The Type II Protein Secretion System of *Legionella pneumophila* Promotes Growth at Low Temperatures

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The gram-negative bacterium *Legionella pneumophila* grows in both natural and man-made water systems and in the mammalian lung as a facultative intracellular parasite. The PilD prepilin peptidase of *L. pneumophila* promotes type IV pilus biogenesis and type II protein secretion. Whereas pili enhance adherence, *Legionella* type II secretion is critical for intracellular growth and virulence. Previously, we observed that *pilD* transcript levels are greater in legionellae grown at 30 versus 37°C. Using a new *pilD::lacZ* fusion strain, we now show that *pilD* transcriptional initiation increases progressively as *L. pneumophila* is grown at 30, 25, and 17°C. *Legionella pilD* mutants also had a dramatically reduced ability to grow in broth and to form colonies on agar at the lower temperatures. Whereas strains specifically lacking type IV pili were not defective for low-temperature growth, mutations in type II secretion (*lsp*) genes greatly impaired the capacity of *L. pneumophila* to form colonies at 25, 17, and 12°C. Indeed, the *lsp* mutants were completely unable to grow at 12°C. The growth defect of the *pilD* and *lsp* mutants was complemented by reintroduction of the corresponding intact gene. Interestingly, the *lsp* mutants displayed improved growth at 25°C when plated next to a streak of wild-type but not mutant bacteria, implying that a secreted, diffusible factor promotes low-temperature growth. Mutants lacking either the known secreted acid phosphatases, lipases, phospholipase C, lysophospholipase A, or protease grew normally at 25°C, suggesting the existence of a critical, yet-to-be-defined exoprotein(s). In summary, these data document, for the first time, that *L. pneumophila* replicates at temperatures below 20°C and that a bacterial type II protein secretion system facilitates growth at low temperatures.

The genus *Legionella* was recognized in 1977, after the isolation of *L. pneumophila* from patients that had succumbed to a form of pneumonia now known as Legionnaires' disease (29). The genus currently includes 49 species of gram-negative bacteria that inhabit aquatic environments (24, 62). Human infection occurs after the inhalation of contaminated water droplets originating from aerosol-generating devices (13, 24), and disease follows from bacterial invasion of lung macrophages (16, 80). *L. pneumophila* is now a common cause of community- and hospital-acquired pneumonia (13, 24).

Shortly after its discovery, *L. pneumophila* was detected in virtually all of the 267 freshwater habitats (i.e., lakes, ponds, rivers, creeks, swamps, and wet soil) examined in the United States (27, 28). The ubiquity of *L. pneumophila* in freshwater has been confirmed throughout the world (10, 14, 20, 39, 44, 58, 83, 84) and, in recent years, the organism has been found in marine and estuarine environments (34, 58, 59, 63). In artificial water systems, *L. pneumophila* is similarly widespread, existing in some areas within the plumbing systems of 60% of large and small public buildings, as well as in private residences (1, 45). The broad distribution of *L. pneumophila* is partly due to the organism's capacity to survive at 4 to 63°C (7, 27, 28, 34, 39, 89). However, since the bacterium grows best in the laboratory at 32 to 37°C (11, 42, 43, 91), there are few data on its physiology at lower temperatures (52). In its aquatic habitats, *L. pneumophila* exists planktonically, sessile within biofilms, as an intracellular parasite of protozoa, and as a viable but noncul-

turable entity (7, 18, 23, 33, 38, 50, 54, 63, 64). The definition of the bacterial factors that promote intracellular infection and low-temperature growth is key to fully understanding the natural history of legionellosis (16, 24, 80).

In previous studies (47, 48), our investigators determined that the PilD prepilin peptidase is required for the formation of *L. pneumophila* pili, surface appendages that enhance attachment to macrophages, amoebae, and biofilms (76; C. E. Lucas, E. Brown, T. S. Forster, R. Murga, R. M. Donlan, N. P. Cianciotto, Y. Abu Kwaik, and B. S. Fields, Abstr. Am. Soc. Microbiol. 102nd Annu. Meet., abstr. Q-258, p. 422, 2002). In later work (69), our group determined that PilD also promotes *L. pneumophila* type II secretion, a form of protein export that others and we have shown to be critical for intracellular growth (31, 68, 70). In the gram negatives, PilD promotes pilus biogenesis and protein secretion by cleaving and then methylating distinct sets of pilin-like proteins (pseudopilins) that form either the type IV pilus scaffold or the type II secretion apparatus (2, 12, 56, 57, 66, 73, 79). Using a murine model of pneumonia, our investigators recently showed that PilD and type II protein secretion, but not type IV pili, play major roles in Legionnaires' disease (70). Secreted activities associated with the *L. pneumophila* type II system include protease, acid phosphatase, RNase, lipase, phospholipase A, phospholipase C, and lysophospholipase activities (4–6, 25, 26, 31, 69, 70). Importantly, the *Legionella* type II secretion pathway (Lsp) remains the only type II system linked to intracellular infection (70, 71, 74).

