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*); 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 restested 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 < 0.05* on day 38), the mutant exhibited markedly reduced numbers at 17°C and below (*P < 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
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 wild-type strain 130b and its secretion mutants for infection of Acanthamoeba castellanii (ATCC 30234) and Hartmannella vermiformis (ATCC 50237) at 22 to 25°C. Briefly, 10^4 CFU of bacteria were added to wells containing 10^5 amoebae, and then at various times, the numbers of bacteria per coculture were determined by plating on buffered charcoal yeast extract (BCYE) agar (38, 40). The relative bacterial efficiency of plating on BCYE agar at 17°C, as observed on three independent occasions and as exemplified in Fig. 5, was determined by plating aliquots on BCYE agar.

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.

**TABLE 1.** Legionella species and their low-temperature growtha

<table>
<thead>
<tr>
<th>Species</th>
<th>Strainb</th>
<th>Source (reference)</th>
<th>17°C growthc</th>
<th>Proteased</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. pneumophila</td>
<td>BAA-74</td>
<td>Clinical (15, 52)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>L. anisa</td>
<td>35292</td>
<td>Environmentala (22)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>L. longbeachae</td>
<td>33462</td>
<td>Clinical (37)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>L. micdadei</td>
<td>33218</td>
<td>Clinical (25)</td>
<td>--</td>
<td>-</td>
</tr>
<tr>
<td>L. moravica</td>
<td>43877</td>
<td>Environmental (63)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>L. oakridgensis</td>
<td>33761</td>
<td>Environmentalb (40)</td>
<td>--</td>
<td>-</td>
</tr>
<tr>
<td>L. parisienis</td>
<td>35299</td>
<td>Environmentalb (7)</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

*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.

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. 10^4-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, NU272, 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
determined 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 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. antisa, L. longbeachae, and L. moravica also grew comparably to L. pneumophila at the two low temperatures. L. cincinnatiensis, L. erythra, L. feeiit, 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. oekridgensis displayed 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. oekridgensis 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
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°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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