIASSVTLI	MAQRLARKLC	DNCKA.PEVV	PEAELELGF	TQQQLAAGRF
FimN	YNIASSVNLI	MAQRLARRLC	NNCKIRDRKH	HTEELLALGF	HEEDL.DDLK
PilF	FNIASSVSLI	MAQRLARRLC	SSCKQEVERP	SASALKVEGF	TDEDLAKDVK
LPilB	LNIASSVTLI	IAQRLARKLC	NQCKAVRDDF	THQGLIELGF	KESDLV.NLK
Consensus	-NIA*SV-LI	-AQRL-R-LC	---CK----	---L---GF	-----L----
	501				550
PPilB	LYSPVGCDC	K.NGYKGRVG	IYEVKNTPA	LQRIIMEEGN	SIEIAEQARK
TapB	LFKPVGCKDC	S.GGYKGRVG	IYIIMLMSEN	IAKLIQMGAN	SLQIAAIAQK
FimN	IYAPKGCERC	SYQGYGRAG	IYQVPISEA	IAELILKNA	AAETAEQCKL
PilF	LYGAVGCDRC	RGQGYKGRAG	YEVMPISSE	MQRVMNNGT	EVGLIDVAYK
LPilB	LYKAVGCEQC	T.SGYRGRVG	LFEVLPMTKE	LGQLIMSGGN	SLDILKLAQS
Consensus	**---GC-C-	---GY-GR-G	***-----	---I*----	---I----
	551				580
PPilB	EGFNDLRTSG	LLKAMQGITS	LEEIVNRVTKD		
TapB	EGMRTLRIISG	LEKARIGVTS	LAELINRVTTN		
FimN	EGYDLRQAA	LNKVKQGLTS	IAEIVLRVTSE		
PilF	EGMVDLRRAG	LLKIMQGITS	LEEVNTANTND		
LPilB	EGMLTIQPSG	IEKVKEGITT	IEEVNRVTVTD		
Consensus	EG---*--*	*-K---G*T-	*-E*---T--		

FIG. 2. Alignment of the deduced amino acid sequence of the *L. pneumophila* PilB protein (LPilB) with homologs from *P. aeruginosa* (PPilB), *A. hydrophila* (TapB), *D. nodosus* (FimN), and *N. gonorrhoeae* (PilF). The positions and identities of amino acids common to all five proteins are indicated on the last

mophila PilD and *P. aeruginosa* PilD (43% identity and 58% similarity) is less than that observed between *L. pneumophila* PilB or PilC and its respective homologs (Fig. 3). However, the *L. pneumophila* protein did contain a conserved tetracysteine domain which is thought to be important for the correct folding of the peptidase (Fig. 3) (38, 45). Overall, the G+C percentage of the *L. pneumophila* pilB, pilC, and pilD genes was 36.6%. This value is fairly close to the 39% G+C content associated with the *L. pneumophila* genome (6), suggesting that this locus is not a recent acquisition (43). Although we have not confirmed that these three ORFs express functional products, these sequence data do indicate that *L. pneumophila* contains a set of genes well known to participate in type II protein secretion. Furthermore, they represent the first recorded instance of a type II secretory system in an intracellular parasite. Given that *L. pneumophila* has pilBCD analogs, we strongly suspect that *L. pneumophila* also possesses the other components of the type II secretory system. Finally, the discovery of pilB, pilC, and pilD in strain 130b strongly suggested that *L. pneumophila* expresses type IV pili.