In contrast to the progress made toward elucidating the components of the type II secretion apparatus, there are few data on the regulation of its genes, although quorum sensing has been linked to *xcp* expression in *Pseudomonas aeruginosa* (15, 73, 74). While demonstrating that *L. pneumophila* piliation

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TABLE 1. *L. pneumophila* strains and their efficiency of plating (EOP) at room temperature

Strain	Mutation(s)	Deficiency	Reference	EOP at 25°C[a]
130b	Wild type	None	22	61 ± 33
NU286	*pilD::lacZ Km*^{r}	Prepilin peptidase	This study	0.032 ± 0.009
NU243	*pilD::Km*^{r}	Prepilin peptidase	47	0.036 ± 0.036
NU272	*pilD::Gm*^{r}	Prepilin peptidase	70	0.003 ± 0.003
NU258	*lspDE::Km*^{r}	Type II secretin and ATPase	69	0.028 ± 0.021
NU275	*lspF::Km*^{r}	Type II membrane protein	70	0.010 ± 0.002
BS100	*pilE$_L$::Km*^{r}	Type IV pilus pilin	76	52 ± 12
NU279	*pilQ::Gm*^{r}	Type IV pilus secretin	70	37 ± 5
NU283	*lspDE::Km*^{r}	Type II secretin and ATPase	70	0.0005 ± 0.0001
	pilQ::Gm^r	Type IV pilus secretin		
GG105	*dotA::Km*^{r}	Type IV secretion	92	57 ± 21
GQ262	*dotDCB::Km*^{r}	Type IV secretion	92	39 ± 27
GN142	*icmGCD::Km*^{r}	Type IV secretion	92	68 ± 7
NU291	*pilD::Gm*^{r}	Prepilin peptidase	This study	0.006 ± 0.006
	icmGCD::Km^r	Type IV secretion		
AA200	*proA::Km*^{r}	ProA metalloprotease	53	73 ± 42
NU254	*map::Km*^{r}	Major acid phosphatase	4	74 ± 26
NU267	*lipA::Km*^{r}	LipA and LipB lipases	6	109 ± 5
	lipB::Gm^r			
NU268	*plcA::Km*^{r}	PlcA phospholipase C	6	87 ± 18
NU270	*plaA::Km*^{r}	PlaA lysophospholipase A	26	80 ± 9

[a] Calculated as follows: 100 × (CFU formed at 25°C in 7 to 10 days/CFU formed at 37°C in 3 days). The results are the means and standard deviations of at least two independent experiments.

is temperature regulated, our laboratory observed an increase in the level of *pilD*-containing transcripts when wild-type legionellae were grown at 30 versus 37°C (48). Thus, we began this study by confirming that *pilD* transcription increases at temperatures below 37°C. In the process, we have uncovered a novel role for type II protein secretion in bacterial growth at low temperatures.

MATERIALS AND METHODS

Bacterial strains and media. *L. pneumophila* serogroup 1 strain 130b (ATCC BAA-74) served as the wild-type strain in this study. Previously described mutants of strain 130b that contain kanamycin resistance (Km^r) or gentamicin resistance (Gm^r) cassette insertions into genes associated with type II protein secretion, type IV piliation, or type IV secretion were also examined (Table 1). Another 130b derivative, NU236, that was studied contains both a promoterless *lacZ* and a Km^r cassette inserted into an iron-regulated gene (*frg*) (37). Legionellae were cultured at 37°C on buffered charcoal yeast extract (BCYE) agar or in buffered yeast extract (BYE) broth (21). A *pilD* mutant of *P. aeruginosa* (i.e., PAK-2B18) containing either vector alone or the complementing *pilD* gene was previously described and was cultured on BCYE agar (77). *Escherichia coli* strain NovaBlue (Novagen, Madison, Wis.), host for recombinant plasmids, was grown on Luria-Bertani agar (8). Antibiotics were added to the media at the following final concentrations (in micrograms per milliliter): ampicillin, 100; chloramphenicol, 6 for *L. pneumophila* and 30 for *E. coli*; gentamicin, 2.5; and kanamycin, 25 for *L. pneumophila* and 50 for *E. coli*.

Construction of an *L. pneumophila* strain containing a *pilD::lacZ* fusion. In order to monitor *pilD* transcription in *L. pneumophila*, we sought to place a promoterless *lacZ* gene under the control of the *pilD* promoter in wild-type strain 130b. Toward that end, the *lacZ*-containing, BamHI fragment of pMC1871 (Pharmacia Biotech, Piscataway, N.J.) was inserted into the BamHI site of pVK3 (85), yielding pVK6, and then the BamHI/BclI fragment of pVK6 was replaced with the BamHI/BclI fragment of pRS551 (75), resulting in pVK10 and a removable, promoterless *lacZ* gene that contains its own translational signal. Next, the SalI/XbaI fragment of pVK10 that contains *lacZ* and a downstream Km^r gene was isolated, Klenow treated, and ligated into the NarI site of *pilD* in pML219 (48), creating pMS10. Finally, an *L. pneumophila* strain (i.e., NU286) containing the *pilD::lacZ* fusion was isolated upon natural transformation (26) of strain 130b with pMS10. The genotype of the strain was verified by PCR and Southern hybridization analysis (data not shown). Genomic DNA was isolated from *L. pneumophila* as previously described (22).

Construction of an *L. pneumophila* strain lacking both type II and type IV

secretion. For the construction of a strain lacking both PilD and Dot/Icm, pGD::Gm (70) containing *pilD::Gm*^{r} was introduced by transformation into *icmGCD*::Km^r mutant GN142 (92), and kanamycin-resistant, gentamicin-resistant clones were isolated. The genotype of the double mutant (i.e., NU291) was verified by PCR, and its phenotype was verified by plating on egg yolk agar (5, 26, 70) (data not shown).

