NORTHWESTERN UNIVERSITY

Type II Secretion and Secreted Ppiases Promote Low Temperature Growth and Survival in *Legionella pneumophila*

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Integrated Graduate Program in the Life Sciences

By

Anna Maria Söderberg

EVANSTON, ILLINOIS

JUNE 2008

ABSTRACT

Type II Secretion and Secreted Ppiases Promote Low Temperature Growth and Survival in *Legionella pneumophila*

Anna Maria Söderberg

PilD is an enzyme that processes prepilins that are part of the type II protein secretion apparatus and the type IV piliation machinery. Using a *Legionella pneumophila pilDlacZ* fusion strain to measure transcription, we observed a 20% increase in β-galactosidase levels at 30°C vs. 37°C. At 25°C and 17°C vs. 37°C, this difference was 3-fold and 6-fold, respectively, indicating a temperature regulation of *pilD*. *L. pneumophila pilD* mutants had a severe growth defect in BYE broth and on BCYE plates at 12-25°C, but not at 30-37°C. This growth defect was due to the loss of the type II secretion and not the type IV pilus apparatus. Since a heavy streak or a sample of supernatants from strain 130b could restore the growth of the *lsp* mutants on low-temperature BCYE plates, we hypothesize that a type II dependent exoprotein(s) promotes low temperature growth and survival. The severe growth defect of the *lsp* mutants was partially due to the presence of iron in the media since growth at low temperatures improved significantly on BCYE plates without the iron supplement. In addition, type II secretion is important for long time survival in sterile tap water at low temperatures since strain 130b

shows greater survivability than the *lsp* mutants after extended incubation at 4-17°C. To isolate secreted proteins important for growth at lower temperatures, we compared supernatants from strain 130b grown in BYE broth at 17°C and 37°C on 2D gels. Two protein spots repeatedly had higher intensities on the 17°C, 2D gel. Mass Spectrometry data identified the proteins in these spots as peptidyl proline isomerases (ppiases) encoded by the *L. pneumophila* genes *lpg1962* and *ppiB*. A *ppiB* mutant had a growth defect in BYE broth and up to a two log reduction in CFUs on BCYE plates at 17°C. An *lpg1962*:*ppiB* double mutant, but not the individual single mutants, had a growth defect in *A. castellanii* when amoeba infections were done at 22°C but not at 35°C. Since ppiases can modify the activity of other proteins, it is possible that these ppiases act by regulating the activity of other secreted proteins, a subset of which might be secreted through the type II secretion system.

TABLE OF CONTENTS

FIGURES & TABLES

INTRODUCTION

PART I - *LEGIONELLA PNEUMOPHILA*

Legionnaires' disease was first identified after an outbreak that occurred in July of 1976 at an American Legion Convention in Philadelphia, killing 29 out of 182 infected individuals (74). The source of the disease remained a mystery for months until Joseph McDade at the Centers for Disease Control and Prevention finally isolated the gram negative bacterium responsible for the outbreak (157). Indirect fluorescent antibody tests from stored patient sera collected from earlier outbreaks of pneumonia indicated that this was not a new disease. In fact, the earliest case of Legionnaires' disease identified in the literature occurred in 1947, when an organism was isolated from a sick guinea pig that had been inoculated with the blood of a patient with a febrile respiratory illness (156). In addition, other outbreaks in which Legionnaires' disease was identified from stored sera were one outbreak in 1957, associated with a meat plant in Austin, Minnesota and one outbreak in 1965, at St. Elizabeth's hospital in Washington D.C. (173, 228). Also, in 1974, an outbreak of Legionnaires' disease occurred at a convention of the Independent Order of Odd Fellows at the same hotel in Philadelphia as the American Legion met at in 1976 (227). Outbreaks of Legionnaires' disease continue to occur multiple times a year. The most severe outbreak in recent years occurred at a flower show in the Netherlands in 1999, where 231 people become ill and 21 died (54, 226).

Legionellosis is usually acquired in the community where it accounts for 2-15% of the acquired pneumonia that requires hospitalization (31). Outbreaks account for <5% of those cases, while the rest is caused by sporadic Legionnaires' disease (153). Most of the cases are not recognized, and therefore in the USA only 1000 cases of Legionnaires' disease are reported every year even though 20,000 cases are thought to occur (1, 153). Symptoms of Legionnaires' disease include fever, malaise, myalgia, rigors, headache and confusion followed by dyspnea and nonproductive cough (235). Those who get sick are usually of advanced age or have compromised host defenses in the lungs due to cigarette smoking, lung disease or immunosuppression (153). This is the reason why the mortality rate can approach 50 % when Legionnaires' disease occurs in a hospital setting (31, 153). The most isolated species is *Legionella pneumophila*, which in one study of reported cases between 1980-89 was shown to be present in 71.5% of all isolates (153). In addition to causing severe pneumonia, *L. pneumophila* can cause a mild, self-limiting flu-like illness called Pontiac fever. It was named after Pontiac, Michigan where in 1968, 95% of the workers at a health department building became sick with fever, headache, myalgia and malaise (81).

L. pneumophila was initially grown on the yolk sacs of embryonated eggs, but it was soon found that Muller Hinton agar supplemented with hemoglobin and IsovitaleX could support growth of *L. pneumophila* (65). After it was learned that iron and L-cysteine were the necessary components in hemoglobin and in IsovitaleX, respectively, the Feeley-Gorman agar was developed (65). This agar medium allowed for a more rapid growth of the organism (65). Later, the starch in the media was replaced with activated charcoal, and the amino acid source was changed from casein to yeast

extract (64). Further additions of N-(2-Acetamido)-2-aminoethanesulfonic Acid (ACES) buffer and alpha-keto glutaric acid to this medium resulted in the buffered charcoal yeast extract (BCYE) medium used today (58).

Currently, there are 52 species in the genus *Legionella*, roughly half of which have been shown to cause infections in humans (68, 135, 137, 176). These *Legionella* species have been found in almost all freshwater habitats examined, including lakes, ponds, rivers, creeks, swamps, wet soil, marine and estuarine environments (72, 73, 95, 172, 218). *Legionella* species are similarly widespread in artificial water systems, in some cases reported in 60% of the plumbing systems in large and small public buildings, as well as in private residences (4, 141). Transmission of Legionnaires' disease occurs by inhalation of aerosols generated from air-conditioners, showers, cooling towers, whirlpool spas, humidifiers, grocery store mist machine, and other devices (68, 119, 150, 163, 247). Person to person spread has never been reported (60). Even though *L. pneumophila* has been isolated from natural water sources at temperatures of 4-63°C (72, 73), it has only been shown to replicate in the laboratory at temperatures between 20-50°C (22, 136, 207, 243, 249). However, the bacteria grow best at a temperature of 32-37°C (133, 136, 249).

L. pneumophila has been show to replicate within two species of ciliated protozoa and 14 species of amoebae, including *Acanthamoeba* and *Hartmannella* amoebae (18, 68, 242). In low nutrient environments, *L. pneumophila* might enter a viable non culturable state (114, 141, 219). *L. pneumophila* can also survive inside biofilms (68, 211) but can only replicate in these biofilms when amoebae are present (52, 165). In addition, *L. pneumophila* can survive planktonically without replication for

long periods of time outside a host (68, 214). Some bacteria like *Flavobacterium breve* and *Fisherella* sp. have the ability to produce factors that stimulate *L. pneumophila* growth (233, 244).

Since *L. pneumophila* cannot spread from person to person, it is thought that the capacity of *L. pneumophila* to establish infection in humans is a consequence of it being able to infect amoebae (18, 68, 159, 242). The reasons for this are many, including the fact that *L. pneumophila* has been found in protozoa from water sources implicated as the source of outbreaks (18) and that the capacity of water samples to support replication is dependent on protozoa (165, 242). In addition, *L. pneumophila* rendered nonculturable due to low nutrient conditions are resuscitated by exposure to amoebae (219), and preculturing of *L. pneumophila* in amoebae make them 100-fold more invasive for epithelial cells and 10-fold more invasive for macrophages (41).

The life cycle of *L. pneumophila* in macrophages and amoebae are very similar; e.g., entry in both cases can occur through coiling phagocytosis (25, 107). In addition, in both hosts, the bacteria create vacuoles that do not acidify or fuse with lysosomes but become surrounded by endoplasmic reticulum (ER) (2, 25). Many factors that promote survival and replication in macrophages are also important for growth in amoebae. For example, Gao et al. isolated 89 transposon mutants that were defective both in protozoan and mammalian phagocytes (76). Therefore, the same strategies that work in amoebae also work in macrophages. However, not all proteins are needed for growth in all hosts; e.g., the HtrA/DegP stress-induced protein is only important for growth in mammalian cells but not in *Acanthamoeba polyphaga* (179).

In its natural environment *L*. *pneumophila* alternates between growth inside a host and the subsequent survival in the outside environment. The bacteria therefore have a biphasic life cycle consisting of a replicative growth phase and a virulent transmissive phase (28). The transmissive phase is induced in response to amino acid depletion inside a host after extensive multiplication of the bacteria (28). At this point, *L*. *pneumophila* activates RelA, a guanosine 3',5'-bispyrophosphate (ppGpp) synthetase which leads to accumulation of the alarmone, ppGpp in the bacterial cell (91). PpGpp has been shown to initiate similar responses important for surviving periods of starvation in many other different bacteria like *Escherichia coli*, *Bacillus subtilis*, and *Myxococcus xanthus* (91). When ppGpp accumulates, the two-component regulator LetA/LetS and the alternative sigma factor (RpoS) are activated (92). This leads to the induction of the transmissive phase characteristics needed for escape from a spent host and for survival in the environment until the next host is found. These characteristics include increased stress resistance, motility, cytotoxicity, sodium sensitivity, and the ability to evade lysosomal endosome fusion (160).

The replicative state is characterized by noninfectious, sodium resistant and non-motile bacteria that are actively replicating (28). In contrast to the wealth of information known about transmissive phase regulation, not much is know about what regulates the replicative phase except that the RNA binding protein, CsrA, which is global repressor of transmissive traits, is active during this phase (161).