The genes required for pilin secretion are often adjacent to the type IV pilin gene. For example, in *P. aeruginosa*, pilA is located 192 bp upstream from pilB (29). Therefore, in an attempt to locate an *L. pneumophila* pilin gene, we sequenced the regions directly upstream of pilB (2 kb) and downstream of pilD (300 bp). The DNA sequences flanking pilB and pilD did not contain the pilin gene and did not have significant similarity to genes in the GenBank database (Fig. 1 and data not shown). However, Stone and Abu Kwaik report in this issue the discovery of an *L. pneumophila* 130b gene (pilE_L) that is required for the production of long pili and whose predicted product has strong homology to type IV pilins (44). Using a digoxigenin-labeled, 2-kb ClaI fragment from pBJ120 which contains pilE_L (44), we probed a Southern blot containing DNAs from all of our pilBCD plasmids and cosmids as well as a 130b control. No hybridization was observed except for one band in strain 130b (data not shown), indicating that there are at least two distinct regions of the *L. pneumophila* chromosome involved in type IV pilin biosynthesis. The arrangement of the *L. pneumophila* pilin biosynthetic genes thus appears to be most like that of *D. nodosus* (Fig. 4). However, chromosomal mapping and transcriptional analysis of both the pilBCD locus and pilE_L will be necessary to establish how similar the organizations of pilin biosynthetic genes are in these two pathogens.

In other organisms, the pilB-, pilC-, and pilD-like genes are often arranged in an operon (Fig. 4). In *L. pneumophila*, pilC began only 8 bp past the end of pilB, and pilD followed only 48 bp past pilC, suggesting that these three ORFs are also co-transcribed (Fig. 1). To confirm this hypothesis, we hybridized RNA isolated from *L. pneumophila* by using the Trizol reagent (Gibco-BRL) with a pilB-specific probe (Fig. 1). Since the legionellae exist in aquatic environments as well as in the mammalian lung, and since the expression of their flagella is greater at 30°C than at 37°C (34), we assessed the expression of pilB in 130b grown at both 30 and 37°C. The Northern blot

line by the conserved letter, whereas conservative amino acid changes are indicated on this line by asterisks. The position of the conserved nucleotide-binding domain (Walker sequence) is in boldface within the consensus sequence. Other species expressing PilB homologs include *Xanthomonas campestris*, enteropathogenic *E. coli*, *Klebsiella pneumoniae*, and *Vibrio cholerae* (data not shown). The sequences for all PilB analogs were obtained from GenBank at NCBI. For protein alignments, we used programs within the Genetics Computer Group Sequencing Analysis Software package (GCG, Madison, Wis.).