Extracellular growth determinations. In order to compare the extracellular growth of *L. pneumophila* strains at different temperatures, equal numbers of CFU of wild-type and mutant bacteria taken from log-phase BYE cultures grown at 37°C were inoculated into 40 ml of BYE broth (in a 125-ml flask) and then incubated with shaking (225 rpm) at 37, 30, 25, and 17°C. The extent of bacterial growth was assessed by measuring the optical density of the cultures at 660 nm. Additionally, bacteria taken from 3-day old, 37°C BCYE plates were resuspended in water, diluted, and plated for isolated colonies on BCYE agar incubated in air at 37, 25, 17, 12, and 4°C. The efficiency of plating at 25, 17, 12, or 4°C was determined by dividing the number of CFU obtained at the low temperature on various days by the number of CFU obtained at 37°C. Plates incubated at 37°C were counted on day 3, whereas those at 25, 17, and 12°C were scored on days 7 to 10, 20 to 21, and 70, respectively. The low-temperature growth of *P. aeruginosa* strains was similarly tested on BCYE agar. To begin to determine if secreted factors facilitate low-temperature growth, a heavy streak of wild-type legionellae taken from BCYE agar that had been incubated at 37°C was added to one side of a BCYE plate unto which had been previously spread ca. 10^5 CFU of a type II secretion mutant, also derived from a fresh 37°C plate. The plates were then incubated at 25°C in air and observed after 7 to 10 days for accelerated growth of the secretion mutant at the low temperature. Control plates received either no added streak or a streak of the secretion mutant. A parallel set of plates was stored at 37°C and analyzed after 3 days of incubation.

Enzymatic assays. Filter-sterilized supernatants from late-exponential-phase cultures grown in BYE broth at 37, 25, and 17°C were tested for enzymatic activities as previously described. Tartrate-sensitive and tartrate-resistant acid phosphatase activities were monitored by the release of *p*-nitrophenol (*p*-NP) from *p*-NP phosphate in 200 mM sodium acetate (pH 5.5) in the absence or presence of 5 mM tartrate (4). To measure alkaline phosphatase activity, the hydrolysis of *p*-NP phosphate was performed in 100 mM Tris, pH 10 (4). Lipolytic activities were determined by *p*-NP palmitate hydrolysis (5, 6), and protease activity was ascertained by azocasein hydrolysis (5). To quantitate *lacZ* expression in *L. pneumophila* gene fusion strains, β-galactosidase activity was measured in 0.1-ml aliquots taken from BYE broth cultures (see above) as previously described (37), and enzyme levels were reported in standard Miller units (8).
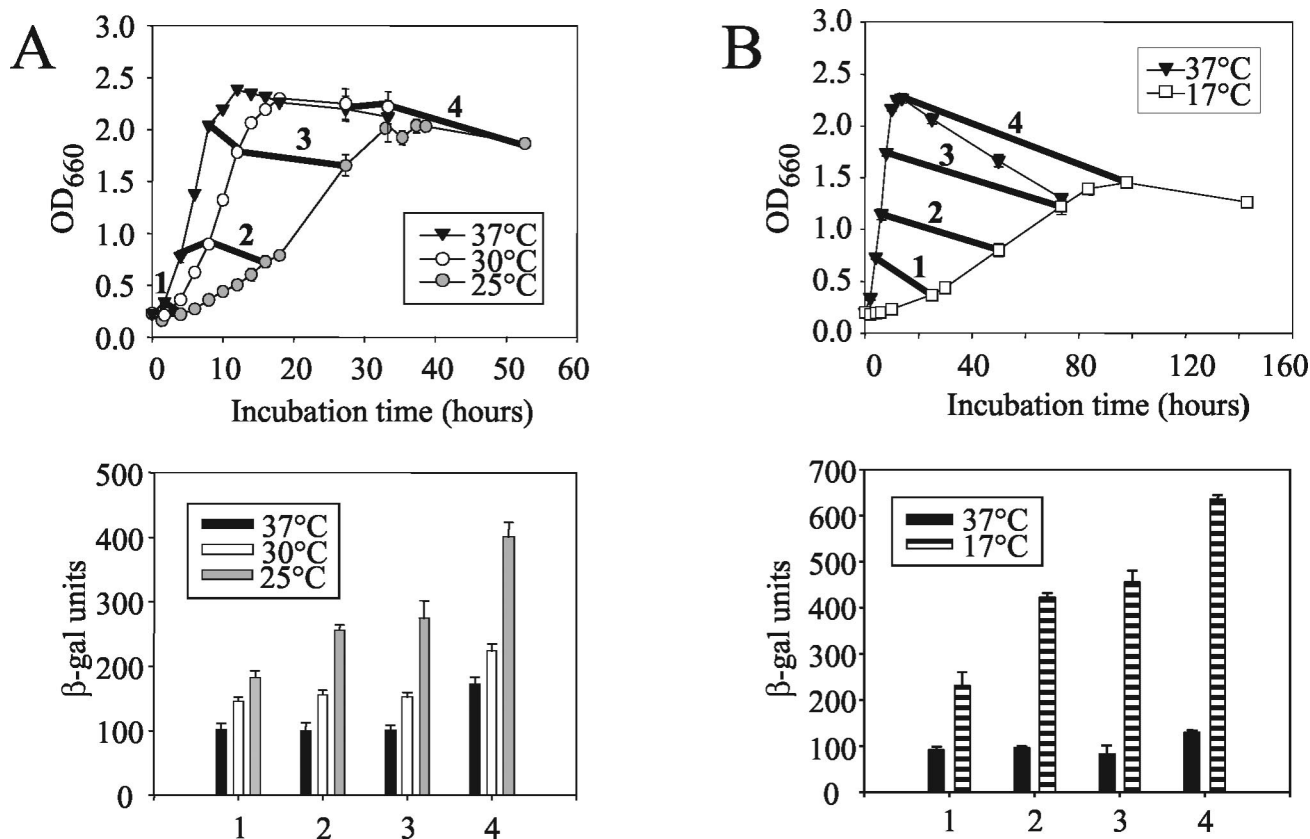
FIG. 1. Effect of temperature on the growth of and β-galactosidase production by an *L. pneumophila* strain containing a *pilD*::*lacZ* gene fusion. Log-phase NU286 bacteria were inoculated into BYE broth and then incubated, as noted, at 37, 30, and 25°C (A) or 37 and 17°C (B). The growth of the cultures was monitored spectrophotometrically (top panels) and, at four indicated time points (labeled as 1, 2, 3, and 4) when the various cultures had achieved comparable stages of growth, the amount of β-galactosidase was examined (bottom panel). The results presented are the means and standard deviations from four samples and are representative of at least two independent experiments.

