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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-Tn*10* 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-Tn*10* 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-Tn*10* insertions in our iron uptake mutants (31). More specifically, 5 μg of genomic DNA was digested with *Hin*dIII, an enzyme which cuts once within the mini-Tn*10*, 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′-GTG ACGACTGAATCCGGT) that recognize sequences on either side of the transposon's *Hin*dIII site as well as a primer (5′-C CTTAACTTAATGATTTTTAC) 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 *Hin*dIII 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-Tn*10* 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-*

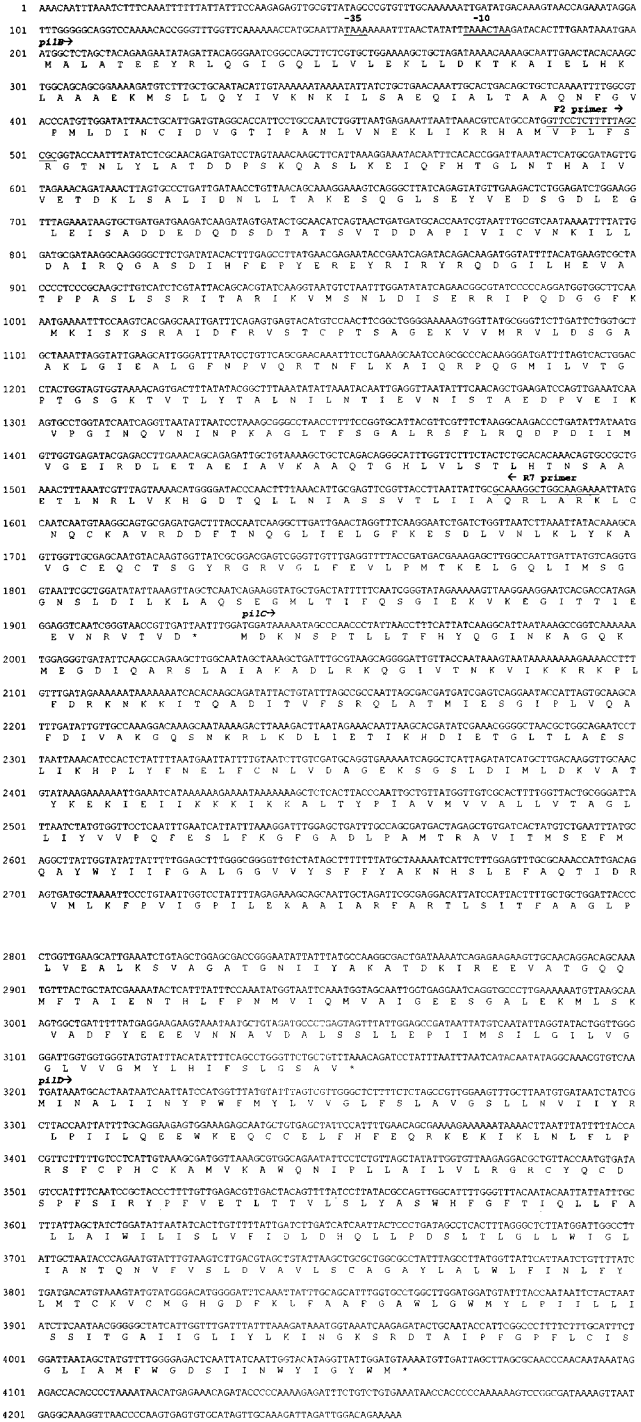

FIG. 1. Nucleotide sequence of the *L. pneumophila pilBCD* genes. The deduced amino acid sequences of the three ORFs and the termination codons (*) are indicated. The direction of transcription-translation of each ORF is indicated by a horizontal arrow. The possible binding sites for the alternative 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.).