	1							50
PPiID	MPLLDYLASH	PLAFVLCAI	.LLGLLVGSF	LNVVHRLPK	MMERNWKAEA			
TapD	MALLLELA.H	GLPWLYFSLV	FLFSLMIGSF	LNVVHRLPI	MLEREWQAEY			
FimP	.MLISELLQF	PLGIFVVG..	.LFSLMVGSF	LNVVIYRVPV	MMDREKQYA			
NPiID	..MSDLS..	VLSPFVAVLA	AVLGLLVGSF	LNVVIYRVPV	MMERGWTFVA			
LPiID	..MINALII	NYPWFMYLVV	GLFSLAVGSL	LNVVIYRPLI	ILQEWEKQC			
Consensus	-----*	-----N	*--L--*GS-	LNV**--R-P-	---			
	51							100
PPiID	REALGLEPEP	.KQAT...YN	LVL PNSACPR	CGHEIRPWEN	IPLVSYLALG			
TapD	RSYFNPDDEG	VDEPP...YN	LMVPRSCCPH	CNHPITALEN	IPLLSWLWLR			
FimP	WQVFHGEDSV	CPEIPKQRFN	LLVPASRCPH	CGHRIRAIEN	IPVISWLFK			
NPiID	KEHLNL...P	LTDDSRTPN	LMKPDSCCPK	CRVPIRAWQN	IPIVSYLLLR			
LPiID	CBLFHFEQ..	.RKEKIKLN	LFLPRSFCPH	CKAMVKAWQN	IPLLAILVLR			
Consensus	-----*	-----N	L--P-S-C-P-	C---*-----N	IP***-L-L-			
	101							150
PPiID	GKCSSCKAAI	GKRYPLVELA	TALLSGYVAN	HFGFTWQAGA	MLLLTWGLLA			
TapD	GRCRCQAPI	SARYPLVELL	TALLSVAVAM	TLAPGWGTLA	ALLLTWVIVA			
FimP	GKCSGCGAAI	SARYLLVELL	TAALSIVAVF	HYHDFLSLGF	ALVFTWTLIA			
NPiID	GKASCQCQTKI	SIRYPLIEEL	TGVLFLGVAV	QYGSWITLGF	GLILTAFLIS			
LPiID	GRYQCDSPPF	SIRYPFVETL	TTVLSLYASW	HFGFTIQLLF	ALLAITWILS			
Consensus	G*C--C*-*	--RY--*E--	T*L-----	-----	-L-----L--			
	151							200
PPiID	MSLIDADHQL	LPDVLVLPPL	WGLIANHFG	LFASLDDALF	GAVFGYLSLW			
TapD	LTFLDLKML	LPDQLTLPPL	WGLLFLNLG	GFVSLGDAVI	GAMAGYLVLV			
FimP	LCFIDAEHQL	LPDRLTLPPL	WGLILAAALFN	VFINLESSVI	GAMIGYLSLW			
NPiID	LTFLDEDTQY	LPDSMTLPPL	WGLIFLNLDG	GFVPLQSAVL	GAVAGYSSLV			
LPiID	LVFLDLHQL	LPDSLTLGLL	WGLIANTQN	VEVSLDVAVL	SCAGAYLAW			
Consensus	---ID-*---	LPD--*--L-L*	W-G-*-*---	-F--L--**-	-----Y--LW			
	201							250
PPiID	SVEWLFKLVT	GREGMGYGDF	KLLAMLGAWG	GWQILPLTIL	LSSLVGAI..			
TapD	SLYWAFKLLT	GREGMGYGDF	KLLAALGAWL	GWQALPIVLL	LSSLVGAF..			
FimP	SVYWLFKLIT	GREGMGYGDF	KLLACLCAWQ	GAWMLPIILF	SAAILGMIYA			
NPiID	LLCAVYKLLT	GKTGMNGDF	KLIAALGAWL	GISALPVLIF	VSSLIGLVAA			
LPiID	LFINLFYIMT	CKVCMHGDF	KLFAAFGAWL	GWMLPIILL	ISSITGAI..			
Consensus	-----*L*T	-*-MG-GDF	KL-A---AW-	G---LP*---	***-G----			
	251							300
PPiID	LGVIMLRLRN	AESGTFIPFG	PYLAIAWGIA	LLWGDQITRT	YLQFAGEK..			
TapD	MGIGLILRLN	HQS.KPIFFG	PYLAIAWGIA	LLWGDSTIRW	YL..TNFL..			
FimP	LGIGLRM...	...GMPFG	PFLAIAWLT	FLYGAQIGQL	FGYPPA...			
NPiID	IVMRV....	.AKGRHFAPG	PALTVSGWII	FTANDSVWRA	VNWWTHPVR			
LPiID	IGLYLKLING	KSRDTAIPFG	PFLCISGLIA	MFWDGSIINW	YIGYWM....			
Consensus	*-*****	-----FG	P-L-***G-	-----	-----			

FIG. 3. Alignment of the deduced amino acid sequence of the *L. pneumophila* PilD protein (LPiID) with homologs from *P. aeruginosa* (PPiID), *A. hydrophila* (TapD), *D. nodosus* (FimP), and *N. gonorrhoeae* (NPiID). The positions and identities of amino acids common to all five proteins are indicated on the last line by the conserved letter, whereas conservative amino acid changes are indicated on this line by asterisks. The conserved tetracysteine domain is highlighted in boldface within the consensus sequence. Other species expressing *L. pneumophila* PilD homologs include *Xanthomonas campestris*, enteropathogenic *E. coli*, *Klebsiella pneumoniae*, and *Vibrio cholerae* (data not shown). The sequences for all PilD analogs were obtained from GenBank at NCBI. For protein alignments, we used programs within the Genetics Computer Group Sequencing Analysis Software package (GCG).

