

Importance of Type II Secretion for Survival of *Legionella pneumophila* in Tap Water and in Amoebae at Low Temperatures[∇]

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***Legionella pneumophila* type II secretion mutants showed reduced survival in both tap water at 4 to 17°C and aquatic amoebae at 22 to 25°C. Wild-type supernatants stimulated the growth of these mutants, indicating that secreted factors promote low-temperature survival. There was a correlation between low-temperature survival and secretion function when 12 additional *Legionella* species were examined.**

Legionella pneumophila is widespread in natural and man-made water systems (8, 21, 29, 39, 41, 47, 53, 64). In these habitats, *L. pneumophila* exists planktonically, within protozoa, and in biofilms (14, 35, 36, 41, 45). The ubiquity of *L. pneumophila* is also a result of the organism's ability to survive at many temperatures, including ones as low as 4°C (21, 29, 62, 64). *L. pneumophila* is an important pathogen of humans, with the inhalation of contaminated water droplets originating from aerosol-generating devices resulting in Legionnaires' disease (16). Given the manner in which infection occurs, it is important to better understand how legionellae survive in water, in protozoa, and at low temperatures. Recently, we found that *L. pneumophila* type II protein secretion is critical for growth in rich broth or agar at 12 to 25°C but not in medium at 30 to 37°C (56). Operative in many gram-negatives (9), type II secretion is a multistep process in which proteins are translocated across the inner membrane in a Sec- or Tat-dependent manner, recognized in the periplasm, and then delivered to the T2S apparatus, whereupon a pilus-like structure "pushes" proteins through a dedicated outer membrane pore or secretin (28).

To investigate the connection between type II secretion and low-temperature survival under conditions that more closely mimic natural habitats, we compared wild-type serogroup 1 strain 130b (Table 1) and its type II secretion mutants for persistence in tap water incubated at 37°C, 25°C, and 17°C. We used three mutants: NU258, containing a mutation in the genes encoding the type II outer membrane secretin (*lspD*) and the inner membrane ATPase (*lspE*); NU275, containing a mutation in the gene for the inner membrane platform protein (*lspF*); and NU272 mutated in the gene encoding the pseudopilin peptidase (*pilD*) (51). Tap water was obtained from laboratory sinks and filter sterilized. Following growth at 37°C in buffered yeast extract (BYE) broth to late log phase (56), wild types and mutants were inoculated into flasks containing 50 ml of the tap water, and then the cultures were incubated with shaking. As with other wild-type *L. pneumophila* (30, 41, 42, 54, 57), 130b persisted in low-temperature tap water for extended times (Fig. 1). Also similar to previous work (27), the recovery

of CFU was maintained for a longer period at low temperatures below 37°C. But across the 17 to 37°C range, the secretion mutants behaved differently than their parent (Fig. 1). At 37°C, the mutants displayed a greater recoverability than 130b between days 7 and 20 ($P < 0.05$). In a similar vein, at 25°C, the mutants were recovered more than the wild type was between days 126 and 141, although the differences were not statistically significant. But at 17°C, the situation reversed: between days 49 and 161, there was less recovery of the mutants ($P < 0.05$). These data imply that type II secretion mutants have reduced survival in tap water at 17°C. That independently derived mutants, inactivated for three different genes, representing three different transcriptional units, including two that are solely dedicated to type II secretion (51), behaved similarly indicated that this survival defect was due to the loss of type II secretion function versus second-site mutations. To confirm that type II mutants have reduced survival in water at low temperatures, we retested the *lspF* mutant in a new water sample incubated at 37°C, 17°C, 12°C, and 4°C. This experiment was started 20 months after the first in order to see if the survival differences were peculiar to certain water samples or not. Again, 130b persisted for long periods of time, especially at temperatures below 37°C (Fig. 2). Indicating that the tap water had changed appreciably, the recovery of 130b was fully maintained at 17°C for 318 days rather than gradually declining over 161 days. Strain 130b was also fully maintained at 12°C, while at 4°C a gradual decline was seen. We suspect that these changes in survival were a manifestation of changing biocides, but details on the treatments used in our local environment were not available to us. Regardless of the extended recovery of 130b, the secretion mutant distinguished itself in the same way that it had in the first experiment. While briefly showing greater survival at 37°C (P of <0.05 on day 38), the mutant exhibited markedly reduced numbers at 17°C and below (P of <0.05 from day 20 on) (Fig. 2). The mutant phenotype increased in magnitude as temperatures went from 17°C to 4°C. That the large declines in mutant recoverability at low temperatures were due to corresponding losses in viability was verified by examining samples with Live/Dead staining (Molecular Probes, Eugene, OR). These data indicate that an intact type II secretion system is needed for the optimal survival of *L. pneumophila* in environmental waters at low temperatures.