RESULTS

Effect of low-temperature growth on *pilD* expression. Based upon Northern blotting and RT-PCR analyses, the level of *pilD* transcripts is higher when *L. pneumophila* is grown on BCYE agar at 30 versus 37°C (48). To further investigate the effect of temperature on *pilD* expression, we constructed a derivative of *L. pneumophila* strain 130b that contains a promoterless *lacZ* gene inserted into *pilD*, and then we assayed β-galactosidase levels in legionellae grown at different temperatures. As expected, the *pilD*::*lacZ* fusion strain, NU286, had the secretion and infectivity defects of previous *pilD* mutants (data not shown). To control for growth stage, the *pilD*::*lacZ* strain was cultured in BYE broth and enzyme comparisons were made using bacteria taken from the same stage of growth. The fusion strain produced 50% more β-galactosidase at 30 versus 37°C, and when the bacteria were grown at 25°C, the enzyme levels increased by two- to threefold (Fig. 1) ($P < 0.05$, Student's *t* test). NU286 cells grown at 17°C had β-galactosidase levels that were often sixfold greater than that produced by 37°C-grown bacteria (Fig. 1) ($P < 0.05$). In contrast to the results obtained with the *pilD*::*lacZ* fusion strain, *L. pneumophila* strain NU236, which contains *lacZ* fused to an *frg* gene (37), did not show elevated β-galactosidase when grown in BYE at 17 versus 37°C (Fig. 2). Thus, the increases in *pilD*::*lacZ* ex-

pression, which were seen in at least eight trials, were not simply the result of the changes in growth rate that were associated with temperature reduction, nor were they an artifact of using a *lacZ* reporter gene. To be sure that the increased β-galactosidase levels observed at the low temperature were also not an artifact of assessing *pilD*::*lacZ* expression in a PilD-negative strain, we repeated the assay using NU286 containing an intact *pilD* gene cloned into pMD1. The complemented *pilD*::*lacZ* fusion strain expressed the same increase in β-galactosidase activity at 17°C ($P < 0.05$) as the original NU286, as well as NU286 containing the pMMB2002 vector (Fig. 3). Taken together, the gene fusion data indicate that *pilD* expression is influenced by temperature, with increases in transcription initiation occurring at low temperatures.

Role of *pilD* in *L. pneumophila* growth at low temperatures. The increases in *pilD* expression at 17 to 30°C suggested that PilD is critical for survival as temperatures decline and that a *pilD* mutant would have a growth disadvantage at low temperatures. Hence, wild-type and *pilD*::*lacZ* mutant bacteria were incubated in BYE broth at 37, 30, 25, and 17°C, and differences in growth were determined spectrophotometrically (Fig. 4). As seen with our previous *pilD* mutant NU243 (47), the fusion strain grew as the wild type did at 30 and 37°C (Fig. 4A and B). However, at 25°C and especially at 17°C, the *pilD* mutant
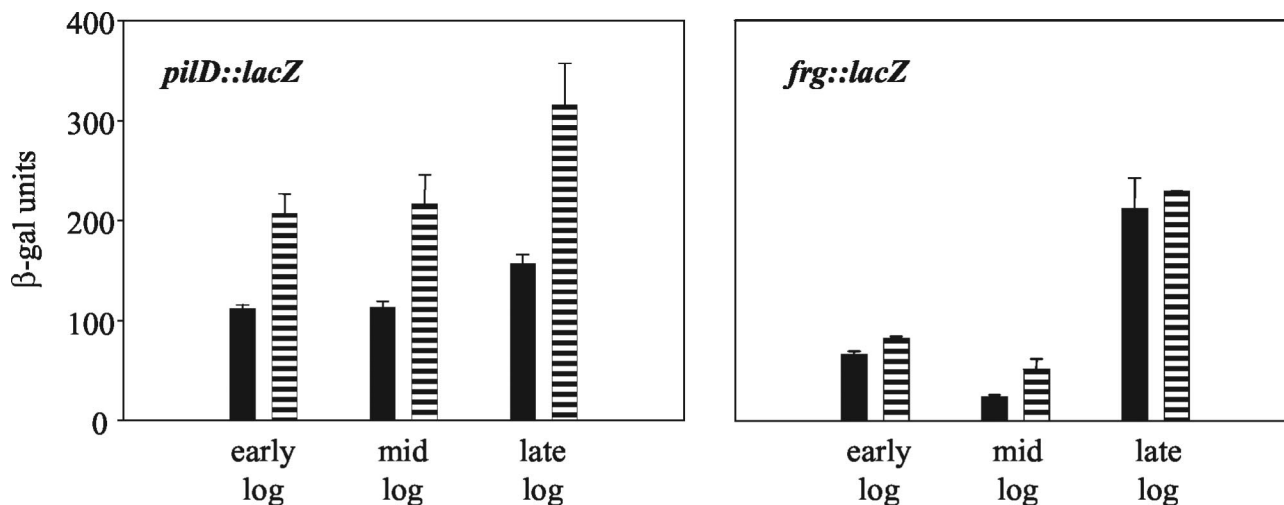
FIG. 2. β-Galactosidase production by *pilD*::*lacZ* and *frg*::*lacZ* fusion strains grown at 37 and 17°C. Log-phase NU286 (left panel) and NU236 (right panel) bacteria were inoculated into BYE broth and then incubated at 37°C (black bars) and 17°C (lined bars). When the cultures reached early, mid-, and late log phase, the amount of produced β-galactosidase was recorded. The results presented are the means and standard deviations from duplicate samples and are representative of two independent experiments.