L. *pneumophila* can be internalized into a host through a pseudopod coil, or through "conventional" phagocytosis which can be mediated by opsonization by complement (25, 80, 107, 177). Within 5 minutes of this internalization event, ER

vesicles and mitochondria attach to the phagosomal surface (232). In the following 10 minutes, the thickness of the phagosomal membrane changes to resemble the thickness of the attached ER vesicles (232). These *L*. *pneumophila*-created vacuoles differ from conventional phagosomes in that they do not acidify or fuse with lysosomes (106, 108). By 6 hours, most of the ER vesicles have been replaced by ribosomes, and the vacuole now resembles the rough ER (232). Within this protected niche, the bacteria can undergo conversion into a replicative state, and bacterial replication begins after a lag phase of 6-10 hours (105, 224). By 24 hours, the vacuole is full of hundreds of bacteria, and lysis of the infected cell is evident (224). The type IV secretion system in *L*. *pneumophila* is absolutely crucial for this intracellular growth (169). More than 30 known effectors are secreted through this system (169). These effectors have been found to be important for all stages of the intracellular lifecycle including the ability to avoid immediate delivery to the endosomal pathway, the ability to associate with the endoplasmic reticulum, and the ability to establish the replicative vacuole (160, 169).

PART II - SECRETION

SECRETION IN GRAM NEGATIVE BACTERIA

Protein secretion in gram negative bacteria can be divided into Sec dependent and Sec independent secretion pathways. In Sec independent secretion, proteins are secreted directly from the cytoplasm into the extracellular milieu using the type I, type III, or type IV secretion systems (14, 44, 104). In Sec dependent secretion, proteins are secreted

first through the inner membrane using the Sec pathway, and then through the outer membrane by the type II or type V secretion systems (96, 121).

SEC PATHWAY

Many proteins in bacteria are translocated through the inner membrane using the general secretion pathway (Sec pathway) (49). Proteins destined for secretion through this pathway are made with a signal sequence of 18-30 amino acid, consisting of a positively charged amino terminal region, a central hydrophobic region, and a more polar carboxy-terminal region (49, 239). The positions at the -1 and -3 positions from the cleavage sites are always amino acids with small uncharged side chains with a strong preference for alanine (239). Most proteins secreted through the Sec-pathway are targeted to the translocase by the molecular chaperon SecB that helps to stabilize the unfolded state of the proteins (49). This is true for all proteins except those with a very hydrophobic central region and all inner membrane proteins (49). These proteins are instead targeted to the Sec pathway by binding to the signal recognition particle (SRP) (49). When the SecB/protein complex reaches the inner membrane, the signal peptide will bind to SecA, which is an ATP dependent motor protein that drives the translocation reaction (49). This leads to conformational changes in SecA that allow for translocation of the protein through a proteinaceous channel made up by the heterotrimeric membrane complex SecYEG (49). At this point, the signal peptide is removed by an inner membrane signal peptidase thereby releasing the protein into the periplasmic space (49).

TAT PATHWAY

Proteins can also be transported through the inner membrane by the twin arginine translocation (Tat) pathway (23, 241). This pathway translocates already folded proteins that are targeted to the Tat pathway by having a signal peptide with two conserved arginines (175). This signal peptide is recognized by the TatBC complex which then associates with TatA, allowing translocation through a channel formed by TatA (23, 175). This pathway is present in *L*. *pneumophila* and has been shown to be important for export of phospholipase C, biofilm formation, growth under iron limiting conditions, and intracellular survival in amoebae and macrophage-like cells (48, 195).

SEC DEPENDENT PATHWAYS

TYPE II SECRETION

Proteins secreted through type II secretion (T2S) are first translocated through the inner membrane through the Sec or Tat pathways, at which point their signal peptide is cleaved off. In the periplasm, the proteins are folded before they are secreted through the type II secretion system into the extracellular milieu (121).

T2S is present in many but not all gram-negative bacteria, including many of the γ–proteobacteria and some of the α, β, and δ-proteobacteria (38). The T2S system secretes many degradative enzymes needed for nutrient acquisition such as lipases, phospholipases, phosphatases, cellulases, and amylases (38). In addition, some pathogens use T2S to secrete toxins; e.g., *Vibrio cholerae* secretes cholera toxin through the T2S (203).

T2S is made up of a core of 12 proteins (i.e. T2S CDEFGHIJKLM and O) that forms a complex that spans the entire cell envelope, though the exact configuration and order of assembly of the subunits is still unclear (38, 121). The inner membrane complex consists of T2SE, T2SF, T2SL and T2SM (69, 121). T2SM is a small inner membrane protein with a short cytoplasmic domain, one transmembrane helix and a periplasmic domain (121). T2SF is an inner membrane protein that interacts with T2SE and T2SL (69). T2SE is thought to function as a hexamer that powers protein secretion through ATP hydrolysis and is associated with the cytoplasmic membrane through interactions with T2SL (69, 121). T2SL has a large cytoplasmic domain, one single transmembrane helix and a smaller periplasmic domain (121). T2SL interacts with T2SE, T2SF, and T2SM to create a stable complex in the inner membrane (69, 121).

T2SO, the prepilin peptidase, is an inner membrane protein with 8 trans-membrane domains that functions as a bifunctional aspartic acid protease that cleaves off the leader peptide on the pseudopilins, T2SGHIJK, and N-methylates their newly generated N-terminus (121, 180). T2SGHIJK are referred to as psuedopilins since they have homology to the pilin subunit in type IV pili (121). Their leader peptide consists of 6-7 positively charged amino acids followed by a stretch of about 20 hydrophobic residues (121). The cleavage site is found immediately before the hydrophobic domain with a highly conserved glycine residue at -1 position (121). T2SG makes up the bulk of the pilus structure (121). The exact functions of T2SHIJK are not known, though it is thought that T2SK might terminate pilus assembly, and T2SI might initiate pilus assembly or anchor the pilus to the cell envelope (121).

The outer membrane complex consists of T2SC and T2SD. T2SC is anchored in the inner membrane but spans the periplasmic space and interacts with T2SL, T2SM and T2SD (121). In this function, T2SC might act as a transducer of energy from the inner to the outer membrane or might have a function in substrate specificity (121). T2SD forms an oligomeric ring structure in the outer membrane through which the secreted proteins reach the extracellular milieu (121).

Proteins secreted through the T2S do not have a distinctive signal sequence (69, 202). Instead, it is thought that the secretion signal involves distal regions of the protein that are brought together after protein folding or a high beta sheet content within the protein (69, 202).

Not much is known about the regulation of the T2S genes, except in *Pseudomonas aeruginosa*, the T2S genes are regulated by quorum sensing, and in *Erwinia amylovara*, expression of the T2S gene, *hofQ*, is upregulated at lower temperatures (33, 84).

The type II secretion machinery is homologous to the type IV pilus (T4P) apparatus, but only four proteins, T2SD, T2SE, T2SF and T2SO, have a high degree of homology between the systems (178). Even though the main function of the T4P is to produce pili, secretion thought the T4P has been shown in several bacteria; e.g., in *Francisella tularensis*, several proteins, including two chitinases, a chitin binding protein, a protease (PepO), and a beta-glucosidase (BglX) are secreted through the T4P (89). In addition, in *V. cholerae*, a colonization factor (TcpF) is secreted through the T4P (130), while a protease is secreted in *Dichelobacter nodosus* (128).

L. *pneumophila* has a functional T2S (38, 90, 196, 198). Proteins believed to be secreted through the T2S in *L*. *pneumophila* include proteins with protease, aminopeptidase, chitinase, RNase, tartrate-sensitive and tartrate-resistant acid phosphatase, lipase, phospholipase A, phospholipase C, and lysophospholipase A activities (10-12, 51, 70, 90, 144, 196, 197, 198). In addition, a 2D gel analysis of secreted protein profiles between the parental strain 130b and a T2S mutant revealed another 15 proteins that might be substrates for the *L*. *pneumophila* T2S (51).

The T2S in *L*. *pneumophila* is important for growth in *H*. *vermiformis*, *A*. *castellanii*, macrophages, and the lungs of A/J mice (90, 198). Most of the known type II secreted enzymes do not have a role in intracellular growth except the zinc metalloprotease, ProA, which is important for growth in *H*. *vermiformis*, and the chitinase which is important for growth in the A/J mouse (51, 197). In addition, *L*. *pneumophila* has T4P, but no secretion has so far been shown to occur through it (198, 220).

TYPE V SECRETION

Autotransporter and two partner secretion are both placed into the type V secretion (T5S) group (96). Many proteins secreted through this system are adhesins and proteases but others include proteins with lipolytic or actin polymerization activities (96, 117). The autotransporter protein is a bifunctional protein containing both the transported protein and the outer membrane pore through which the protein is translocated in one protein (96, 229). The N-terminus of these proteins contain a signal sequence, which is cleaved off during transport through the Sec pathway in the inner

membrane, followed by the passenger domain that will be transported through the pore in the outer membrane (96, 229). This is followed by an alpha helical linker region before the translocation domain at the C-terminus, which usually has 250-300 residues and forms a 12-14 stranded beta barrels after insertion into the outer membrane (96, 229). After secretion through the translocation pore, the passenger domain can remain attached to the bacterial surface or be released into the extracellular milieu by proteolytic cleavage (96, 229).

In two partner secretion, the passenger part and the translocator part are translated as two different proteins. Both proteins have a signal peptide and are transported through the inner membrane through the Sec pathway. The passenger protein has a 110 residue conserved domain that is recognized by the transporter protein and provides specificity to this system. These genes are usually found in an operon (96, 229). In *L. pneumophila*, genes encoding a putative T5S system have only been found in the genome of one (Paris) of the four sequenced strains (32).

SEC INDEPENDENT PATHWAYS

TYPE I SECRETION

In type I secretion (T1S), the ABC-transporter dependent pathway, the translocator complex is composed of three proteins that span the inner and outer membranes and the periplasmic space (53, 104). Those proteins include an ABC transporter located in the inner membrane, which provides energy for protein transport through ATP hydrolysis (53, 104). The second protein is a membrane fusion protein with

a short cytoplasmic domain, a lipid anchor in the inner membrane followed by a large periplasmic domain which forms a pore that links the inner and outer membrane components together (53, 104). The final protein is an outer membrane protein which forms a long water filled channel through the outer membrane and most of the periplasmic space (53, 104).