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           1                                                  50
   PPilB   ...MNDSIQL SGLSRQLVQA NLLDEKTALQ AQTQAQRNKL SLVTHLVQNK
    TapB   ....MTSSPN SGLALSLAAS SLLSESDSQR YLSQAKAQRK PFVTFLIENE
    FimN   ......MSEY DELIAYMVKN KVATPEQLKE VQISVQKKRG NFLQQLLSDG
    PilF   ........MS VGLLRILVQN QVVTVEQAEH YYNESQAGK. EVLPMLFSDG
   LPilB   MALATEEYRL QGIGQLLVLE KLLDKTKAIE LHKLAAAEKM SLLQYIVKNK
Consensus  ---------- --*---*--- ---------- ---------- --*--*----

           51                                                 100
   PPilB   LVSGLALAEL SAEQFGIAYC DLNSLDRESF PRDAISEKLV RQHRVIPLWR
    TapB   ILDSKALADF CELEYGVPLL DLAAFDLAEI PQKYLNQKLI EKHHVLPIYT
    FimN   LLDASKLSKI NREVYNLPIV ALRELNIRRE LTAQFKEAVV RKHTAMPVYA
    PilF   VISPKSLAAL IARVFSYSIL DLRHYPRHRV LMGVLTEEQM VEFHCVPVFR
   LPilB   ILSAEQIALT AAQNFGVPML DINCIDVGTI PANLVNEKLI KRHAMVPLFS
Consensus  **----*--- ---------- -*-------- ---------- ------P*--

           101                                                150
   PPilB   RGNKLFVGIS DAANHQAIND VQFSTGLTT. EAILVEDDKL GLAIDKLFEN
    TapB   QGHTLYIAMS DPTNVSALED FGFSFGLHT. EALLVEENKL TTAIGKLLES
    FimN   NGGRLFIATI DPNNRSMLEE FKYQGKFTSV EPIIANLDAI EALIEEHYAG
    PilF   RGDKVFFAVS DPTQMPQIQK TVSAAGI.AV ELVIVEDDQL AGLLDWVGSR
   LPilB   RGTNLYLATD DPSKQASLKE IQFHTGLNT. HAIVVETDKL SALIDNLL.T
Consensus  -G--**---- D--------- ---------- --**-----* ---*------

           151                                                200
   PPilB   ATD..GLAGL ..DDVDLEGL DVGVKETSGQ EDTGAE.ADD APVVRFVNKM
    TapB   DQDALGMEDI ..DESEISEL EVSDENSRLD ESVNTT.DDD APIVKYINKI
    FimN   LGGMDDLFDD ETEEKDLDAI SNALGGLDAN E.......EE APVVRFVTGM
    PilF   STSLLQELGE GQEEEEESHTL YIDNEE.... .......AED GPVPRFIHKT
   LPilB   AKESQGLSEY VEDSGDLEGL EISADDEDQD SDTATSVTDD APIVICVNKI
Consensus  ---------- ---------* ---------- ---------- -P*---*---

           201                                                250
   PPilB   LLDAIKGGSS DLHFEPYEKI YRVRFRTDGM LHEVAKPPIQ LASRISARLK
    TapB   MMDAIKRGAS DLHFEPYETK YRIRFRIDGI LHEIATPPVN LANRFSARLK
    FimN   LLDAIRTGAS DLHFEPYETT YRVRFRRDGV LQEVAAPPPS IATRITARLK
    PilF   LSDALRSGAS DIHFEFYEHN ARIRFRVDGQ LREVVQPPIA VRGQLASRIK
   LPilB   LLDAIRQGAS DIHFEPYERE YRIRYRQDGI LHEVATPPAS LSSRITARIK
Consensus  *-DA**-G*S D*HFE-YE-- -R*R*R-DG- L-E*--PP-- *-----*R*K

           251                                                300
   PPilB   VMAGLDISER RKPQDGRIKM RVSK.TKSID FRVNTLPTLW GEKIVMRILD
    TapB   VMARLDIAER RLPQDGRIKL KLSR.NKSMD MRVNTLPTMW GEKIVIRLLD
    FimN   VMADLDIAEK RVPQDGRIKL YVSD.TKAID FRVNSLPTLW GEKIVLRILD
    PilF   VMSRLDISEK RIPQDGRMQL TFQKGGKPVD FRVSTLPTLF GEKVVMRILN
   LPilB   VMSNLDISER RIPQDGGFKM KISK.SRAID FRVSTCPTSA GEKVVMRVLD
Consensus  VM*-LDI*E- R-PQDG--** ---------D -RV---PT-GEK*V-R*L-