Amoebae are natural hosts for *L. pneumophila*, and the

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TABLE 1. *Legionella* species and their low-temperature growth^a

Species	Strain ^b	Source (reference)	17°C growth ^c	Protease ^d
<i>L. pneumophila</i>	BAA-74	Clinical (15, 52)	+++	+
<i>L. anisa</i>	35292	Environmental ^d (22)	+++	+
<i>L. brunensis</i>	43878	Environmental (63)	—	—
<i>L. cincinnatiensis</i>	43753	Clinical (58)	++	+
<i>L. erythra</i>	35303	Environmental (7)	++	+
<i>L. feeleii</i>	35072	Clinical (26)	++	+
<i>L. hackeliae</i>	35250	Clinical (7)	—	—
<i>L. londiniensis</i>	49505	Environmental (12)	—	—
<i>L. longbeachae</i>	33462	Clinical (37)	+++	+
<i>L. micdadei</i>	Stanford-R	Clinical (48)	—	—
	33218	Clinical (25)	—	—
	31B	Clinical (48)	+	—
<i>L. moravica</i>	43877	Environmental (63)	+++	+
<i>L. oakridgensis</i>	33761	Environmental ^c (40)	—	—
<i>L. parisiensis</i>	35299	Environmental ^c (7)	++	+

^a +, modest growth; ++, good growth; +++, robust growth; —, no growth.
^b Except for *L. micdadei* 31B and Stanford-R, the designations refer to ATCC numbers.

^c The relative bacterial efficiency of plating on BCYE agar at 17°C, as observed on three independent occasions and as exemplified in Fig. 5.

^d Casein hydrolysis, as observed at 37°C on at least three independent occasions.

^e Other isolates of these species have been linked to human infection (32, 33, 59).

coincidence of amoebae and legionellae has been verified in low-temperature waters (43, 44). Thus, to determine if the importance of type II secretion at low temperatures is manifest in a mimic of environmental intracellular growth, we compared 130b and its secretion mutants for infection of *Acanthamoeba castellanii* (ATCC 30234) and *Hartmannella vermiformis* (ATCC 50237) at 22 to 25°C. Briefly, 10⁴ CFU of bacteria were added to wells containing 10⁵ amoebae, and then at various times, the numbers of bacteria per coculture were determined by plating on buffered charcoal yeast extract (BCYE) agar (38, 50, 51). Upon the infection of acanthamoebae, 130b increased in numbers 10³- to 10⁴-fold over a 10-day period (Fig. 3A). In