Proteins secreted through T1S vary greatly in size from 19-800 kDa. The nature of the secretion signal, located in the C-terminal end, is still poorly understood, but most proteins that are secreted have glycine-rich repeats and all except one are very acidic with a pI of approximately 4. Proteins secreted through T1S include proteases, phosphatases, glucanases, nucleases, lipases, and toxins. The most studied protein is the pore forming toxin hemolysin A from *E. coli* (53, 104).

One T1S, encoded by the *lssXYZABD* locus, has been characterized in *L. pneumophila* strain Corby (118). In addition, from the completed sequencing of the genomes of *L. pneumophila* strain Philadelphia-1, Paris, and Lens, it is evident they all have the Lss T1S system. However, it is not known what proteins are secreted through these T1S systems (32, 34).

TYPE III SECRETION

More than 25 species of gram negative bacteria, both pathogens and symbionts, have type III secretion systems (T3S) (44). This secretion system allows bacteria to dock to and deliver effectors across the membrane of the host through a needle-like complex (44, 75). Among them, they secrete more than a 100 different kinds of effectors that function to reprogram the target cell to benefit the bacterium (44). The T3S apparatus

consists of two ring structures in the outer and inner membrane joined together by a rod with an attached needle protruding from the bacterial surface (44, 75). This needle is about 60 nm long with an inner diameter of 2-3 nm (44). Proteins secreted by the T3S have a signal sequence located in the first 20-30 amino acids, though the exact nature of this signal sequence is not known (75).

T3S is thought to be evolutionary related to the bacterial flagella transport machinery, and the two systems share a core of 8 conserved proteins (44, 122). The primary function of the flagella transport machinery is motility, but in some cases, proteins are also secreted through it (122). Proteins secreted through the flagella transport machinery include YplA, a virulence-associated phospholipase in *Yersinia enterocolitica* (250), the virulence proteins FlaC and Cia in *Campylobacter jejuni* (132, 216), and hemolysin BL and phosphatidylcholine-preferring phospholipase C in *Bacillus thuringiensis* (79). *L. pneumophila* does not have T3S. However, the bacteria are flagellated, but no proteins have been shown to be secreted through this apparatus (99).

TYPE IV SECRETION

Secretion pathways related to bacterial conjugation are referred to as type IV secretion (T4S) (14). Some T4S systems transfer DNA to other bacteria or release/take up DNA from the environment, while other T4S are dedicated to protein secretion (14). T4S spans both membranes and the periplasmic space creating a channel through which the DNA or protein are translocated from the cytoplasm to the extracellular milieu (14, 37). An exception to this rule is the T4S in *Bordetella pertussis* in which the pertussis toxin

passes the inner membrane using the Sec pathway and then the outer membrane using the T4S (14). T4S can be divided into type IVA and type IVB, depending on sequence homologies between the groups, where type IVA T4S is related to the VirB/D4 T4S of *Agrobacterium tumefaciens* and type IVB T4S is related to the Dot/Icm T4S of *L*. *pneumophila* (37). The type IVA T4S in *A*. *tumefaciens* is the most studied system and is encoded by the genes *virB1* to *virB11* and *virD4* (37).

The type IVB secretion system in *L*. *pneumophila* is encoded by 25 genes that are split into 2 different regions. Not much is known about the architecture of the type IVB T4S, though one recent study showed a five-protein core consisting of the inner membrane proteins DotF and DotG interacting with the outer membrane proteins DotC, DotD, and DotH (209). In *L*. *pneumophila*, this secretion system is required for growth in amoebae, macrophages, and A/J mice, and more than 30 effectors have are secreted through it (169).

In addition, *L*. *pneumophila* has a type IVA system encoded by the *lvh* genes (210). This T4S is not important for intracellular replication at 37°C, but *lvh* mutants have an approximately 100-fold defect in entry and intracellular replication when they are pre-grown at 30°C (189). This *lvh* T4S also has an important role for both entry into amoebae after *L*. *pneumophila* has been water stressed and intracellular replication after infected amoebae that have been encysted are returned to the replicative trophozoite stage (17).

TYPE VI SECRETION

The recently discovered type VI secretion (T6S) system is present in many gram negative bacteria; e.g., *V*. *cholera*e, *P*. *aeruginosa*, *Yersinia pestis*, *E*. *coli*, and *Salmonella enterica* (186). This secretion system is thought to secrete proteins from the cytoplasm into the host cells (187). The protein sequence needed for transport through this system is unknown but does not involve a cleavable signal peptide (187). A protein with unknown function, Hcp, is secreted through T6S in many bacteria (186). *L*. *pneumophila* does not appear to have T6S, since it is lacking at least two genes involved in T6S; i.e., *vasA* and *vgrG-2*. But, since the components of this secretion system are still being defined, it is hard to conclude this for certain (187).

PART III – GROWTH AT LOW TEMPERATURES

When bacteria are shifted from a higher to a lower temperature, certain changes must occur for the bacteria to survive. These include changes in lipid composition to make the membranes more fluid, changes in the transcription/ translation machinery to allow for protein synthesis, and changes in protein expression to adapt the bacteria for growth at a lower temperature (27, 35, 148, 182, 230, 240, 246).

Bacteria modulate membrane fluidity by altering fatty acid composition. This can be done by shortening the length of the fatty acid chains, using branched and cyclic fatty acids or by introduction of a *cis* double bond into the fatty acids (35). In addition, it has been reported that *E*. *coli* also modifies its lipid A at low temperatures by

exchanging laurate with an unsaturated fatty acyl chain; i.e. palmitoleate on the lipid A moiety (240).

Branched fatty acids are commonly used by Gram-positive bacteria, though some Gram-negative bacteria can have a high proportion of branched fatty acids (246). One such example is *Legionella* species, which have a branched fatty acid composition of 35-85% depending on species (125, 138). Branched fatty acids are synthesized by turning the amino acids valine, leucine and isoleucine into their corresponding keto acids which subsequently are attached to fatty acids by FabH, thereby creating a branched fatty acid (148).

E. *coli* uses an anaerobic de novo pathway for unsaturated fatty acid production. In this pathway, FabA introduces a *cis* bond between the third and fourth carbon on short fatty acids (usually C10). Then FabB elongates the fatty acid tail to the desired length (46). Another pathway involving fatty acid desaturation is commonly used in cyanobacteria and *B*. *subtilis*. In this pathway, saturated fatty acids are converted to unsaturated fatty acids by the activity of an acyl-lipid desaturases in the membrane that inserts a double bond into previously synthesized fatty acid chains (46). Different kinds of desaturases insert double bounds at different positions of the fatty acids (35). Most organisms only have one pathway for production of unsaturated fatty acids; e.g. *E*. *coli* and *B*. *subtilis*. Other bacteria use more than one pathway; e.g. *P*. *aeruginosa* has both the Fab and the desaturase pathway, while *Synechocystis* cyanobacteria express several desaturase genes, *desA*, *desB* and *desD* (46, 162).

In *B*. *subtilis* a two component signal transduction system DesK/DesR is responsible for the perception and transduction of the low temperature signal (151).

When the bacteria encounter low temperatures, a higher rigidity in the membrane leads to a change in the conformation of DesK. DesK can then activate the response regulator DesR through phosphorylation which leads to an increase in *des* gene transcription. After the membrane becomes more fluid due to higher content of unsaturated fatty acids, DesK dephosphorylates DesR, and *des* gene transcription is decreased (151). In *Synechocystis*., Hik33 and the response regulator Rer1 influence gene expression of the *desD* gene in a similar manner but in contrast to the *B*. *subtilis* desK/R regulator, Hik33/Rer1 also regulates genes involved in transcription and translation (201).

Since lower temperatures cause stabilization of secondary structures in RNA and DNA, a transient inhibition of protein synthesis occurs due to reduced transcription and translation efficiencies when bacteria are switched to a lower temperature (182). This growth inhibition is referred to as the acclimation phase and can last for one to several hours depending on the temperature (88). The cold shock response has been extensively studied in *E*. *coli* where sixteen proteins are cold shock inducible (182, 230). These proteins can be divided into two groups: the class I proteins which are present at very low levels at 37°C and are induced to very high levels after a shift to lower temperatures, and the class II proteins, which are present at 37°C and have a <10-fold induction after cold shock (182, 230). Both class I and class II proteins are involved with cold adapting the transcription and translation machinery and include nucleases, helicases, and other ribosome-associated components. These proteins include CspA, CspB and CspG, which function as RNA/DNA chaperones, CsdA, which is a ribosome associated protein that has RNA unwinding capabilities, NusA, which is involved in

termination of transcription, and IF-2, which is an initiation factor (182, 230). After some time, the ribosome acquires additional proteins that allow it to function at lower temperature, and protein synthesis of a new set of proteins now occurs allowing the bacteria to adapt to life at a lower temperature (182, 230).

Cold-adapted enzymes must compensate for the reduction in chemical reaction rates since a reduction of 10°C in growth temperature will cause biochemical reaction rates to decline 2-3 times (162). Therefore, cold-adaptive proteins have evolved to have an increased flexibility in protein structure by decreasing the number of arginine and proline residues, decreasing the amount of weak interactions like aromatic interactions and hydrogen bonds or by increasing the number of glycine residues (66, 199). This leads to enzymes with good activities at lower temperatures but also a much reduced thermal stability at higher temperatures (66, 162, 199).

No cold active secreted enzymes have so far been isolated from mesophilic bacteria but several have been reported from organisms living in the cold. These secreted cold adapted proteins include proteases in *Leucosporidium antarcticum*, *Colwellia psychrerythraea,* and *Methanococcoides burtonii***,** lipases from *Moraxella* spp. and a metagenomic library, a xylanase form a *Penicillium* strain, cellobiohydrolase from *Penicillium chrysogenum*, an alpha-amylase from *Alteromonas haloplanctis,* and a cellulase from *Pseudoalteromonas haloplanktis* (62, 67, 78, 110, 111, 115, 205, 236). Unfortunately, none of these secreted proteins have been shown to be needed for growth at low temperatures.