revealed a transcript of sufficient size (i.e., approximately 4 kb) to include all three ORFs, but only from the bacteria grown at 30°C (Fig. 5). No transcripts were detected in the bacteria grown at 37°C. To determine whether low-level reverse transcription occurred at 37°C, we used a more sensitive reverse transcrip-

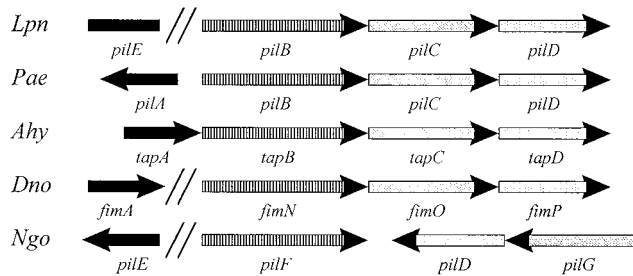


FIG. 4. Organization of type II secretory/pilus biogenesis genes in *L. pneumophila* (*Lpn*), *P. aeruginosa* (*Pae*), *A. hydrophila* (*Ahy*), *D. nodosus* (*Dno*), and *N. gonorrhoeae* (*Ngo*) (19, 22, 29, 35). Note that the orientation of the *Lpn* pilin gene with respect to the *Lpn pilBCD* operon is unknown. Common shading of the bars indicates homology between the proteins.

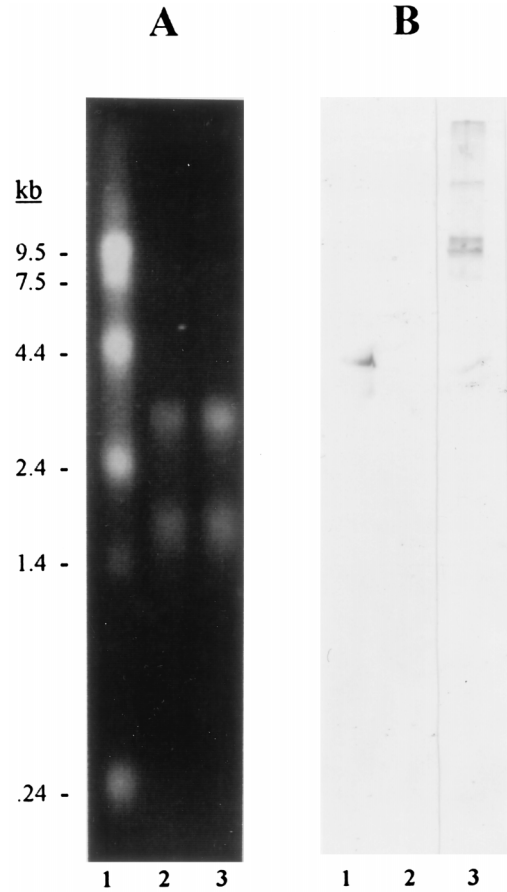


FIG. 5. Temperature-dependent expression of *pilBCD*. Five micrograms of bacterial RNA along with RNA size markers (Gibco-BRL) was electrophoresed through a 1% agarose-formaldehyde gel and then transferred onto nitrocellulose. After baking, the blot was incubated overnight at 50°C with a digoxigenin-labeled probe in the manufacturer's recommended hybridization solution (Boehringer Mannheim). After high-stringency washing, the hybridized probe was detected colorimetrically. Duplicate samples were stained with ethidium bromide to visualize the integrity and concentration of RNA. (A) Ethidium bromide-stained agarose-formaldehyde gel containing RNA markers (lane 1) and RNA from *L. pneumophila* grown at 30°C (lane 2) and 37°C (lane 3). Note that the 30°C sample contains no more, and likely less, RNA than the 37°C sample. (B) Northern blot hybridized with the *pilB*-specific probe. Samples include RNA isolated from *L. pneumophila* grown at 30°C (lane 1) and 37°C (lane 2) and *E. coli* XL1Blue (pML218) grown at 37°C (lane 3). The larger bands evident in lane 3, panel B, are most likely due to transcripts initiating from a vector promoter(s).