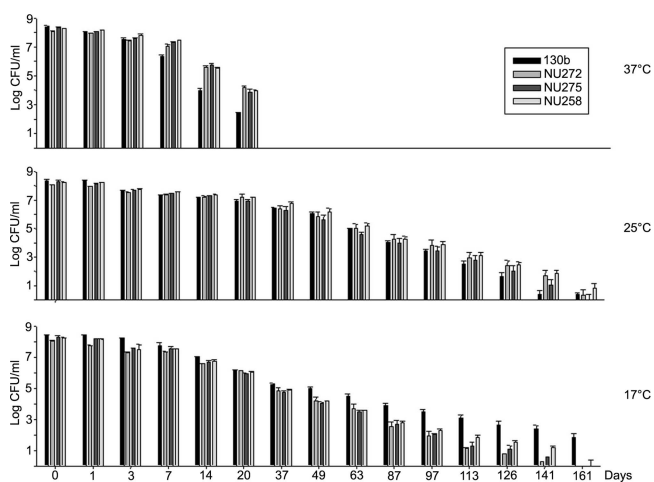


FIG. 1. Survival of wild-type and type II secretion mutant *L. pneumophila* in tap water at 37°C, 25°C, and 17°C. Wild-type strain 130b, *pilD* mutant NU272, *lspF* mutant NU275, and *lspDE* mutant NU258 were inoculated at comparable levels into sterile tap water, and then the samples were incubated at either 37°C (top), 25°C (middle), or 17°C (bottom). On the indicated days, the numbers of CFU in the samples, presented as means and standard deviations, were determined by plating aliquots on BCYE agar.

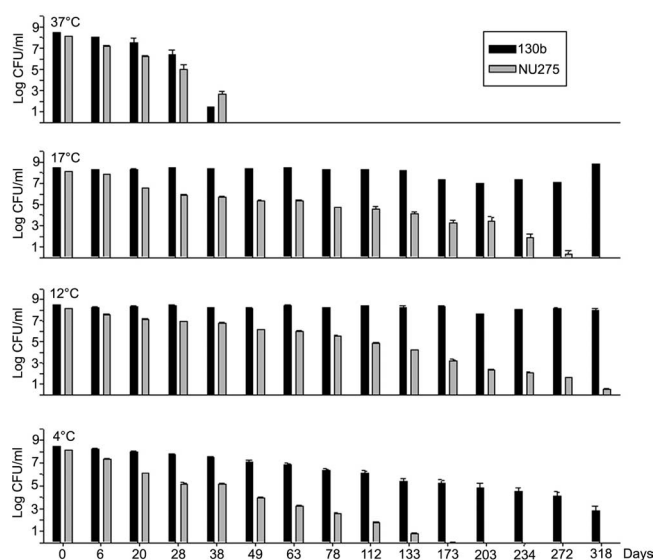


FIG. 2. Survival of wild-type and *lspF* mutant *L. pneumophila* at 37°C, 17°C, 12°C, and 4°C. Wild-type strain 130b and *lspF* mutant NU275 were inoculated at comparable levels into a second sample of sterile tap water and then incubated at the indicated temperatures. At the noted time points, the numbers of CFU in the samples, presented as means and standard deviations, were determined by plating aliquots on BCYE agar.

marked contrast, *lspF* mutant NU275 did not exhibit any evidence of growth, although the parent and the mutant showed like reductions in survival when incubated in medium alone. A similar situation was observed when bacteria infected the *hartmannellae* (Fig. 3B). Whereas 130b grew ca. 10⁴-fold, *pilD* mutant NU272 and *lspDE* mutant NU258 did not increase significantly during the 6-day infection. Amoebal entry assays, done as previously described (24, 60), revealed that 130b, NU275, and NU258 have comparable rates of entry into both *A. castellanii* and *H. vermiformis* at 22°C and 37°C (data not shown). Taken together, these data indicate that functional type II secretion is needed for the infection of amoebae at low temperatures and more specifically for intracellular replication.

To further investigate the mechanism of low-temperature survival, 130b was grown at 17°C, and then cell-free culture supernatants were tested for their ability to restore low-temperature growth to an *lspF* mutant. The supernatants greatly stimulated the growth of the mutant when inoculated onto BCYE agar at 17°C (Fig. 4). Exposure to BYE broth that had been incubated at 17°C did not likewise stimulate growth (data not shown). Some mutant colonies arose at low temperatures independently of wild-type stimulation at a lower frequency (Fig. 4); these colonies likely have suppressor mutations. That wild-type supernatants, but not medium controls, rescue mutant growth at low temperatures indicates that a secreted bacterial factor(s) and/or a medium component(s) that is modified by growing legionellae can promote low-temperature survival. Since our type II secretion mutant undergoes a greater degree of leakage than the wild type does when incubated in BYE broth at the low temperature (55), it was not possible for us to reliably use 17°C mutant supernatants in this assay in order to