appeared defective for growth (Fig. 4C and D). Indeed, at 17°C, the mutant culture displayed both a reduced growth rate and a lower maximum optical density. As another way of identifying differences in low-temperature growth, we compared wild type and the *pilD*::*lacZ* NU286 mutant for their ability to form colonies on BCYE agar incubated at 25°C. After 8 days at room temperature, the wild type exhibited 61% ± 33% (mean ± standard deviation) of the number of CFU that was seen on control plates incubated at 37°C for 3 days (Table 1). In contrast, the number of mutant CFU at 25°C was only 0.032% ± 0.009% of that seen at 37°C (Table 1), indicating that the mutant's efficiency of plating at the lower temperature is ca. 2,000-fold lower than that of wild type ($P < 0.001$). When the incubation period was prolonged to 14 days, the number of mutant colonies increased but was still 900-fold less than wild type (data not shown). When a swabful of bacteria representing hundreds of mutant colonies that had grown up at room temperature were replated, they still showed poor growth at the lower temperature, indicating that their deficiency in plating was not an artifact of being transferred from an optimal (i.e., 37°C) to suboptimal (i.e., 25°C) growth condition but was a heritable trait. Thus, the *L. pneumophila pilD* mutant had a greatly reduced ability to grow at 25°C. That three different types of *pilD* mutant (i.e., NU286, NU243, and NU272) behaved similarly (Table 1) suggested that this phenotype was due to the mutation of *pilD* and not a spontaneous second-site mutation. Importantly, wild-type and mutant bacteria expressing a plasmid copy of *pilD* had comparable efficiencies of plating at 25°C; i.e., 41 and 35% of CFU at 37°C, respectively (Table 2), implying that the growth defect was specifically due to loss of PilD. Together, these data indicate, for the first time, that a PilD protein preferentially promotes growth at low temperatures.

To begin to determine whether other bacterial PilD proteins promote growth at low temperatures, we compared a *pilD* mutant of *P. aeruginosa* and its complemented derivative (77) for their ability to grow on BCYE agar at 37, 25, and 12°C.

After 3 days at 25°C, the strain containing an intact *pilD* gene yielded 90.1% ± 11.8% of the number of CFU obtained at 37°C in 1 day. In contrast to the behavior of the *Legionella* mutant, the *Pseudomonas pilD* mutant displayed a normal ef-
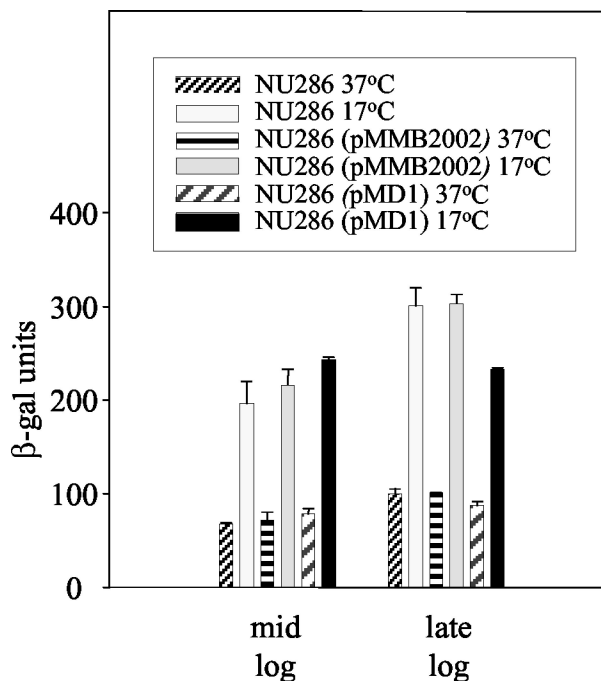