However, some cell-associated proteins or factors have been shown to be important for growth in the cold. Trehalose is a disaccharide that is thought to stabilize

cell proteins and lipid membranes in response to various harsh environmental conditions including exposure to cold. In *E.coli*, trehalose synthesis is induced upon cold exposure, and a mutant deficient in trehalose production lost viability faster than the parental strain at 4°C (123).

In *L. monocytogenes*, carnitine, which like trehalose functions in cryotolerance, is not synthesized by the cell and therefore needs to be transported from the outside. In the cold, a mutant in the chill activated carnitine transporter, OpuC, grew slower than the parental strain in the presence of carnitine (9). *Listeria monocytogenes* also has an oligopeptide binding protein (OppA) required for bacterial growth at low temperatures (24).

In *Synechococcus* sp. PCC 7942, the heat shock proteins HtpG and ClpB both have a role in cold acclimation (109, 185). The ClpB content of the cell increases 5-fold when cultures are shifted from 37°C to 25°C, and the HtpG protein was induced at 16°C but not 30°C. These data correlate with the fact that both a *clpB* mutant and an *htpG* mutant had growth defects at the lower temperature (109, 185). In addition, the histidine utilization operon in the Antarctic psychotroph *Pseudomonas syringae* is upregulated at low temperatures, while in *Serratia marcescens* expression of the outer membrane protein ompF is upregulated at 28°C vs. 42°C (20, 126).

In recent years, more detailed studies have been made where selective capture of transcribed sequences (SCOTS), DNA microarrays, or 2D gel analysis have been used to acquire a more thorough understanding of the amount of change in gene expression needed for cold adaptation. In *E. coli*, at 1 and 5 hours after cold shock, expression in genes involved with cold-shock response, motility, sugar transport and

metabolism are upregulated (183). In *L*. *monocytogenes*, bacteria grown to mid log at 10°C had an upregulation in genes involved in motility, amino acid metabolism, cell surface alterations, and degrative metabolism (147). In addition, *B*. *subtilis* grown at 15°C for 70 hours had an induction of 279 genes and a reduction of 301 genes (27). Thus, for bacteria to successfully adapt to growth at low temperatures several changes including membrane remodeling and up or down regulation of many genes must occur.

PART IV – PPIASES AND THEIR FUNCTION

There are 3 different families of peptidyl prolyl *cis*/*trans* isomerases (ppiases); i.e. the cyclophilins, the FKBPs and the parvulins (83). These families have no amino acid similarity between the groups and do not share a similar structure, but they all have the ability to rotate the bond in between any amino acid (Xaa) and the amino acid proline (56, 83). Proline is unique among amino acids in that its ring structure allows it to readily exist in both the *cis* and *trans* peptide bond conformation (7, 8). This poses a problem in protein folding, since the Xaa-Pro *cis*/*trans* isomerization reaction becomes a rate limiting step that at 25°C would take minutes to occur without help (57, 83). The acceleration of protein folding by ppiases is well documented (82, 83, 127). There is also evidence that an additional function of ppiases is to regulate enzyme activity. NMR and X-ray chrystallography data suggest that the Xaa-Pro bond can exist in both the *cis* and *trans* form in the native protein structure of some proteins. (7, 8) and since the *cis* to *trans* rotation is a 180 degree rotation this can have a big effect on the overall protein structure (7). One example of a protein in which these conformational changes occur is a lipase in *Candida rugosa*, which has a loop that can flip up or down over the active

site depending on the *cis* or *trans* conformation of the proline bond (7). Another example is the bacteriophage MS2 coat protein, which also has proline in a loop structure that can bend depending on the *cis*/*trans* conformation (7). In some cases, the interaction with the ppiase responsible for these switches and its effect on the protein structure has been studied in more detail; e.g. with the nonreceptor tyrosine kinase, Itk, which participates in T cell activation (43). This protein has been shown to interact with cyclophilin A, and the switch from *cis* to *trans* form causes a conformational change in Itk that then allows it to interact with its signaling partners (43). Other ppiases have also been shown to have a role in controlling processes such as the mitotic cycle (248) and channel gating (149).

Ppiases are found in all organisms, and in bacteria these proteins can be cytoplasmic, periplasmic, or secreted (83, 93, 129, 212). Some ppiases have been shown to have a role in infection. For example, the secreted ppiase HP0175 in *Helicobacter pylori* induces apoptosis in gastric epithelial cells (19). Additionally, the surfaced exposed SlrA in *Streptococcus pneumoniae* is involved in colonization of the upper airways (98). Finally, Mip in *L. pneumophila*, which can exists as a surface exposed or a secreted form (50, 212), is important for infection in guinea pigs (131). The ppiase activity of the Mip protein has recently been indicated in the release or activation of a type II secretion exoprotein with p-nitrophenol phosphorylcholine (pNPPC) activity and in making collagen more susceptible to degradation by a serine protease (50, 245). Mip is therefore one example of a surface exposed/secreted ppiase that might regulate the activity of other proteins.

The *cis*/*trans* isomerization reaction is more limiting when the temperature is low since most reactions occur more slowly at lower temperatures (162). It is therefore not surprising that many cell associated ppiases have been shown to be upregulated during cold exposure; e.g. RotA in *E*. *chrysanthemi*, trigger factor and PpiA in *E*. *coli*, a FKBP ppiase in *Thermococcus*, PpiB in *B*. *subtilis*, and a FKBP ppiase in the psychrotrophic bacterium *Shewanella* sp. SIB1 (86, 116, 124, 183, 184, 225). This indicates an important role for cell associated ppiases at low temperatures. However, a function for a secreted ppiase in cold shock and cold adaptation has so far not been reported in the literature.

MATERIALS & METHODS

Bacterial strains

L. *pneumophila* serogroup 1 strain 130b (ATCC BAA-74) served as wild-type strain and was used for mutagenesis in this study (63, 101). Previously described mutants of strain 130b with mutations in genes associated with type II protein secretion, type II exoproteins, type IV piliation, or type IV secretion are described in **Table 1**. The construction of the chitinase mutant NU318 was described elsewhere (51). Mutants lacking iron regulated genes (NU235 and NU236), which both contain a promoterless *lacZ* gene, were previously described (101). Other *Legionella* species used appear in **Table 2**. A *pilD* mutant of *P*. *aeruginosa* (i.e. PAK-2B18) containing either vector alone or the complementing *pilD* gene was previously described (222). *E*. *coli* (DH5α and DH5α lambda pir) was used as a host for recombinant plasmids (Invitrogen, Carlsbad, Calif.).

Bacteriological media

Legionella species were routinely cultured on buffered charcoal yeast extract (BCYE) agar which contains 10 g/L ACES buffer, 10 g/L yeast extract, 2.4 g/L KOH, 1 g/L alpha-ketoglutaric acid, 15 g/L agar, 1.5 g/L activated charcoal, 0.4 g/L L-cysteine, and 0.25 g/L ferric pyrophosphate, with the pH adjusted to 6.85-6.95 (58, 144). Buffered yeast extract (BYE) broth was made essentially as the agar containing medium except for the omission of charcoal and sterilization by membrane filtration (0.2 micron) instead of

Table 1. *L. pneumophila* strain130b and its derivatives used in this study

Strain	Relevant genotype	Deficiency	Reference
130b	wild type strain	None	(63)
NU286	*pilD::'lacZ* aphA***	Prepilin peptidase	This study
NU243	*pilD::aphA*	Prepilin peptidase	(144)
NU272	*pilD::aacC1****	Prepilin peptidase	(198)
NU258	*lspDE::aphA*	Type II secretin and ATPase	(196)
NU259	*lspG::aphA*	Type II pseudopilin	(198)
NU275	*lspF::aphA*	Type II membrane protein	(198)
BS100	*pilE$_L$::aphA*	Type IV pilus pilin	(220)
NU279	*pilQ::aacC1*	Type IV pilus secretin	(198)
NU283	*lspDE::aphA*	Type II secretin and ATPase	(198)
	pilQ::aacC1	Type IV pilus secretin	
AA474	*lvhB9::aphA*	Type IV secretion	See below
AA405	*dotG::aphA*	Type IV secretion	See below
GG105	*dotA::aphA*	Type IV secretion	(251)
GQ262	*dotDCB::aphA*	Type IV secretion	(251)
GN142	*icmGCD::aphA*	Type IV secretion	(251)
NU291	*pilD::aacC1*	Prepilin peptidase	This study
	icmGCD::aphA	Type IV secretion	
AA200	*proA::aphA*	ProA metalloprotease	(158)
NU254	*map::aphA*	Major acid phosphatase	(10)
NU267	*lipA::aphA*	LipA and LipB lipases	(12)
	lipB::aacC1		
NU268	*plcA::aphA*	PlcA phospholipase C	(12)
NU270	*plaA::aphA*	PlaA lysophospholipase A	(71)

Type IV protein secretion mutants of strain 130b that contain a mini-Tn*10phoA* (155) insertion in *dotG* (AA405) or *lvhB9* (AA474) were kindly provided by N.C. Engleberg, University of Michigan. * promoter-less *lacZ* gene ***aphA* encodes kanamycin resistance ****aacC1* encodes gentamicin resistance

Table 2. *Legionella* species used in this study

Species	Strain*	Pathogen
L. pneumophila 130b	BAA-74	Yes
L. pneumophila Philadelphia-1	33152	Yes
L .pneumophila Oxford	43110	Yes
L. pneumophila Concord	35096	Yes
L. pneumophila 82A3105	43736	Yes
L. pneumophila 1169-MN-H	43703	Yes
L. anisa	35292	Yes
L. birminghamensis	43702	Yes
L. bozemanii	33217	Yes
L. brunensis	43878	No
L. cherrii	35252	No
L. cincinnatiensis	43753	Yes
L. erythra	35303	Yes
L. feeleii	35072	Yes
L. gratiana	49413	No
L .hackliae	35250	Yes
L. israelensis	43119	No
L. jamestowniensis	35298	No
L. jordanis	33623	Yes
L. londinensis	49505	No
L. longbeachae	33462	Yes
L. micdadei	31B	Yes
L. micdadei	33218	Yes
L. micdadei	Detroit-1	Yes
L. micdadei	Stanford-R	Yes
L. moravica	43877	No
L. oakridgensis	33761	Yes
L. parisiensis	35299	Yes

*The strain designations represent American Type Culture Collection (ATCC) strain numbers. For the source of the *L. micdadei* strains not at ATCC and a reference for all strains listed above see references (5, 171, 217).

autoclaving. Low-iron BCYE agar was made by omitting the 0.25 g/L ferric pyrophosphate iron supplement. In one experiment, bacteria were grown in chemically defined media (CDM) as previously described (5). Bacteria were occasionally patched onto egg yolk plates made as the BCYE plates except with substitution of starch instead of charcoal and containing an additional 5% (vol/vol) egg yolk (11, 71). *E. coli* were grown at 37°C on LB agar (13). Antibiotics were added to the media at the following final concentrations (μg per ml): ampicillin, 100; chloroamphenicol, 6 for *L. pneumophila* and 30 for *E. coli*; gentamicin, 2.5; and kanamycin, 25 for *L. pneumophila* and 50 for *E. coli*. Unless otherwise noted, chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo.).