           301                                                350
   PPilB   SSSAQMGIDA LGYEEDQKEL YLAALKQPQG MILVTGPTGS GKTVSLYTGL
    TapB   SSAARLNIEQ LGFDDRQKAQ YLRALSKPQG MILVTGPTGS GKTVSLYTGL
    FimN   SSAAKLNIEI LGFEPDQKQQ YLDAISKPQG LVLVTGPTGS GKTVSLYTGL
    PilF   SDAASLNIDQ LGFEPFQKKL LLEAIHRPYG MVLVTGPTGS GKTVSLYTCL
   LPilB   SGAAKLGIEA LGFNPVQRTN FLKAIQRPQG MILVTGPTGS GKTVTLYTAL
Consensus  S--A-*-I*- LG-E--Q--- -L-A*--P-G **LVTGPTGS GKTV-LYT-L

           351                                                400
   PPilB   NILNTTDINI STAEDPVEIN LEGINQVNVN PRQGMDFSQA LRAFLRQDPD
    TapB   NILNTTEVNI STAEDPVEIN LPGVNQVQVN PKAGLTFASA LRSFLRQDPD
    FimN   NILNKPTVNI STAEDPVEIN LPGINQVNVN PKTGLDFSAA LKAFLRQDPD
    PilF   NILNTESVNI ATAEDPAEIN LPGINQVNVN DKQGLTFAAA LKSFLRQDPD
   LPilB   NILNTIEVNI STAEDPVEIK VPGINQVNIN PKAGLTFSGA LRSFLRQDPD
Consensus  NILN----NI -TAEDP-EI- *-G*NQV-*N ---G*-F*-A L**FLRQDPD

           401                                                450
   PPilB   VIMVGEIRDL ETAEIAIKAA QTGHMVMSTL HTNSAAETLT RLLNMG.VPA
    TapB   VVMVGEIRDL ETAEIAIKAA QTGHLVLSTL HTNSAAETLT RMMNMG.VPA
    FimN   IIMVGEIRDI TTAEIAVKAA QTGHLVLSTL HTNDVPQTIA RLVNIG.IEP
    PilF   IIMVGEIRDL ETADIAIKAA QTGHMVFSTL HTNNAPATLS RMLNMG.VAP
   LPilB   IIMVGEIRDL ETAEIAVKAA QTGHLVLSTL HTNSAAETLN RLVKHGDTQL
Consensus  **MVGEIRD* -TA*IA*KAA QTGH*V-STL HTN----T*- R*---G----

           451                                                500
   PPilB   FNLATSVNLI IAQRLARKLC SHCKK.EHDV PKETLLHEGF .PEELIGTFK
    TapB   FNIASSVTLI MAQRLARKLC DHCKA.PEVV PEAELLELGF TQQQLAAGLR
    FimN   YNIAASVNLI MAQRLARRLC NNCKIRDRKH HTEELLALGF HEEDL.DDLK
    PilF   FNIASSVSLI MAQRLLRRLC SSCKQEVERP SASALKEVGF TDEDLAKDWK
   LPilB   LNIASSVTLI IAQRLARKLC NQCKAVRDDF TNQGLIELGF KESDLV.NLK
Consensus  -NIA*SV-LI -AQRL-R-LC --CK------ ----L---GF ----L-----

           501                                                550
   PPilB   LYSPVGCDHC K.NGYKGRVG IYEVVKNTPA LQRIIMEEGN SIEIAEQARK
    TapB   LFKPVGCKDC S.GGYKGRVG IYEIMLMSEN IAKLIMQGAN SLQIAAIAQK
    FimN   IYAPKGCERC SYQGYRGRAG IYQVVPISEA IAELILKNAS AAEIAEQCLK
    PilF   LYGAVGCDRC RGQGYKGRAG VYEVMPISEE MQRVIMNNGT EVGILDVAYK
   LPilB   LYKAVGCEQC T.SGYRGRVG LFEVLPMTKE LGQLIMSGGN SLDILKLAQS
Consensus  **---GC--C ---GY-GR-G ***----*-- ----I*---* ---I------