FIG. 3. β-Galactosidase production by the *pilD*::*lacZ* fusion in the presence and absence of an intact *pilD* gene. Log-phase NU286, NU286 containing the vector pMMB2002 (70), and NU286 containing a complementing *pilD* gene on pMD1 (70) were inoculated into BYE broth and then incubated at 37°C (hatched and lined bars) and 17°C (gray and black bars). When the cultures reached mid-log and late log phase, the amount of produced β-galactosidase was recorded. The results presented are the means and standard deviations from duplicate samples and are representative of two independent experiments.
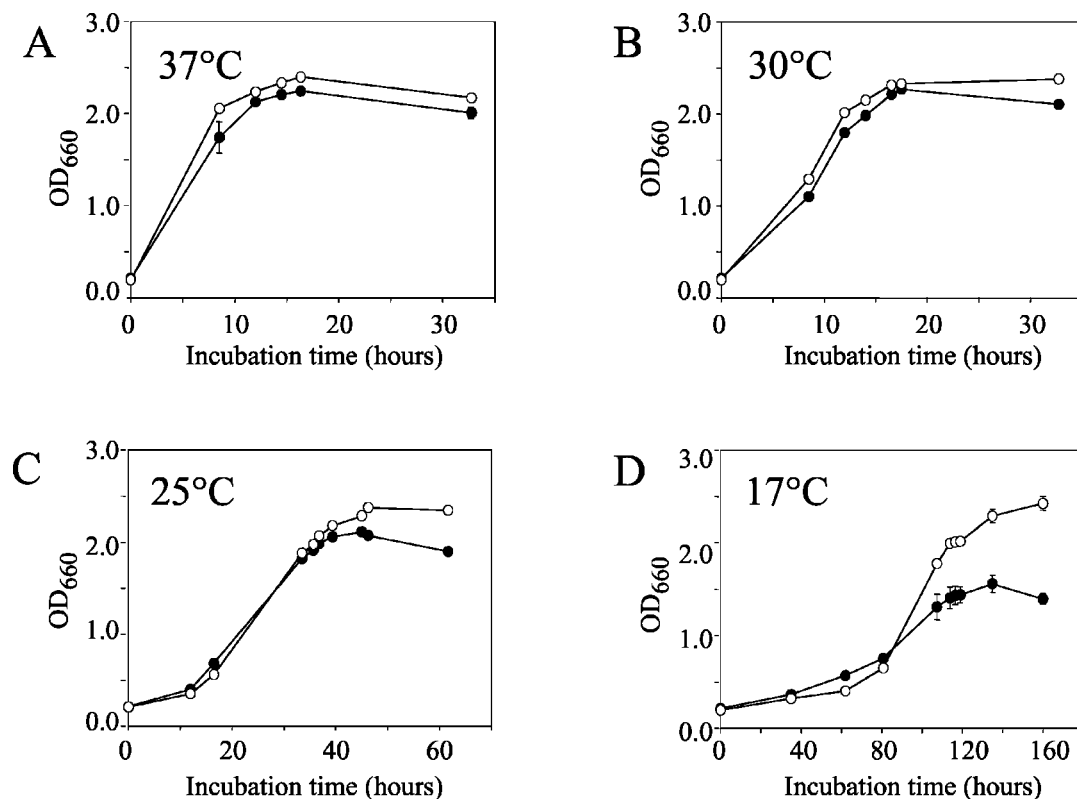
FIG. 4. Effect of temperature on the growth of wild-type and *pilD* mutant *L. pneumophila*. Log-phase 130b (○) and NU286 (●) bacteria were inoculated into BYE broth and then incubated at 37°C (A), 30°C (B), 25°C (C), and 17°C (D). The growth of the strains was monitored by recording the optical density of the cultures at the various times. The apparent differences between the wild-type and mutant cultures at 25 and 17°C were statistically significant ($P < 0.05$; Student's *t* test). The results presented are the means and standard deviations from duplicate samples and are representative of at least two independent experiments.

ficiency of plating at 25°C; i.e., 110% ± 18%. Both PilD⁺ and PilD⁻ *Pseudomonas* strains showed a 40 to 46% efficiency of plating when incubated at 12°C for 10 days.

Role of type II protein secretion in *L. pneumophila* at low-temperature growth. To begin to understand the role of PilD at low temperatures, we sought to determine whether the defect displayed by the *L. pneumophila pilD* mutants could be ascribed to the loss of type II protein secretion and/or the loss of type IV pilus biogenesis. In gram negatives, type II secretion is a two-step process (30, 55, 60, 71, 73). Initially, proteins destined for secretion are carried across the inner membrane by the Sec system but, in several cases, they are transported by the twin-arginine translocation system (88). After removal of their signal peptide, the proteins enter the periplasm, where they form disulfide bonds, fold, or oligomerize. The exoproteins then enter the type II secretion apparatus, ultimately exiting the cell through an outer membrane pore (secretin). The secretion apparatus consists of greater than 12 proteins, including inner membranes proteins, an ATP-binding protein, the PilD-dependent pseudopilins that span the periplasm, and the outer membrane secretin (55, 71, 73). In order to address the contribution of type II secretion, we made use of our previously described *L. pneumophila lspDE* and *lspF* mutants, which lack the LspD secretin and LspE ATPase, and the inner membrane LspF protein, respectively (69, 70). The type IV pilus apparatus has much similarity to the type II secretion

apparatus, possessing, in addition to the pilin subunits, an ATPase, the pseudopilins, and an outer membrane secretin (55, 71). In order to examine the role of the pilus, we employed a *pilE*$_L$ mutant that lacks pilin and a *pilQ* mutant that lacks the secretin (70, 76). To gauge the role of type II secretion and type IV piliation in *L. pneumophila* low-temperature growth, we examined the *lspDE*, *lspF*, *pilE*$_L$, and *pilQ* mutants for their relative growth on BCYE agar at 25°C. Whereas the *pilE*$_L$ and *pilQ* mutants had an efficiency of plating that was identical to

TABLE 2. Complementation analysis of *L. pneumophila pilD* and *lspF* mutants

Strain[a]	Mutation	Plasmid gene	EOP at 25°C[b]
130b(pMMB2002)	Wild type	Cm^r vector	59 ± 9
130b(pMD1)	Wild type	*pilD* in pMMB2002	41 ± 25
130b(pMF1)	Wild type	*lspF* in pMMB2002	55 ± 16
NU272(pMMB2002)	*pilD::Gm^r*	Cm^r vector	0.010 ± 0.014
NU272(pMD1)	*pilD::Gm^r*	*pilD* in pMMB2002	35 ± 23
NU275(pMMB2002)	*lspF::Km^r*	Cm^r vector	0.056 ± 0.071
NU275(pMF1)	*lspF::Km^r*	*lspF* in pMMB2002	51 ± 6

[a] Strains containing the chloramphenicol resistance (Cm^r) vector pMMB2002 and the complementing plasmids pMD1 and pMF1 were previously described (70).