tase PCR assay. Briefly, total RNA was first treated with RNase-free DNase for 2 h at 37°C. The RNA was then precipitated, and the DNase treatment was repeated three times until control PCRs indicated no residual DNA contamination. After addition of random hexamers, reverse transcriptase, and RNase inhibitors to 1 µg of *L. pneumophila* RNA, the reaction mixtures were incubated at 42°C for 1 h, followed by 10 min at 94°C. With the cDNA as template, PCR was performed with *pilB*-specific primers (Fig. 1). We detected the expected 1,048-bp PCR product, indicating that *pilB* mRNA exists within bacteria grown at 37°C (data not shown). Taken together, these data suggest that, although the *L. pneumophila pilBCD* genes are similar both in their predicted products and in their organization to type II secretory genes in other gram-negative bacteria, they are unique in terms of their regulation. The difference between the levels of *pilB* expression at 30 and

37°C suggests that *pilBCD* is regulated in a manner similar to that observed with the *L. pneumophila* flagellin gene, i.e., transcriptional control by the alternative σ^{28} -like RpoF factor (16). In support of this notion, the promoter region of the *pilBCD* operon appears to possess some elements of the σ^{28} consensus sequence (Fig. 1).

The increase in the level of *pilBCD* transcripts at 30°C suggested that piliation in *L. pneumophila* is also controlled by temperature. To address this hypothesis, we grew strain 130b on buffered charcoal yeast extract agar for 72 h at either 30 or 37°C and then examined bacteria by electron microscopy. To visualize pili on the surface of *L. pneumophila*, we employed a slight variation of the method described by Ruffolo et al. (42). Briefly, 100 μ l of sterile phosphate-buffered saline was placed on isolated colonies of strain 130b, and then Formvar-coated copper grids (Ladd Industries, Burlington, Vt.) were placed gently onto the wetted colonies. After 2 min, the grids were removed, and excess saline was wicked off with Whatman no. 3 filter paper. Bacteria adherent to the grid were stained with 10 μ l of 1% phosphotungstic acid (PTA; Sigma Chemical Co., St. Louis, Mo.) for 1 min, after which the PTA was carefully removed with filter paper, and the grids were allowed to air dry for several minutes. Finally, stained bacteria were visualized on a JEOL JEM-100 CxII transmission electron microscope at 60 kV. When grown at 30°C, on average 5 to 10% of bacteria had pili, with many unattached pili also present on the grids, but on rare occasions up to 50% of the bacteria could be seen to possess pili. Typically, we saw only one pilus per cell that was of a length, diameter, and position comparable to those observed by others (40, 44) (Fig. 6A). Bacteria with multiple pili radiating from their surfaces were also noticed (Fig. 6B). It is possible that these multiple pilin strands can form a cohesive bundled pilus as seen with the bundle-forming pilus of enteropathogenic *Escherichia coli* (12). In contrast, we did not see pili on bacteria grown at 37°C, and only rarely could a flagellum be found (Fig. 6C). This temperature-dependent expression of pili was observed in three independent experiments, with hundreds of bacteria being examined on each occasion. The lower incidence of piliation at 37°C in our study compared to others is likely due to differences in growth conditions. For example, Rodgers et al. observed piliated *L. pneumophila* when strains were grown on enriched blood agar (41). Similarly, Stone and Abu Kwaik examined strain 130b after growth in static buffered yeast extract broth, a method differing from ours in O₂ concentration, the presence of agar, and the general stage of bacterial growth (44). Currently, it is unknown whether the temperature-induced alteration in piliation results simply from the observed changes in *pilBCD* transcription or also requires changes in *pilE_L* expression. Other temperature-regulated pili include the type IV bundle-forming pilus and the M pilus of *E. coli* (21, 48). Whereas the M pilus, like the *L. pneumophila* pilus, is minimally expressed at 37°C, the bundle-forming pilus is hyperexpressed at the elevated temperature. Although temperature-regulated piliation is not novel, this is, to our knowledge, the first demonstration of temperature-dependent expression of type II secretory genes.