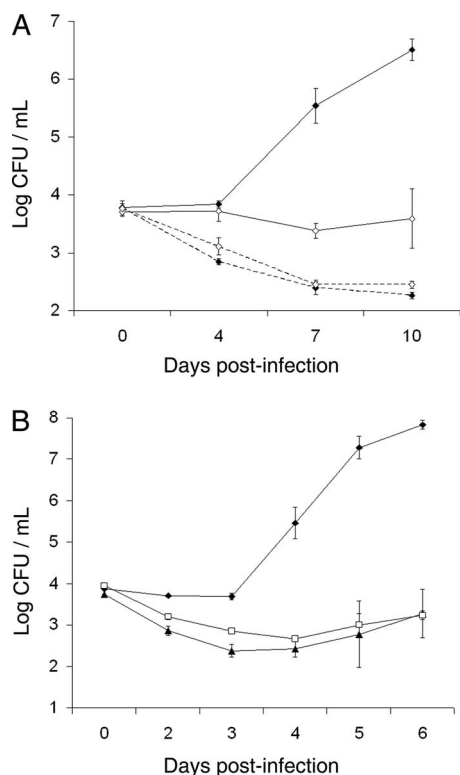


FIG. 3. Growth of wild-type and type II secretion mutant *L. pneumophila* in low-temperature cultures of amoebae. (A) *A. castellanii* was infected with wild-type strain 130b (◆) or *lspF* mutant NU275 (◇) and then incubated at 22°C. On the indicated days, the numbers of bacteria in the cultures were determined by plating. In parallel, the survival of the parent and the mutant in tissue culture medium alone was assessed (dashed lines). The values presented are the means and standard deviations of the results from four infected wells. On days 4, 7, and 10, the recovery of the *lspF* mutant was significantly less than that of parental 130b ($P < 0.05$; Student's *t* test). Similar results were obtained from four additional experiments, although in some of those experiments, the net growth of wild-type strain 130b was ca. 10^4 -fold versus ca. 10^3 -fold. (B) *H. vermiformis* was infected with wild-type strain 130b (◆), *pilD* mutant NU272 (▲), or *lspDE* mutant NU258 (□) and then incubated at 25°C. At the indicated times, the numbers of bacteria in the cultures were determined by plating. The values presented are the means and standard deviations of the results from four infected wells and is representative of two independent experiments. On days 2 through 6, the recovery of the mutants was significantly less than that of strain 130b ($P < 0.05$; Student's *t* test).

determine whether the growth-stimulatory substance(s) is type II dependent or not.

The *Legionella* genus contains 51 species, besides *L. pneumophila*, and nearly one-half of the species are implicated in disease (13). To begin to understand the behavior of the other legionellae at low temperatures, we compared 14 strains, representing 12 species (Table 1), for growth on BCYE agar at 37°C, 22°C, and 17°C (Fig. 5). With the exception of *L. hackeliae*, all strains grew like *L. pneumophila* did at 37°C. *L. anisa*, *L. longbeachae*, and *L. moravica* also grew comparably to *L. pneumophila* at the two low temperatures. *L. cincinnatiensis*, *L. erythra*, *L. feeleeii*, and *L. parisiensis* grew like *L. pneumophila* did at 22°C but showed modest reductions in survival at 17°C. In contrast, *L. brunensis*, *L. hackeliae*, *L. londiniensis*, *L. micdadei* strains 33218 and Stanford-R, and *L. oakridgensis* dis-