[b] Efficiency of plating, calculated as 100 × (CFU formed at 25°C in 7 to 10 days/CFU formed at 37°C in 3 days). The results are the means and standard deviations from two independent experiments.

that of wild type, the *lsp* mutants showed the type of plating defect that was seen with the *pilD* mutants (Table 1). Indeed, the *lspDE* mutant and the *lspF* mutant had efficiencies of plating of 0.028% ± 0.021% and 0.010% ± 0.002%, respectively (Table 1). The growth defect of the *lspF* mutant was eliminated when the intact *lspF* gene was reintroduced on a plasmid (Table 2). These data indicate that *L. pneumophila* type II protein secretion system, but not type IV piliation, is required for optimal bacterial growth at 25°C and that the importance of PilD in low-temperature growth is due mainly, if not completely, to its role in promoting the assembly of the type II secretion apparatus. An *L. pneumophila* double mutant (i.e., NU283) lacking both *lspDE* and *pilQ* was also impaired for low-temperature growth (Table 1). The fact that the double mutant appeared more defective than single *lsp* mutants suggests that PilQ and/or the pilus apparatus may have a dispensable role in low-temperature growth. Unlike the PilD and Lsp mutants, three previously described *L. pneumophila dot/icm* mutants (92) grew normally at the lower temperatures (Table 1), indicating that the *Legionella* type IV secretion system is not required for low-temperature growth. Furthermore, a newly constructed double mutant containing mutations in both *pilD* and *icmGCD* (*dot FEP*) (86) displayed a reduced ability to grow at the lower temperatures that was similar to that seen for mutants lacking just type II secretion (Table 1).

To determine whether type II secretion promotes bacterial growth at temperatures below 25°C, we compared strain 130b and its *lspF* mutant for their growth on BCYE agar at 17, 12, and 4°C. In the two experiments done at 17°C, wild-type legionellae formed countable colonies in 20 to 21 days, achieving 28% ± 16% of the number of CFU obtained at 37°C. In contrast, the *lspF* mutant had an efficiency of plating at 17°C that was only 0.0006% ± 0.0004%, a defect that was complemented by reintroduction of *lspF* on pMF1. At 12°C, strain 130b formed colonies after 46 days, and by day 72 it achieved 0.2% of the number of CFU seen on 37°C plates. Strikingly, the *lspF* mutant never formed colonies at 12°C. Neither WT nor mutant formed colonies at 4°C. Thus, the *L. pneumophila* type II protein secretion becomes increasingly more important as temperatures decrease.

Theoretically, the reduced ability of Lsp mutants to grow at low temperature could be due to the loss of growth-promoting factors that are released into the extracellular milieu and/or a cell-associated defect such as an alteration in outer membrane function. Beginning to distinguish between these possibilities, we observed that the *lspDE* mutant formed thousands of colonies at 25°C in only 8 to 9 days when plated next to a heavy streak of wild-type bacteria (Fig. 5), implying that a secreted and/or diffusible factor(s) is able to restore growth to the mutant. Such a streak of wild type did not stimulate increased or more rapid colony formation on 25°C plates inoculated with wild type, nor did it enhance mutant growth when the plates were incubated at 37°C (data not shown). Since *lspDE* and *pilD* mutant streaks did not similarly enhance growth (Fig. 5 and data not shown), the stimulatory factor appears to be type II dependent. As an initial attempt toward identifying the factor, we tested mutants lacking specific Lsp-dependent activities for their abilities to grow on BCYE agar at 25°C. The *map*, *proA*, *plaA*, *lipAlipB*, and *plcA* mutants all grew as wild type did

(Table 1), implying that the Map tartrate-sensitive acid phosphatase, ProA (Msp) metalloprotease, PlaA lysophospholipase A, LipA and LipB lipases, and PlcA phospholipase C are not required at low temperatures. In support of this notion, we observed that the level of acid phosphatase, protease, and lipolytic activity toward *p*-NP palmitate in supernatants did not increase when wild-type bacteria were grown at either 25 or 17°C (data not shown). Taken together, these data suggest that a yet-to-be-defined type II-dependent secreted factor(s) helps promote *L. pneumophila* growth at low temperatures.

DISCUSSION

Combined with previous Northern blotting and RT-PCR results (48), our gene fusion data indicate that *pilD* expression is influenced by temperature, with increases in transcriptional initiation occurring at low temperatures. To our knowledge, these data are the first demonstration of *pilD* regulation in any bacterium and the first information on *L. pneumophila* gene expression below 30°C. The observed *pilD* regulation may explain, at least in part, the increased piliation we see when *L. pneumophila* are grown at 30°C as opposed to 37°C (48). The *pilD* promoter region has a putative binding site for σ^{28} factor FliA (48), suggesting that the regulation of *pilD* has similarity with that of the *L. pneumophila* flagellin (*flaA*) gene (36). Indeed, when grown at 30° vs. 37°C, a *flaA*::*lacZ* fusion strain shows elevated β-galactosidase akin to what we saw with the *pilD*::*lacZ* strain (35). In the course of assessing the impact of temperature on gene expression, we also observed that the level of β-galactosidase produced by the *pilD*::*lacZ* fusion strain increased ca. twofold as the culture progressed from log to stationary phase irrespective of temperature (Fig. 1). That *pilD* mutants show a modest reduction in late stationary phase survival is compatible with this result (47). The onset of stationary phase produces many other changes in *L. pneumophila*, including increases in *flaA* expression (32, 35).

We have obtained novel data indicating a role for type II protein secretion in bacterial growth at low temperature. In the process, we have also documented, for the first time, that *L. pneumophila* is capable of extracellular replication at temperatures less than 20°C.