In addition to *L. pneumophila*, the *Legionella* genus contains 40 other species, with half of these being associated with disease (2). Pili have been detected, but not classified, in strains of *L. micdadei*, *L. birminghamensis*, *L. gormanii*, and *L. james-towniensis* as well as strains from *L. pneumophila* serogroups 1 to 6, but not in a strain of *L. longbeachae* (40). Thus, we tested various *L. pneumophila* serogroups and *Legionella* species for hybridization with the *pilB*-specific probe (Table 1). *L. pneumophila* strains representing serogroups 2 to 5 and 8 to 14 hybridized under high-stringency conditions (permitting ca.

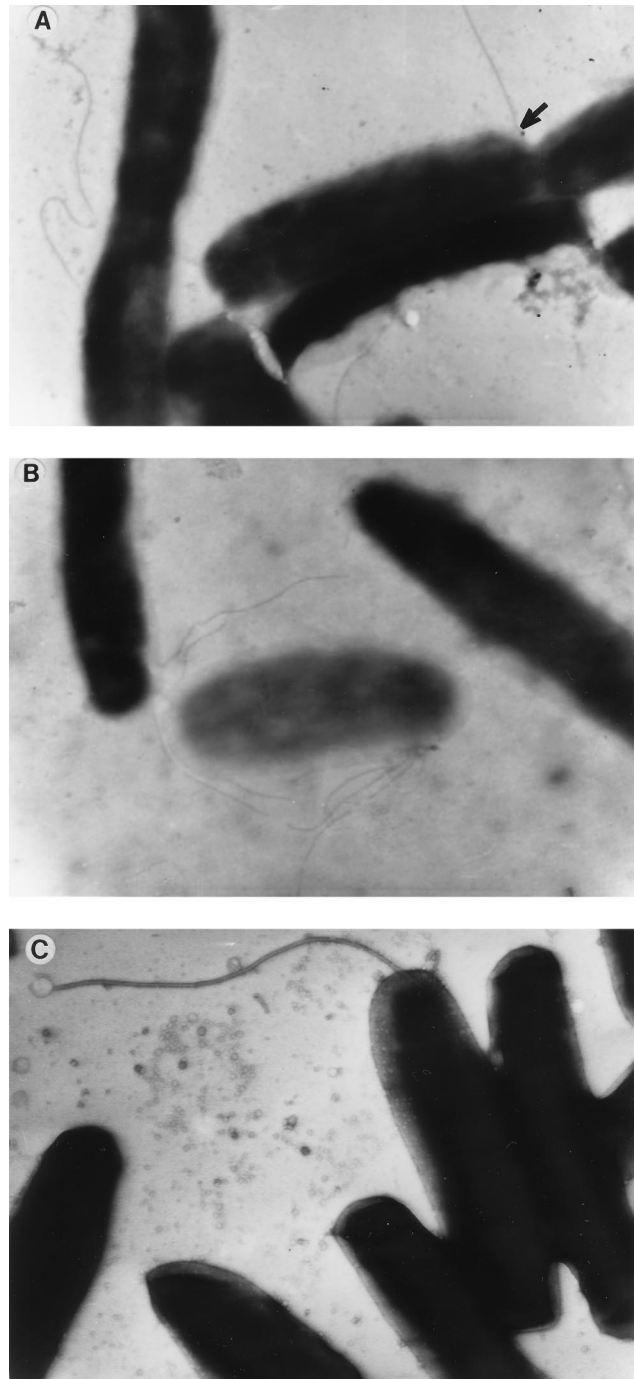


FIG. 6. Temperature-dependent piliation of *L. pneumophila*. Bacteria were grown at either 30°C (A and B) or 37°C (C), stained with PTA, and examined by transmission electron microscopy. (A) Three different bacteria grown at 30°C possess a single pilus. One of the pilus structures may represent the bundling of two or more individual fibers (see arrow for possible fusion point). (B) Multiple pili are seen radiating from two different bacteria also grown at 30°C. (C) One of the bacteria grown at 37°C has a flagellum, but none of the cells have pili. Note the significantly larger diameter of the flagellum in panel C in comparison to the thinner pili in the first two panels. All electron micrographs are at a magnification of ca. $\times 17,000$.

10% base pair mismatch) with the probe, giving a single band that varied in size (data not shown). Similarly, 14 other *Legionella* species tested hybridized with *pilB* DNA, albeit under low-stringency conditions (permitting ca. 30% base pair mis-

TABLE 1. *Legionella* strains^a used in this study

Sp.	Strain	Serogroup	Implicated in disease
<i>L. pneumophila</i>	130b (Wadsworth)	1	Yes
<i>L. pneumophila</i>	ATCC 33154	2	Yes
<i>L. pneumophila</i>	ATCC 33155	3	Yes
<i>L. pneumophila</i>	ATCC 33156	4	Yes