Isolation of *L. pneumophila* genomic DNA

Genomic DNA was isolated from *L. pneumophila* based on a protocol by Neumann, et al. (167). One eight to one fourth of the bacteria on a three day old BCYE plate grown at 37°C were swabbed from the plate and resuspended in 1 ml of Phosphate Buffered Saline (PBS). After a 2 minute centrifugation (16000xg), the pellet was resuspended in 0.5 ml SET (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5). After addition of 50 μl of freshly made 10 mg/ml lysozyme and 5 μl of 100 mg/ml RNase A, the tube was incubated at 37°C for 30 minutes. 60 μl of 10% SDS and 3 μl of 18.6 mg/ml proteinase K were added and the tube was gently inverted before incubation with occasional gentle inversion at 55°C for 2 hours. Then, 210 μl of 6 M NaCl was added and the content of the tube was gently mixed. After addition of 700 μl chloroform, the tube was incubated at room temperature for 30-60 minutes on a rotating wheel. Following a 10 minute

centrifugation at 16000xg, the aqueous phase was transferred to a new tube using a blunt end pipette tip. The DNA was precipitated by adding 1 volume of isopropanol followed by centrifugation at 16000xg for 10 min. Finally, the pellet was washed with 70% ethanol, dried and resuspended in 50 µl TE (10 mM Tris·Cl pH 7.4, 1 mM EDTA pH 8.0).

Electroporation of plasmid DNA into *L. pneumophila*

Electrocompetent *L. pneumophila* were made by suspending two plates of freshly grown bacteria in 10-15 ml of sterile water (40). After a 10 minute centrifugation at 4300xg (5500 RPM, Beckman JA-14 rotor), the pellet was resuspended in 200 ml of sterile, ice-cold 10% glycerol and centrifuged again for 25 minutes. The glycerol wash and centrifugation was repeated. Finally, the bacteria were resuspended in 500 µl of 10% glycerol, resulting in a suspension of 10^{11} colony-forming units (CFU)/ml. The cells either were used immediately or were stored as 50 µl samples at -70°C. Electroporation of DNA into 50 µl electrocompetent *L. pneumophila* was performed using the BIORAD Gene Pulser Xcell and an electric pulse of 2.4kV (BIORAD, Hercules, CA) (40). The cells were then incubated in 2 ml of BYE for 2 hours while shaking at 37°C before plating onto the appropriate BCYE medium.

Sequence analysis

DNA sequencing was performed by the Northwestern University Biotechnology Laboratory, and primers were obtained from Integrated DNA Technologies (Coralville, IA). DNA and protein sequences were analyzed using Lasergene software (DNASTAR,

Madion, WI). Database searches were done using programs based on the BLAST algorithm (6). Predictions for signal peptides were determined using SignalP (21, 168). Signal peptides are short sequences (approximately 20 amino acids long), at the N-terminal end of the proteins, which are cleaved off when the proteins are transported through the inner membrane (168). These signal peptides have a common structure with a positively charged n-region, followed by a hydrophobic h-region and a neutral but polar c-region (168). The cleavage site is characterized by the (-3, -1) rule indicating that the amino acids at the -1 and -3 position relative to the cleavage site must be small, neutral residues (168). SignalP uses two networks to recognize these signal peptides and their cleavage site (168). The accuracy of these predictions are 95% (21). Predictions of protein localization was determined using PSORTb (77, 188). PSORTb is a program used to localize proteins to one or more of the five primary localization sites in Gram-negative bacteria; i.e., the cytoplasm, the inner membrane, the periplasm, the outer membrane and the extracellular space (77). The program uses several analytical modules for these predictions including the prescence of alpha-helical transmembrane regions, the presence of motifs known to exist in proteins localized to a certain compartment, BLAST search to a small database of proteins of known localizations and a signal peptide prediction (77). These PSORTb predictions have an overall precision rate of 97%, while the programs precision rate for predicting extracellular proteins is 94% (77).

Gene cloning

For plasmids used in this study see **Table 3.**

Table 3. Plasmids used in this study

Plasmid name	Relevant characteristics	Relevant gene and plasmid	Origin
pMMB2002	Cm^R vector used for complementation	*cat*[a]	(198)
pMD1	*pilD* gene cloned into pMMB2002	*piD*	(198)
pMF1	*lspF* gene cloned into pMMB2002	*lspF*	(198)
pMB3	*ppiB* gene cloned into pMMB2002	*ppiB*	This work
pEH40	Plasmid containing mini-Tn*10* transposon	'*lacZ*[b]*::aphA*[c]	(101)
pMM237	Plasmid containing mini-Tn*10* transposon	'*phoA*[d]*::aphA*	(155)
pX1918GT	Gm^R gene	Gm^R gene *aacC1*	(208)
PMB2190	Km^R gene	Km^R gene *aphA*	(87)
pGemT Easy	Cloning vector		Promega
pRE112	Counterselectable plasmid with *sacB* gene		(61)
pGD:Gm	*pilD* gene with Gm^R cassette inserted into the NarI site cloned into pGemT Easy	*pilD*::*aacC1*[e] in pGemT Easy	(198)
pUC18	Cloning vector		Invitrogen
pMC1871	BamHI fragment releases promoter-containing *lacZ* gene	*lacZ* gene	Pharmacia
pRS551	BamHI/BclI fragment containing beginning of promoter-less *lacZ* gene	'*lacZ* gene	(213)
pVK6	BamHI digested pMC1871 containing *lacZ* gene cloned into pVK3	*lacZ*	(215)
pVK10	BamHI/BclI fragment containing promoter-less *lacZ* gene from pRS551 fused to *lacZ* gene in pVK6	'*lacZ* cloned into pVK6	(215)
pML219	6-kb fragment containing *pilBCD* in pSU2719	*pilBCD* in pSU2719	(215)
pMS10	SalI/XbaI fragment from pVK10 ligated into the NarI site of *pilD* in pML219	promoter-less *lacZ* inserted into *pilD* gene	This work
pB24	1.6-kb PCR fragment containing *ppiB* cloned into pGemT Easy	*ppiB* in pGemT Easy	This work

pB24K	BamHI digested pB24 with HincII digested Km^R cassette from pMB2190	*ppiB*::*aphA* in pGemT Easy	This work
pB24KS3	NotI digest of pB24K containing *ppiB*:Km^R cloned into pRE112	*ppiB*::*aphA* in pRE112	This work
pB24G2	BamHI digested pB24 with HincII-PvuII digested Gm^R cassette from pX1918GT	*ppiB*::*aacC1* in pGemT Easy	This work
p24GS4	NotI digest of pB24G2 containing *ppiB*:Gm^R cloned into pRE112	*ppiB*::*aacC1* in pRE112	This work
pR11	0.8-kb PCR fragment containing *lpg1962*cloned into pGemT Easy	*lpg1962* in pGemT Easy	This work
pR11K1	AgeI digested pR11 with HincII digested Km^R cassette from pMB2190	*lpg1962*::*aphA* in pGemT Easy	This work
pR11K1S3	NotI digest of pR11K1 containing *lpg1962*:Km^R cloned into pRE112	*lpg1962*::*aphA* in pRE112	This work
pR11G2	AgeI digested pR11 with HincII-PvuII digested Gm^R cassette from pX1918GT	*lpg1962*::*aacC1* in pGemT Easy	This work
pR11G2S3	NotI digest of pR11G2 containing *lpg1962*:Gm^R cloned into pRE112	*lpg1962*::*aacC1* in pRE112	This work

[a] *cat* encodes chloroamphenicol gene

[b] promoter-less *lacZ* gene

[c] *aphA* encodes kanamycin gene

[d] truncated *phoA* gene

[e] *aacC1* encodes gentamicin gene

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 (238), yielding pVK6, and then the BamHI/BclI fragment of pVK6 was replaced with the BamHI/BclI fragment of pRS551 (213), 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 kanamycin resistance (Km^R) gene was isolated, treated with Klenow, and ligated into the NarI site of *pilD* in pML219 (145), creating pMS10. Finally, an *L. pneumophila* strain (i.e., NU286) containing the *pilD*::'*lacZ* fusion was isolated upon natural transformation of strain 130b with pMS10. The genotype of the strain was verified by PCR, Southern hybridization analysis and by patching onto egg yolk plates as previously described (11, 71, 215).

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 (198) containing *pilD::aacC1* was introduced by transformation into *icmGCD*::*aphA* mutant GN142 (251), and kanamycin resistance, gentamyacin resistance (Gm^R) 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 (11, 71, 198) (data not shown).

Construction of *L. pneumophila* strains lacking ppiase genes.