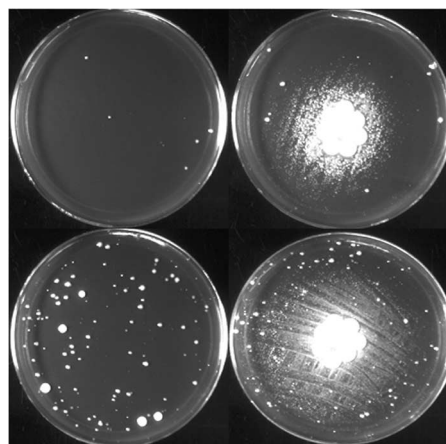


FIG. 4. Low-temperature growth stimulation by *L. pneumophila* supernatants. A total of 10^5 (top row) and 10^6 (bottom row) CFU of *lspF* mutant NU275 was spread onto BCYE agar and then was either overlaid with disks that had been impregnated with 2 ml of cell-free supernatants obtained from 17°C cultures of wild-type strain 130b (right column) or left alone (left column). Images of the growth were obtained after 10 days of incubation at 22°C. Growth stimulation achieved by exposure to wild-type supernatants was also seen in two other independent experiments.

played greatly reduced survival at 22°C and a complete inability to grow at 17°C. *L. micdadei* strain 31B, though clearly showing a reduced capacity to survive at 22 and 17°C, grew better at low temperatures than the other *L. micdadei* strains. As summarized in Table 1, these data indicate that survival and growth at low temperatures are common but not universal among species of *Legionella*. The finding that multiple species of *Legionella* were able to grow on BCYE agar at low temperatures is not surprising, since non-*pneumophila* legionellae have been detected in many waters, including low-temperature ones (29, 64). But the observation that several species of *Legionella* were unable to grow or grew very poorly at 22°C and 17°C was unanticipated. Although some studies have suggested that the makeup of the *Legionella* population is influenced by temperature in warm waters (36, 53, 61), there was no indication in the literature that would have predicted the inability of certain legionellae to survive at low temperatures. Thus, our data indicate, for the first time, that only a subset of *Legionella* species flourish in cold water systems.

When these various species were grown on agar containing casein, as previously described (23, 51), we observed an intriguing correlation between strong growth at low temperatures and protease activity (Table 1). Whereas species that grew relatively well at 17°C had casein-degrading activity, *L. brunensis*, *L. hackeliae*, *L. londiniensis*, *L. micdadei*, and *L. oakridgensis* lacked it. Since caseinolytic activity is indicative of type II secretion in *L. pneumophila* (23, 51), we hypothesize that those *Legionella* species that do not grow well at low temperatures lack aspects of type II secretion. Because an *L. pneumophila proA* mutant specifically lacking casein degradation grows normally at room temperature (56), we suspect that the inability of some legionellae to grow at low temperatures is not simply due to a lack of caseinolytic activity. In support of these hypotheses, past studies have found *L. micdadei* strains, including the ones used here as well as others, to also lack type II-dependent