           551                          580
   PPilB   EGFNDLRTSG LLKAMQGITS LEEVNRVTKD
    TapB   EGMRTLRISG LEKARIGVTS LAEINRVTTN
    FimN   EGYLDLRQAA LNKVKQGLTS IEEVLRVTSE
    PilF   EGMVDLRRAG ILKIMQGITS LEEVTANTND
   LPilB   EGMLTIFQSG IEKVKEGITT IEEVNRVTVD
Consensus  EG---*--*- *-K---G*T- *-E*---T--
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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 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.).

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 *Cla*I 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 cotranscribed (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

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           1                                                   50
   PPilD   MPLLDYLASH PLAFVLCAI. .LLGLLVGSF LNVVVHRLPK MMERNWKAEA
    TapD   MALLLELA.H GLPWLYFSLV FLFSLMIGSF LNVVIHRLPI MLEREWQAEY
    FimP   .MLISELLQT PLGIFFVG.. .LFSLMVGSF LNVVIYRVPV MMDREEKQYA
   NPilD   ...MSDLS.. VLSPFAVPLA AVLGLLVGSF LNVVIYRVPV MMERGWTVFA
   LPilD   ...MINALII NYPWFMYLVV GLFSLAVGSL LNVIIYRLPI ILQEEWKEQC
Consensus  ---------- ---------- -*--L-*GS- LNV**-R-P- ***-------

           51                                                  100
   PPilD   REALGLEPEP .KQAT...YN LVLPNSACPR CGHEIRPWEN IPLVSYLALG
    TapD   RSYFNPDDEG VDEPP...YN LMVPRSCCPH CNHPITALEN IPLLSWLWLR
    FimP   WQVFHGEDSV CPEIPKQRFN LLVPASRCPH CGHRIRAIEN IPVISWLFLK
   NPilD   KEHLNL...P LTDDESRTFN LMKPDSCCPK CRVPIRAWQN IPIVSYLLLR
   LPilD   CELFHFEQ.. ..RKEKIKLN LFLPRSFCPH CKAMVKAWQN IPLLAILVLR
Consensus  ------*--- ---------N L--P-S-CP- C---*----N IP***-L-L-

           101                                                 150
   PPilD   GKCSSCKAAI GKRYPLVELA TALLSGYVAW HFGFTWQAGA MLLLTWGLLA
    TapD   GRCRGCQAPI SARYPLVELL TALLSVAVAM TLAPGWGTLA ALLLTWVLVA
    FimP   GKCSGCGAAI SARYLLVELL TAALSVIVAF HYHDPLSLGF ALVFTWTLIA
   NPilD   GKCASCQTKI SIRYPLIELL TGVLFGLVAW QYGWSWITLG GLILTAFLIS
   LPilD   GRCYQCDSPF SIRYPFVETL TTVLSLYASW HFGFTIQLLF ALLAIWILIS
Consensus  G*C--C-*-- --RY--*E-- T-*L------ ---------- -L-----L--

           151                                                 200
   PPilD   MSLIDADHQL LPDVLVLPLL WLGLIANHFG LFASLDDALF GAVFGYLSLW
    TapD   LTFIDLDKML LPDQLTLPLL WGGLLFNLLG GFVSLGDAVI GAMAGYLVLW
    FimP   LCFIDAEHQL LPDRLTLPLL WLGILAALFN VFINLESSVI GAMIGYLSLW
   NPilD   LTFIDEDTQY LPDSMTLPLI WLGLIFNLDG GFVPLQSAVL GAVAGYSSLW
   LPilD   LVFIDLDHQL LPDSLTLGLL WIGLIANTQN VFVSLDVAVL SCAGAYLALW
Consensus  ---ID-*--- LPD-*-L-L* W-G-*-*--- -F--L--**- -----Y--LW