Based on data from the *L*. *pneumophila* Philadelphia-1 genome found at National Center for Biotechnology Information (NCBI at http://www.ncbi.nlm.nih.gov/), two pairs of primers were designed for amplifying genes from 130b DNA. Mas23 (5'- CGTACG GAGCTCATATTCAG) and Mas25 (5'- TGGTAATATTTTCAATGACTACAGG) yielded a 773-bp fragment containing *lpg1962* and Mas26 (5'- TCTGCAATGAATACGGATGG) and Mas27 (5'- GGTACACAAAAAGTTCTCGC) yielded a 1,552-bp fragment containing *ppiB*. To facilitate construction of *L*. *pneumophila* mutants the PCR fragments encoding *ppiB* and *lpg1962* were ligated into pGem-T Easy (Promega, Madison, Wis.), yielding pB24 and pR11, respectively. To facilitate the generation of double mutants both Km^R, Gm^R genes were inserted into *ppiB* and *lpg1962*. The Gm^R gene was isolated from pX1918GT after HincII and PvuII digestions (208) and was ligated into the single BamH1 site, which cuts 271 bp after the *ppiB* start codon, in pB24 or the single AgeI site, which cuts 292 bp after the *lpg1962* start codon, in pR11 to generate pB24G2 and pR11G2, respectively. A Km^R fragment isolated from pMB2190 upon HincII digestion (87) were inserted into the same sites generating pB24K and pR11K1. The mutated pR11G2 and pR11K1 were then moved from pGem-T Easy as a NotI digested, Klenow treated fragment which then was ligated into the SmaI site of the counterselectable pRE112, to give pR11G2S3 and pR11K1S3, respectively. The mutated pB24G and pB23K were also cloned into pRE112 as described above to generate pB24GS4 and pB24KS3. The pRE112 suicide vector facilitates allelic exchange in *L*. *pneumophila* by virtue of its sacB gene (61). A double mutant was constructed in the Km^R *lspF* mutant NU275 background by electroporating the pB24GS4 or the pR11G2S3 plasmids into

NU275. Double *ppiB*: *lpg1962* mutants were constructed by introducing pB24GS4 into a Km^R *lpg1962* mutant or by introducing pB24KS3 into a Gm^R *lpg1962* mutant. Production of competent 130b cells and electroporation of pR11G2S3, pR11K1S3, pB24GS4 and pB24KS3 into *L. pneumophila* were achieved as previously described. Potential mutants were selected based on chloroamphenicol sensitivity, sucrose resistance, Km^R resistance (pR11K1S3, pB24KS3), and Gm^R resistance (pR11G2S3 and pB24GS4) indicative of the introduction of the mutated gene into the 130b chromosome by homologous recombination. Verification of the mutant genotypes was carried out by PCR with the same primers used for PCR of the genomic DNA.

Complementation analysis.

To facilitate complementation of the *ppiB* mutant, a 1.1kb fragment containing *ppiB* was amplified by PCR from strain 130b using primers Mas27 and Mas28 (5'- TGTTTT GCATGATGTTTGTAAT) and then subcloned into pGem-T Easy. The resulting plasmid was sequentially digested by NsiI, treated with Klenow, and digested by EcoRI. Finally, the resulting 0.9kb product was ligated into EcoRI and SmaI digested pMMB2002 (198) under the control of the tac promoter, generating pMB3. The construction of the *lspF* complementing plasmid pMF1 and the *pilD* complementing plasmid pMD1 has been described elsewhere (198).

Extracellular growth determination at different temperatures

In order to compare the extracellular growth of *L. pneumophila* strains at different temperatures, equal numbers of CFU of the 130b strain and mutant bacteria taken from

mid to late log-phase BYE cultures grown at 37°C were inoculated into BYE broth and then incubated with shaking (225 rpm) at 37°C, 30°C, room temperature (RT), 17°C, or 12°C. Taking the bacteria from mid log phase as compared to late log phase usually made the bacteria grow faster which was more evident in the low temperature cultures. The extent of bacterial growth was assessed by measuring the optical density (OD) of the cultures at 660 nm. Due to different spectrophotometers having different abilities in measuring ODs in late log phase and since the OD660 has been measured on at least 6 different spectrophotometers through the years the maximum OD660 recorded for the 130b strain and mutants is different in experiments done at different times. In addition, bacteria taken from 3-day old 37°C BCYE plates were resuspended in water, diluted, and plated for isolated colonies on BCYE agar incubated 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. In some experiments, bacteria were instead resuspended in water, diluted, and 10µl spots of each dilution were spot plated in rows onto BCYE plates incubated at 37°C and 17°C. Growth was recorded by taking pictures of the plates on day 3 and day 10-25 for 37°C and 17°C, respectively.

Genetic screen to identify factors important for low temperature growth

To identify *L. pneumophila* genes important for low temperature growth, *sacB* containing mini-Tn*10* plasmids pEH40 (101), containing a promoterless *lacZ* gene and a Km^R

cassette, or pMM237 (155), containing a truncated *phoA* gene and a Km^R cassette, were electroporated into 130b. Transformants were selected on BCYE plates containing kanamycin and sucrose after 3 days of growth at 37°C. These colonies were then replica-patched onto two sets of BCYE plates using a toothpick followed by incubation of the plates at 37°C for 3 days or at 17°C for 6 days. For patches with reduced growth at 17°C, the corresponding patch from the 37°C plate was picked. The low temperature growth defect for these mutants were confirmed by plating 10µl spot of several dilutions on two sets of plates that where incubated at 37°C for 3 days or at 17°C for 21 days. To determine the genetic loci potentially important for low temperature growth in *L. pneumophila*, inverse PCR was used to find the genes with Tn*10* insertions as previously described (145). In brief, genomic DNA was digested with HindIII, an enzyme which cuts once within the mini-Tn*10*, and then the restricted DNA was circularized with T4 DNA ligase. PCR products were generated with primers USHIND (5'-TGATTTT GATGACGAGCG) and DSHIND (5'-GTGACGACTGAATCCGGT) that recognize sequences on either side of the HindIII site as well as Tn10-2 (5'-TCATTAG GGGATTCATCA) specific for a sequence in the transposon's inverted repeats. A few mutants were not successfully sequenced by inverse PCR, and genomic DNA from these mutants was therefore digested with PstI or EcoRI before ligation to pUC18. After electroporation into *E. coli*, colonies containing plasmids with genes with Tn*10* insertions were selected for by using the Km^R marker in the transposons. After plasmid isolation, primers Tn10-2 and M13For (5'-TGTAAAACGA CGGCCAGT) that recognize sequences in the transposon end and pUC18, respectively, were used to generate PCR products for sequencing.

Preparation of supernatants from BYE grown bacteria

Supernatants were collected from bacteria grown in BYE broth until mid-log to early stationary phase. In brief, cultures were spun down at 2200g for 30 minutes before the supernatant was sterilized by passage through a 0.2 µm filter and either used immediately or stored at -20°C. If needed, supernatants were concentrated 30-100 fold by passage through a Millipore YM10 ultrafiltration cell (11).

2D gel analysis and protein identification

L. pneumophila were grown in 300 ml broth at 37°C, 17°C or 12°C, and 50 ml supernatants were collected at several time points in the late log to early stationary phase. These supernatants were concentrated 50-fold as described above. Samples were treated with the Ready Prep 2-D Cleanup Kit (BioRad, Hercules, CA) and then applied to a pH 3-10 Ready Strip IPG strip by overnight rehydration (BioRad, Hercules, CA). Isoelectric focusing was done using a PROTEAN IEF cell (BioRad, Hercules, CA) for a total of 50,000 V-hr. The IPG strips were equilibrated for 10 min each in equilibratium buffers I and II and then resolved on a 12% polyacrylamide SDS gel containing a 3 cm 5% stacking gel using a PROTEAN II xi (BioRad, Hercules, CA). The Benchmark (Invitrogen, Carlsbad, California) protein ladder was used as a molecular weight marker. Gels were stained with Coomassie Brilliant Blue G-250 and images were acquired using a Kodak digital camera. Protein spots were excised and submitted for identification to Stanford Mass Spectometry Services (Stanford University, Stanford, CA at http://mass-spec.stanford.edu). The MS/MS spectra were searched using MASCOT (Matrix Science, Boston, MA) by using the bacterial database NCBI-nr. Peptide mass

tolerance was set at 2 Da and fragment mass tolerance was set at 0.8 Da. Unambiguous matches were found by considering the number of peptides matched, the percentage of ORF covered, and the agreement between the experimental and predicted masses and isoelectric points for the protein (51).

Cross-complementation assay using whole cells

To determine if secreted factors facilitate low-temperature growth, a heavy streak of the 130b strain 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. 10^4-10^6 CFU of a type II secretion mutant also derived from a fresh 37°C plate. The plates were then incubated at RT 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.

Cross-complementation assay using low temperature supernatants

The 130b strain was grown in BYE broth at 17°C to late log phase. Sterile supernatants were prepared as previously described (11). Roughly 30 µl supernatants were added to sterile paper discs placed on top of a sterile surface inside a hood. After about 40 minutes when this amount of liquid had dried into the discs, 30 µl was again added per disc. This was repeated 7 times. In the end, 8 discs with a combined total of 2 ml of dried in supernatants were added to plates that had been plated with 10^5 or 10^6

CFUs/ml of *lspF* mutant. Plates were incubated at room temperature and growth promotion was assessed by visual inspections of the plates at different days.

Water survival

Tap water used for the first two water survival experiments (chapter 3) were collected from the same tap in the laboratory, 20 months apart, and was filter sterilized before use. In addition, in a third water survival experiment with the *ppiB* and *lpg1962* mutants (chapter 5) water was again collected from the same tap (28 months after experiment 2) and autoclaved before use. Bacteria from 3 day old plates grown at 37°C were used to start overnight cultures in BYE broth. The bacteria were grown to a similar late log phase before they were centrifuged (2200 g for 30 minutes) and the pellet was resuspended in tap water. After one more centrifugation, the bacteria were resuspended in 50ml of tap water to a concentration of 10^8 CFUs/ml. Cultures were then allowed to incubate with shaking at 225 rpm at 4°C, 12°C, 17°C and 37°C. Survival was monitored at different time points by plating for CFUs on BCYE plates incubated at 37°C. The amount of viable bacteria were also determined by live/dead stain at various time points by mixing 1 ml of water culture with 1 µl of a 1:1 ratio of the dyes SYTO9 and propidium iodine from the Live/Dead BacLight Bacterial Viability kit (Molecular Probes, Eugene, OR). A few µl of this bacteria-dye mix were removed and added to 0.5 ml of dH20 and this solution was then filtered onto black polycarbonate filters (Osmonics, Minnetonka, MN). The filters were allowed to dry for a few minutes in the dark before the amount of live/dead bacteria was determined using fluorescence microscopy (Leica DMR, Wetzlar, Germany).