Previous work on low-temperature growth has focused on "cold-shock" proteins, factors that are induced transiently upon decreases in temperature and reprogram the basic cellular processes of replication, transcription, translation, and protein folding (9, 40, 67, 82, 90). Few studies have characterized those processes that permit long-term growth at low temperature, i.e., cold adaptation (49, 67, 82). Changes in membrane phospholipids, such as increased levels of unsaturated fatty acids, *trans* isomers of the unsaturated fatty acids, and short-chain fatty acids, are critical for maintaining membrane fluidity at low temperature (17, 46, 72, 82). Indeed, in the only previous study on *L. pneumophila* physiology at low temperature, Mauchline et al. found increases in the amount of unsaturated fatty acids in bacteria grown at 24°C (52). Newly recognized forms of cold adaptation are increases in trehalose and palmitoylated lipid A within *E. coli* (41, 87) and solute (e.g., glycine, betaine) uptake in *Listeria monocytogenes* (3, 49). The ability of wild-type *L. pneumophila* to stimulate, in *trans*, low-temperature growth of *lsp* mutants suggests that low-temperature-in-
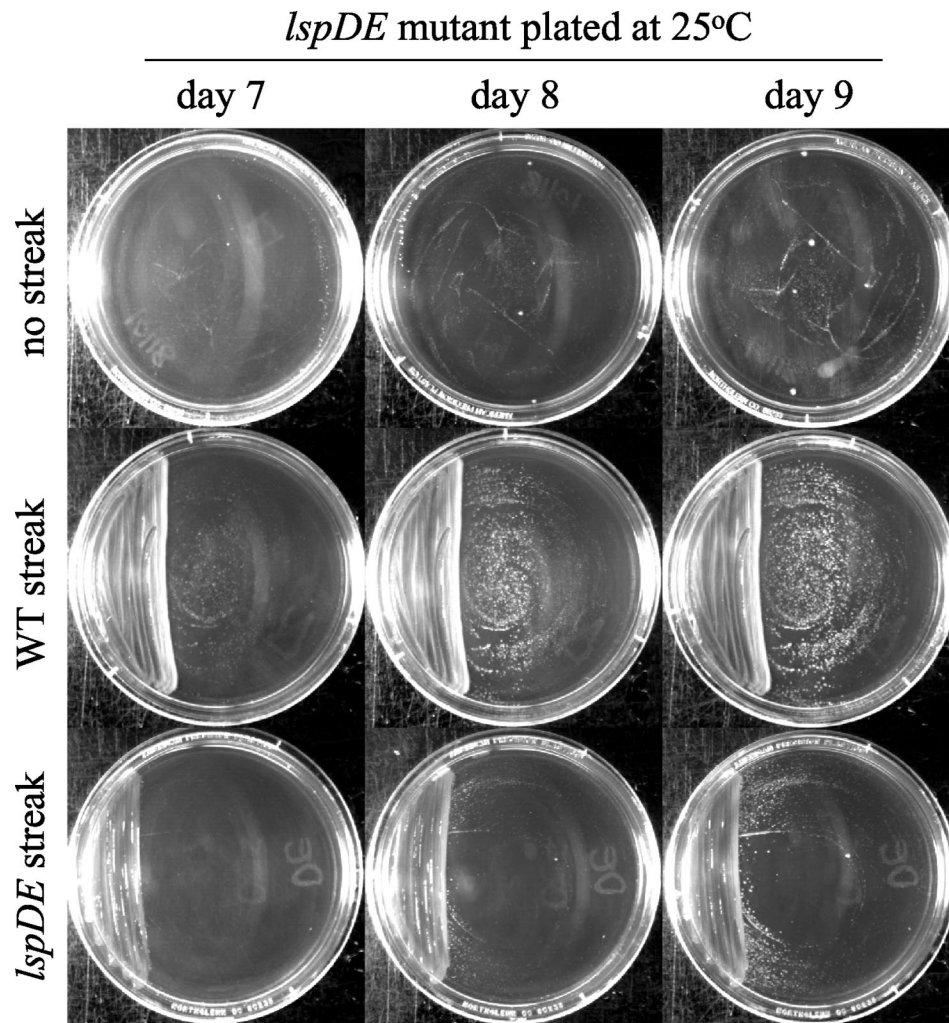
lspDE mutant plated at 25ºC

FIG. 5. The effect of adjacent wild-type bacteria on the low-temperature growth of an *L. pneumophila* type II secretion mutant. Approximately 10^5 CFU of *lspDE* mutant NU258 were plated for CFU on a series of BCYE agar plates. Some plates were then, as indicated, also inoculated in one sector with streaks of wild-type or mutant bacteria. One set of plates (shown here) was incubated at 25°C, and another (data not shown) was stored at 37°C. The picture depicts the 25°C colonial growth of the *lspDE* mutant on days 7, 8, and 9. The results presented are representative of at least two experiments.

duced, type II exoproteins may be another mechanism of cold adaptation. Compatible with this idea, we found, in preliminary experiments, that a number of the proteins present in wild-type culture supernatants change in abundance upon reduction in growth temperature (M. Soderberg and N. P. Cianciotto, unpublished results). Alternatively, the growth stimulation that we observed could be due to a type II-dependent membrane protein which mediates the production of a diffusible factor that enhances growth at the low temperature. Clearly, the further study of the role of Lsp in low-temperature growth will enhance our understanding of *L. pneumophila* persistence in aquatic environments and therefore its transmission to humans. It will also have implications for other bacteria that possess type II secretion systems, including pathogens, such as *Aeromonas hydrophila*, *Burkholderia* spp., *P. aeruginosa*, and *Vibrio* spp. that, like *L. pneumophila*, survive at low environmental temperatures (19, 51, 61, 65, 74, 78, 81).

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