           201                                                 250
   PPilD   SVFWLFKLVT GKEGMGYGDF KLLAMLGAWG GWQILPLTIL LSSLVGAI..
    TapD   SLYWAFKLLT GKEGMGYGDF KLLAALGAWL GWQALPIVLL LSSLVGAF..
    FimP   SVYWLFKLIT GREGMGYGDF KLLACLCAWQ GAWMLPIILF SAAILGMIYA
   NPilD   LLCAVYKLLT GKTGMGNGDF KLIAALGAWL GISALPVLIF VSSLIGLVAA
   LPilD   LFINLFYLMT CKVCMGHGDF KLFAAFGAWL GWMYLPIILL ISSITGAI..
Consensus  -----*-L*T -*--MG-GDF KL-A---AW- G---LP*--- -***-G----

           251                                                 300
   PPilD   LGVIMLRLRN AESGTPIPFG PYLAIAGWIA LLWGDQITRT YLQFAGFK..
    TapD   MGIGLILLRN HQS.KPIPFG PYLAIAGWIA LLWGDSITRW YL..TNFL..
    FimP   LGIGLRM... ....GPMPFG PFLAIAGWLT FLYGAQIGQL FGYFPA....
   NPilD   IVMRV..... .AKGRHFAFG PALTVSGWII FTANDSVWRA VNWWLTHPVR
   LPilD   IGLIYLKING KSRDTAIPFG PFLCISGLIA MFWGDSIINW YIGYWM....
Consensus  *-*------- --------FG P-L-**G--- ------*--- ----------
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FIG. 3. Alignment of the deduced amino acid sequence of the *L. pneumophila* PilD protein (LPilD) with homologs from *P. aeruginosa* (PPilD), *A. hydrophila* (TapD), *D. nodosus* (FimP), and *N. gonorrhoeae* (NPilD). 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).

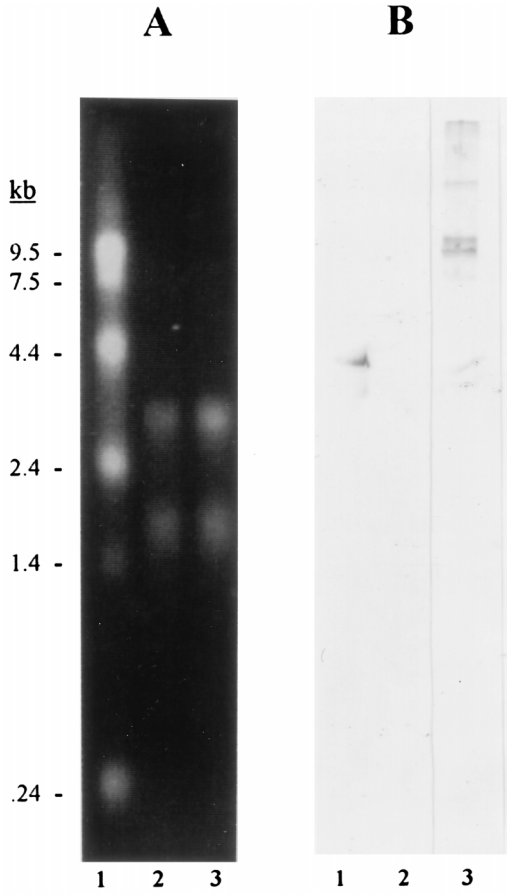

FIG. 5. Temperature-dependent expression of *pilBCD*. Five micrograms of bacterial RNA along with RNA size markers (Gibco-BRL) was electrophoresed through a 1% agarose-formaldehyde gel and then transferred onto nitrocellulose. After baking, the blot was incubated overnight at 50°C with a digoxigenin-labeled 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).