β-galactosidase assay

To quantitate *lacZ* expression in *L. pneumophila* gene fusion strains, β-galactosidase (Bgal) activity was measured by taking aliquots from BYE broth cultures grown at 37°C, 30°C, RT, and 17°C (13, 101). After recording the OD660, 0.1 ml was added to 0.9 ml Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol). The bacteria were then lysed by adding 2 drops of chloroform and 1 drop of 0.1% SDS followed by vortexing for 10 seconds. After 5 minute incubation at 28°C, 0.2 ml of the colorigenic substrate o-nitrophenol-β-D-galactosidase (4 mg/ml) was added to each tube. To stop the reaction, 0.1 ml 1 M Na_2CO_3 was added, and the OD420 and OD550 were recorded. Bgal units were calculated by Miller units: 100 x (OD420- (1.75 x OD550))/ (reaction time in minutes x volume of culture in mls x OD660).

Enzymatic activities in *L. pneumophila* supernatants

Phosphatase activity

Acid phosphatase, tartrate resistant acid phosphatase, and alkaline phosphatase activity were measured as the ability of the sample to release p-nitrophenol (pNP) from p-nitrophenyl phosphate (pNPP) as described previously (10). In brief, 10 µl of supernatants were added to 90 µl 200 mM sodium acetate buffer pH 5.5 containing 0.2% p-NPPhosphate in a microtiter plate. The microtiter plate was incubated for 3 hours at 37°C inside a plastic bag with a small piece of wet Kleenex to prevent

evaporation. Before reading the plates at 410 nm, 1 µl 10 M NaOH was added to each well since pNP is not yellow at an acidic pH. Tartrate resistant acid phosphatase activity was measured as above but with 5 mM sodium tartrate added to the sodium acetate buffer. Alkaline phosphatase activity was assayed as for the acid phosphatase activity but using a 100 mM Tris pH 10 buffer and with the omission of the addition of 1 µl 10M NaOH.

Protease activity

Protease activity was measured using azocasein as a substrate (11). In brief, 100 µl of supernatant was mixed with 100 µl assay buffer (25 mg/ml azocasein in 0.1 M phosphate buffer pH 7.6). The mixture was then incubated at 37°C for 30 minutes, before addition of 800 µl of 5% TCA. After centrifugation at 2000xg for 5 to 10 minutes, the supernatants were transferred to a new tube. Finally, 1 volume of 0.5 N NaOH was added and the absorbance at 440 nm was measured.

Proteolytic and lipolytic activities determined on egg yolk plates

Egg yolk plates are commonly used to detect secreted lipolytic and proteolytic activities (11, 71). On these plates, secreted bacterial phospholipase activities results in an opaque precipitate, lipase activity results in iridescent sheen, and proteolytic activity results in a clearing around the plated bacteria. In *L*. *pneumophila*, this assay can be used to distinguish between the parental strain and mutants lacking type II secretion dependent lipolytic and proteolytic activities (11, 71).

Pulmonary infection of A/J mice with *L. pneumophila*

Intratracheal infection of A/J mice is often used as a model system for acute *L. pneumophila*-induced pneumonia (26). To assess the importance of PpiB and Lpg1962 *in vivo*, competition assays were done, as described previously (191, 198). In brief, six- to eight-week-old, female mice (Jackson Laboratories, Bar Harbor, ME) were inoculated intratracheally with 10^6 CFU of a *ca.* 1:1 ratio of the 130b strain and mutant bacteria. At various time points, infected mice were sacrificed, and lungs were homogenized (Tissue Tearor, Biospec Products, Inc. Bartlesville, OK) in 5 ml of phosphate-buffered saline. Host cell lysis was insured by incubation of the tissue sample with 50 µl of 10% saponin. The numbers of viable bacteria and the ratio of the 130b strain to mutant were determined by plating on both standard and Km^R supplemented BCYE agar.

Intracellular infection of *L. pneumophila* in amoebae

To examine the ability of *L. pneumophila* and its derivatives to grow within a protozoan host at 17°C, 22°C and 35°C *A. castellanii* or *H. vermiformis* were infected as previously described (40, 144, 159, 198). In brief, amoebae were preadapted to the 17°C, 22°C or 35°C temperature by passing them for at least one passage at the respective temperature before infection. Then, 10^4 CFU bacteria, from 3-day old BCYE plates grown at 37°C, were added to wells with $5x10^5$ amoebae. At 0, 24, 48, and 72 hours for the 35°C infection, at 0, 4, 7, 9 and 11 days for the 22°C infection and at 0, 4, 9, 12, 15 and 18 days for the 17°C infection, the numbers of bacteria per coculture were determined by plating serial dilutions on BCYE plates.

H. vermiformis were maintained in Axenic Media 1034 which contains (w/v) 0.1% yeast extract, 0.1% BactoPeptone (BD, Franklin Lakes, NJ), 1.5 μM hemin, 34 μM folic acid, buffered in 130 μM KH_2PO_4 and 180 μM Na_2HPO_4, supplemented with 10% FBS, adjusted to a pH of 6.5. For infection, amoebae were centrifuged at 1100rpm in a Jouan BR4i centrifuge (Jouan, Winchester, VA) for 15 minutes and resuspended in a 1:1 mix of Axenic media without FBS and Puck's saline (110 μM $CaCl_2$, 380 μM KCl, 610 μM K_2PO_4, and 620 μM $MgSO_4$ in 11% (w/v) glucose).

A. castellanii were maintained in PYG media (2% protease bacto peptone, 0.1% yeast extract, 0.1% sodium citrate dihydrate, 0.1 M glucose, 4 mM $MgSO_4$, 0.4m M $CaCl_2$, 0.05 mM $Fe(NH_4)_2(SO_4)_2$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3, pH 6.5). For infection, amoebae were centrifuged at 1100rpm in a Jouan BR4i centrifuge (Jouan, Winchester, VA) for 15 minutes before resuspended in PY media (PYG media without glucose).

Chapter 1

PILD EXPRESSION IN *LEGIONELLA PNEUMOPHILA* IS TEMPERATURE REGULATED

INTRODUCTION

The *pilBCD* operon in *L. pneumophila* was discovered while characterizing an iron acquisition mutant (145). In various gram negative bacteria, PilB, PilC, and PilD are involved in type IV pilus biogenesis and type II secretion (139, 223). PilB is required for pilus extension while PilC is an inner membrane protein that might facilitate pilin translocation (170, 223). The function of PilD, the prepilin peptidase, is to catalyze both the cleavage of the amino-terminal signal sequence and the subsequent N-methylation of the exposed hydrophobic amino-terminal of pilin-like proteins (pseudopilins) (139, 223). While PilB and PilC are only involved in type IV pilus biogenesis, PilD has a dual function since the PilD processed pseudopilins are integral parts of both the type IV pilus apparatus and the type II secretion apparatus (121, 139, 223).

Since *Legionella* can be found in aquatic environments and since the expression of its flagella is higher at 30°C than at 37°C (174), *pilB* expression was tested from strain 130b grown on plates at 30°C and 37°C (145). Northern blot data showed increased levels of a transcript large enough to contain *pilB*, *pilC* and *pilD* from bacteria grown at 30°C (145). This result was confirmed by RT-PCR (145). The increased levels of *pilB* at 30°C suggested that piliation might be greater at the lower temperature (145). Indeed, while no pili could be detected at 37°C, 5-10% of bacteria were piliated at 30°C (145). These data were in conflict with an earlier paper showing pili on *L. pneumophila*

grown at 37°C (192). It was thought the reason for this difference was due to those bacteria being grown at 37°C with added 5% CO_2 (192). This was confirmed to be the case as more than 50% of the bacteria were piliated when *L*. *pneumophila* was grown at 37°C with 5% CO_2 (143). This indicated that the *pilBCD* operon in *L*. *pneumophila* might be regulated by both temperature and the presence of CO_2 (143). However, it was also hypothesized that the CO_2 effect might be due to a decrease in the pH of the media (143).

The *L*. *pneumophila* pilin gene, *pilE*, was first characterized by Abu Kwaiks' lab (220). A *pilE* mutant had a 50% decrease in adherence to human epithelial cells, macrophages and *A*. *polyphaga* but did not have defect in intracellular growth (220). In contrast, a *pilD* mutant had a large intracellular growth defect in macrophages, amoebae, and mice indicating the importance of type II secretion but not type IV piliation for intracellular survival (144, 198).

The components of the type II secretion apparatus have been studied in detail but there are not many data about the regulation of the type II secretion apparatus genes except that quorum sensing has been linked to *xcp* expression in *P*. *aeruginosa* (33, 121). The aim of the present study was to gain new knowledge about *pilD* regulation. This study started by confirming that *pilD* expression is up-regulated at 30°C and in the process gained evidence for an even higher up-regulation of gene expression at even lower temperatures. In addition, the effect of pH on *pilD* expression was briefly examined.

RESULTS

To further investigate the effect of temperature on *pilD* expression a *pilD*::'*lacZ* fusion strain was constructed by inserting a promoterless *lacZ* into the NarI site of *pilD*. This mutant strain was given the designation NU286. In the first β-galactosidase (Bgal) assessment experiment, NU286, together with two strains containing iron regulated *lacZ*-fusions, NU235 and NU236 (101), were grown as patches on BCYE plates at 37°C and 30°C. On day 2, 3, 4, and 5, two patches from each temperature were resuspended in dH_20 and Bgal activity was measured. NU286 bacteria grown at 30°C had a higher Bgal expression than bacteria grown at 37°C on all days tested even though the difference was only about 20-30% (**Fig. 1-1**). In contrast, the two iron regulated genes in NU235 and NU236 had a higher expression at 37°C (**Fig. 1-1**).

In the same experiment, NU235, NU236 and NU286 were also patched on BCYE plates with a pH of 6.2 instead of the normal pH of 6.9. As above, two patches per strain were tested for Bgal activity on day 2, 3, 4 and 5. The Bgal activity from the bacteria grown on the pH 6.2 plates was lower at every time point for all the mutants (**Fig. 1-1**). Since I was interested in pursuing the reasons why *pilD* expression increases, the down-regulation of the gene in response to pH was not pursued. Instead, work was concentrated on showing a possible temperature regulation for *pilD*.