<i>L. pneumophila</i>	ATCC 33216	5	Yes
<i>L. pneumophila</i>	ATCC 35096	8	Yes
<i>L. pneumophila</i>	MDPH ^b	9	Yes
<i>L. pneumophila</i>	MDPH ^b	10	Yes
<i>L. pneumophila</i>	MDPH ^b	11	Yes
<i>L. pneumophila</i>	MDPH ^b	12	Yes
<i>L. pneumophila</i>	B2A3105	13	Yes
<i>L. pneumophila</i>	1169-MN-H	14	Yes
<i>L. birthingamensis</i>	1407-AL-H		Yes
<i>L. erythra</i>	SE-32A		No
<i>L. gormanii</i>	ATCC 33297		Yes
<i>L. feeleii</i>	WO-44C		Yes
<i>L. hackeliae</i>	Lansing 2		Yes
<i>L. israelensis</i>	Bercovier 4		No
<i>L. jamestowniensis</i>	JA-26		No
<i>L. longbeachae</i>	ATCC 33462		Yes
<i>L. micdadei</i>	Rivera		Yes
<i>L. oakridgensis</i>	OR-10		Yes
<i>L. parisiensis</i>	PF-209		Yes
<i>L. sainthelensii</i>	Mount St. Helens 4		Yes
<i>L. santicrucis</i>	SC-63		No
<i>L. spiritensis</i>	MSH-9		No

^a For the origins of these strains and their disease associations, see reference 8, but in the case of *L. micdadei* refer to reference 32 and for *L. parisiensis* see reference 39.

^b Obtained from the Michigan Department of Public Health (MDPH).

match [data not shown]). With the exception of *L. israelensis*, the intensity of the bands from the various species was noticeably weaker than that from *L. pneumophila*, despite equivalent amounts of genomic DNA being analyzed for each sample. Nevertheless, these data indicate that *pilBCD* is conserved in the *Legionella* genus and suggest that many legionellae have the genetic potential to express type IV pili. Finally, since *L. pneumophila* as well as other *Legionella* species secretes enzymes and toxins (11), it is likely that the *pilBCD* operon facilitates *Legionella* growth and pathogenesis in multiple ways.

Nucleotide sequence accession number. The *L. pneumophila pilBCD* sequence is deposited in the GenBank database at the National Center for Biotechnology Information (NCBI) under accession no. AF038655.

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