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 transcription occurred at 37°C, we used a more sensitive reverse transcriptase 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

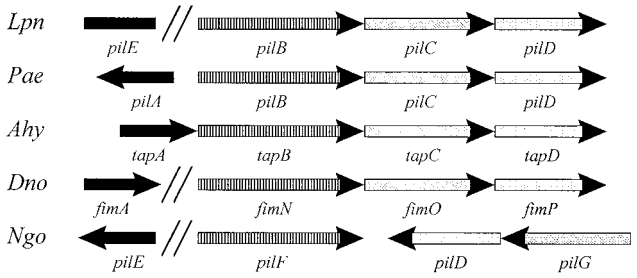

FIG. 4. Organization of type II secretory/pilus biogenesis genes in *L. pneumophila* (*Lpn*), *P. aeruginosa* (*Pae*), *A. hydrophila* (*Ahy*), *D. nodosus* (*Dno*), and *N. gonorrhoeae* (*Ngo*) (19, 22, 29, 35). Note that the orientation of the *Lpn* pilin gene with respect to the *Lpn pilBCD* operon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

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 μl 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 μl 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 O_2 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. jamestowniensis* 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. 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-

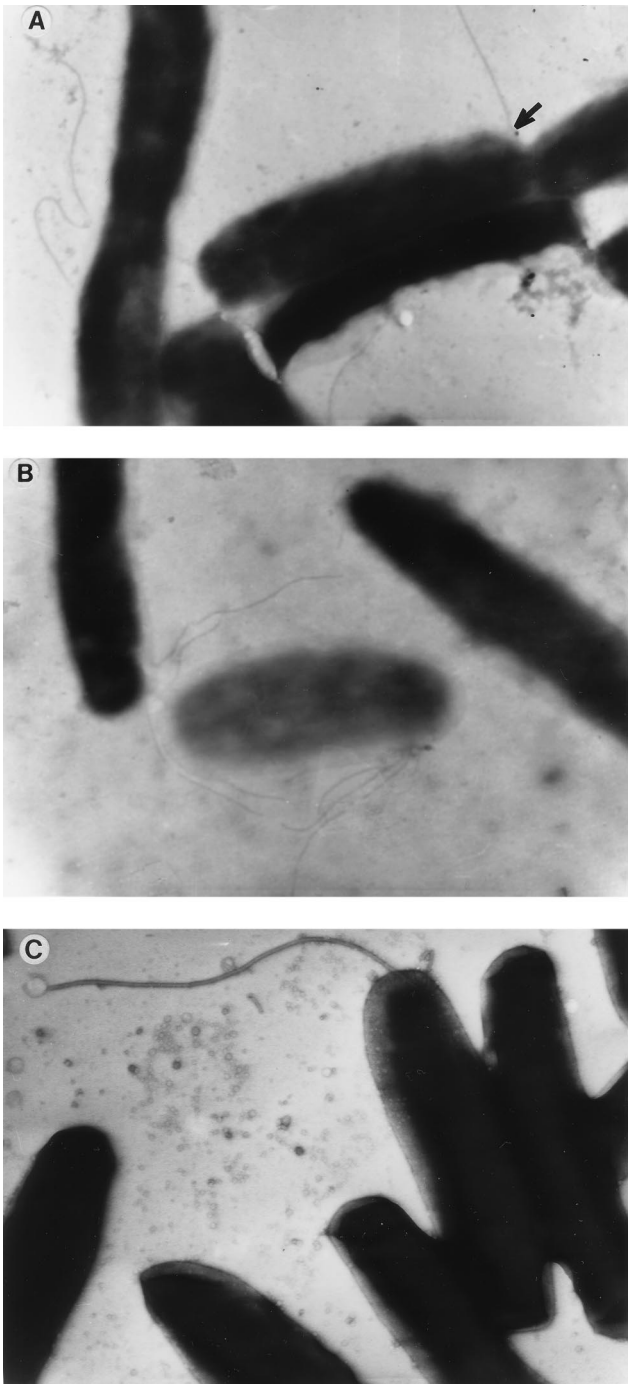

FIG. 6. Temperature-dependent piliation of *L. pneumophila*. Bacteria were grown at either 30°C (A and B) or 37°C (C), stained with PTA, and examined by transmission electron microscopy. (A) Three different bacteria grown at 30°C possess a single pilus. One of the pilus structures may represent the bundling 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. ×17,000.

TABLE 1. *Legionella* strains[a] used in this study

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