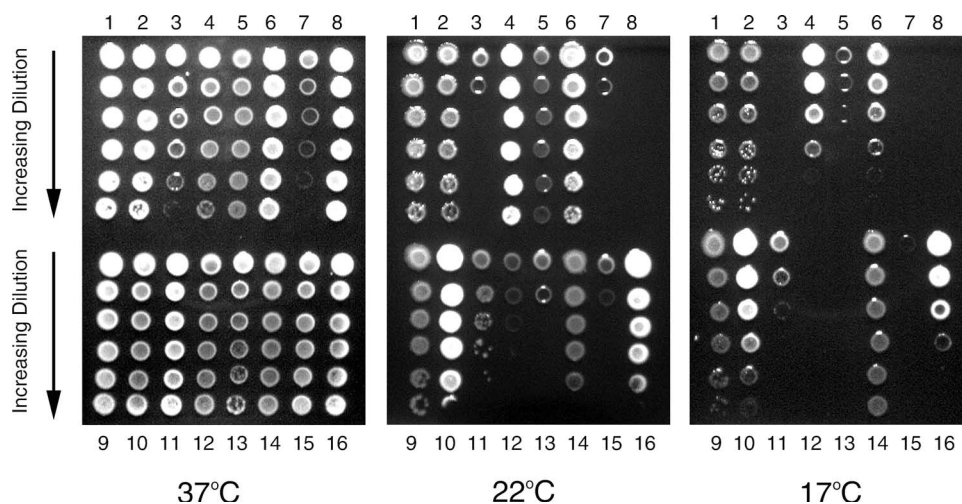


FIG. 5. Low-temperature growth of different *Legionella* species. Tenfold serial dilutions of suspensions of *L. pneumophila* 130b (lanes 1 and 9), *L. anisa* (lane 2), *L. brunensis* (lane 3), *L. cincinnatiensis* (lane 4), *L. erythra* (lane 5), *L. feeleii* (lane 6), *L. hackeliae* (lane 7), *L. londiniensis* (lane 8), *L. longbeachae* (lane 10), *L. micdadei* 31B (lane 11), *L. micdadei* 33218 (lane 12), *L. micdadei* Stanford-R (lane 13), *L. moravica* (lane 14), *L. oakridgensis* (lane 15), and *L. parisiensis* (lane 16) were spotted, in the indicated order, onto BCYE agar and then incubated at either 37°C (left), 22°C (center), or 17°C (right). Images of bacterial growth were then obtained after 3 days for the 37°C plates, 13 days for the 22°C plates, or 15 days for the 17°C plates. Because of the reddish pigmentation exhibited by *L. erythra* (7), the extent of growth in lane 5, center and right, is not fully apparent in the images. These results are representative of two (22°C) or three (37°C, 17°C) independent experiments.

phosphatase, hemolytic, and lipolytic activities (5, 17, 18, 49, 51). Since *L. micdadei* and all other *Legionella* species tested thus far contain *lsp* genes (51), the deficiencies in low-temperature growth and secreted activity exhibited by some *Legionella* species are likely due to a lack of expression of the type II secretion apparatus or the absence of (expression of) individual exoproteins.

In the simplest scenario, type II-dependent effectors facilitate extra- and intracellular survival by allowing the bacterium to acquire nutrients and/or combat harsh conditions, such as oxidative stress, biocides, or bactericidal/static agents made by other microbes. That the role of Lsp in environmental mimics is most manifest at lower temperatures may be because nutrients are less available or toxic factors are more prevalent in that situation. For example, dissolved oxygen and thus the potential for oxidative stress increases with decreasing temperature (1). In the absence of secreted effectors, such as would occur for an *lsp* mutant, increased cell leakage and lysis may ultimately occur. Given what is known of the type II secretome, there are many candidate effectors. For example, when 130b is grown in BYE broth at 37°C, the secretome encompasses more than 25 proteins, including many degradative enzymes as well as proteins with no similarity to known proteins (2–4, 6, 10, 11, 19, 20, 23, 31, 49–51). Also, 2D polyacrylamide gel electrophoresis analysis of supernatants produced during growth at 12 to 17°C revealed other exoproteins that are more pronounced at low temperatures (55). Although more work is needed to identify the critical secreted factors, the new data presented here significantly increase our appreciation for the role that type II secretion plays in *Legionella* ecology. In the past, we and others had shown the importance of Lsp for the infection of host cells and lung tissue at 35 to 37°C (23, 31, 46, 49, 51), while others suggested a role in biofilms at 30°C (34). Thus, we can now state that Lsp is important in the full range of *Legionella* niches, from planktonic and intracellular aquatic niches to the

extra- and intracellular niches in the human host and from low-temperature conditions to high-temperature situations. Current findings also represent the first identification of *Legionella* genes that are necessary for optimal survival in low-temperature aquatic habitats and as such may constitute potential targets for minimizing the risk posed by legionellae in water systems. Finally, based on these latest findings in *Legionella*, it is plausible that the type II secretion systems of other bacteria, including pathogenic species of *Aeromonas*, *Burkholderia*, *Erwinia*, *Pseudomonas*, *Vibrio*, and *Xanthomonas*, are especially critical for persistence in low-temperature water habitats.

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