Bgal levels were next assayed by growing NU236, NU286 and strain 130b in BYE broth at 30°C and 37°C while monitoring the expression at several time points along the growth curve (**Fig. 1-2**). These studies again showed a small difference in Bgal levels between NU286 bacteria grown at the two temperatures in that the Bgal levels for the 30°C grown bacteria were consistently about 20-25% higher than the Bgal

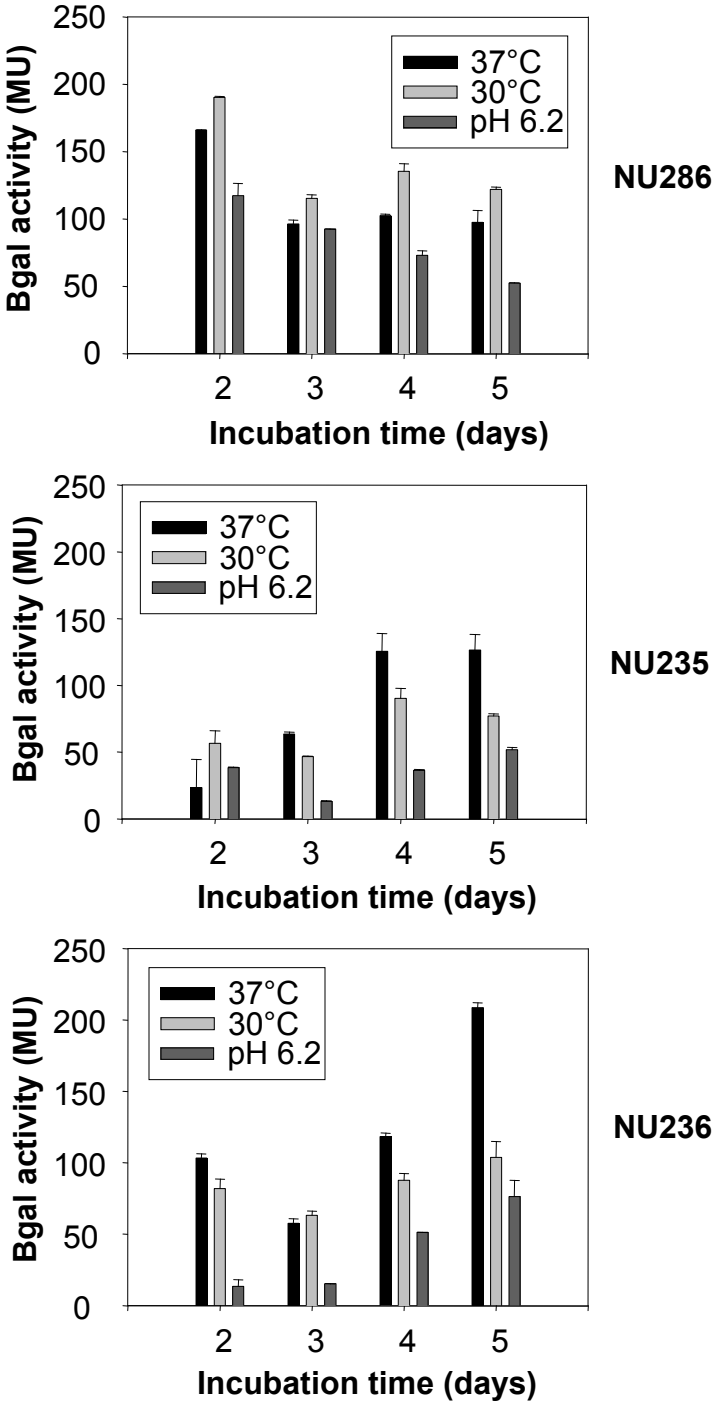

Figure 1-1. Effect of temperature and pH on β-galactosidase production by NU286, NU235 and NU236 grown on plates at 30 and 37°C. *pilD* mutant NU286, and two iron regulated mutants, NU235 and NU236, were grown at 37°C and 30°C on normal BCYE plates or at 37°C on pH 6.2 BCYE plates were resuspended into water on day 2, 3, 4, and 5 and the β-galalctosidase activity was determined as described. The results presented are the means and standard deviations from three samples.

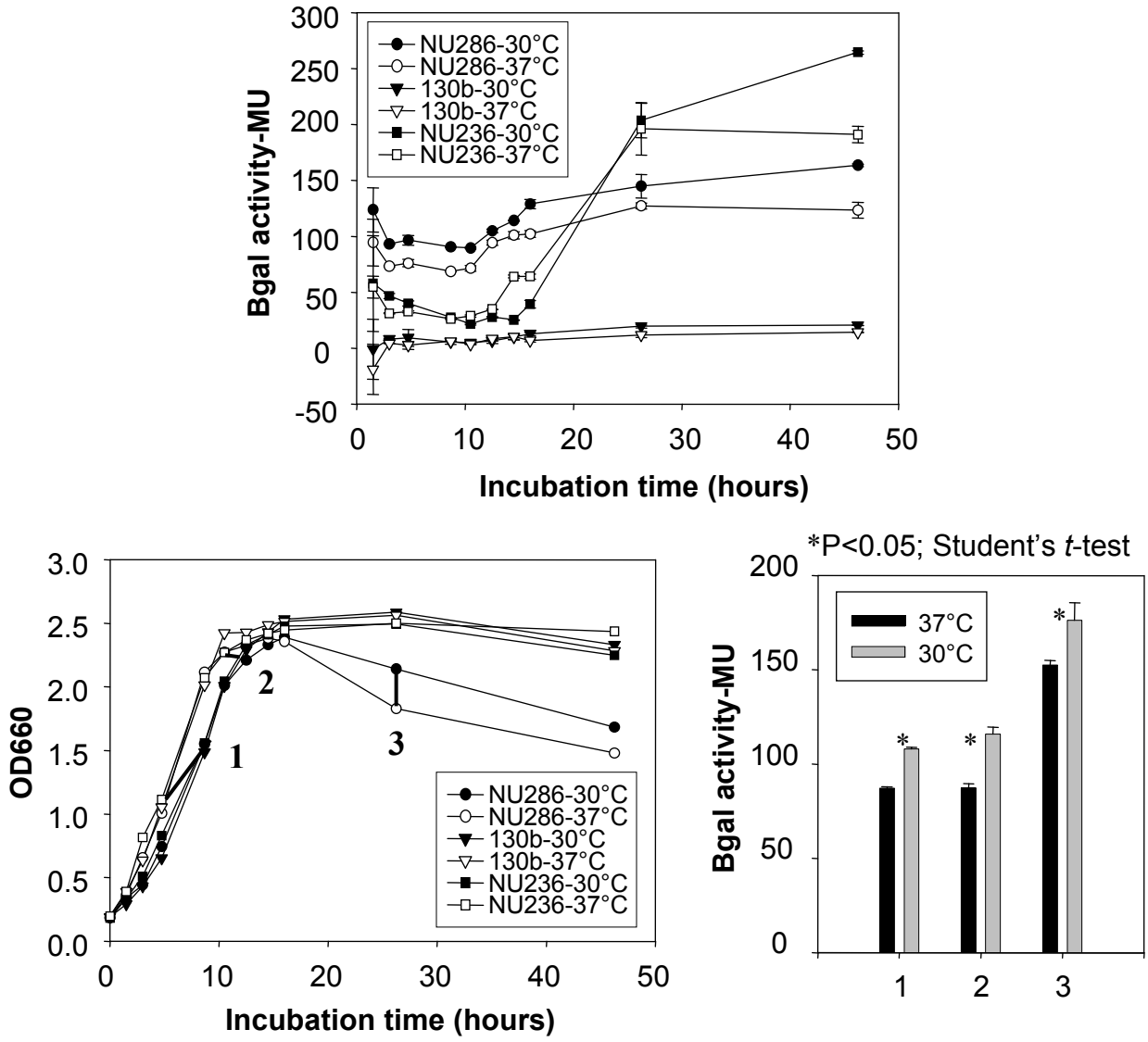

Figure 1-2. Growth of and β-galactosidase production by *L. pneumophila* strain NU286, NU236 and strain 130b at 30 and 37°C. Log-phase *pilD* mutant NU286, iron regulated mutant NU236 and strain 130b bacteria were inoculated into BYE broth and then incubated, as noted, at 37°C and 30°C. The β-galactosidase activity was monitored (top panel) and the growth of the cultures was monitored spectrophotometrically (bottom left) at several time points along the growth curve. Since the expression of β-galactosidase activity was growth dependent comparison between β-galactosidase activity at the two temperatures for the NU286 mutant were done at the three indicated time points (labeled as 1, 2, and 3) when the various cultures had achieved comparable stages of growth (bottom right). The results presented are the means and standard deviations from three samples. Similar results were seen at least 8 times.

levels for the 37°C grown bacteria. This small up regulation was seen in at least 8 separate experiments. In contrast, NU236 did not show a consistent up-regulation of Bgal levels at either temperature and Bgal levels for 130b were close to zero as would be expected. In addition, the Bgal levels at both temperatures also increased slightly during the course of growth indicating that *pilD* expression might be both temperature and growth phase regulated. Because of this growth dependent difference, and since the growth rate at 30°C was slightly slower than the growth rate at 37°C, Bgal levels were compared not at the same time point but between bacteria from the same stage of growth.

These Bgal data were promising but it was possible that the increases in Bgal levels seen at 30°C vs. 37°C actually were due to a growth rate related increase since the growth rates at 30°C were slightly slower than at 37°C. Therefore temperature shift experiments were done where all cultures started out at 37°C but at 2.5 hours half of the cultures were shifted to 30°C (**Fig. 1-3**). If an increase in Bgal levels could be seen before the growth rate of the shifted cultures slowed, it would show that the increase was temperature dependent. Unfortunately, a difference in Bgal levels of 20-30% could not be seen until 3 hours after the shift and at this time point the growth rates of the shifted cultures had already changed. This pointed to a growth rate induced change in *pilD* expression, though one alternative explanation is that the Bgal change is a temperature dependent change, but that the bacteria needs some time to readjust after cold shock treatment. This would not be surprising since all bacteria when adapting to cold shock, go through an acclimation phase which can last from one to several hours (88, 230).

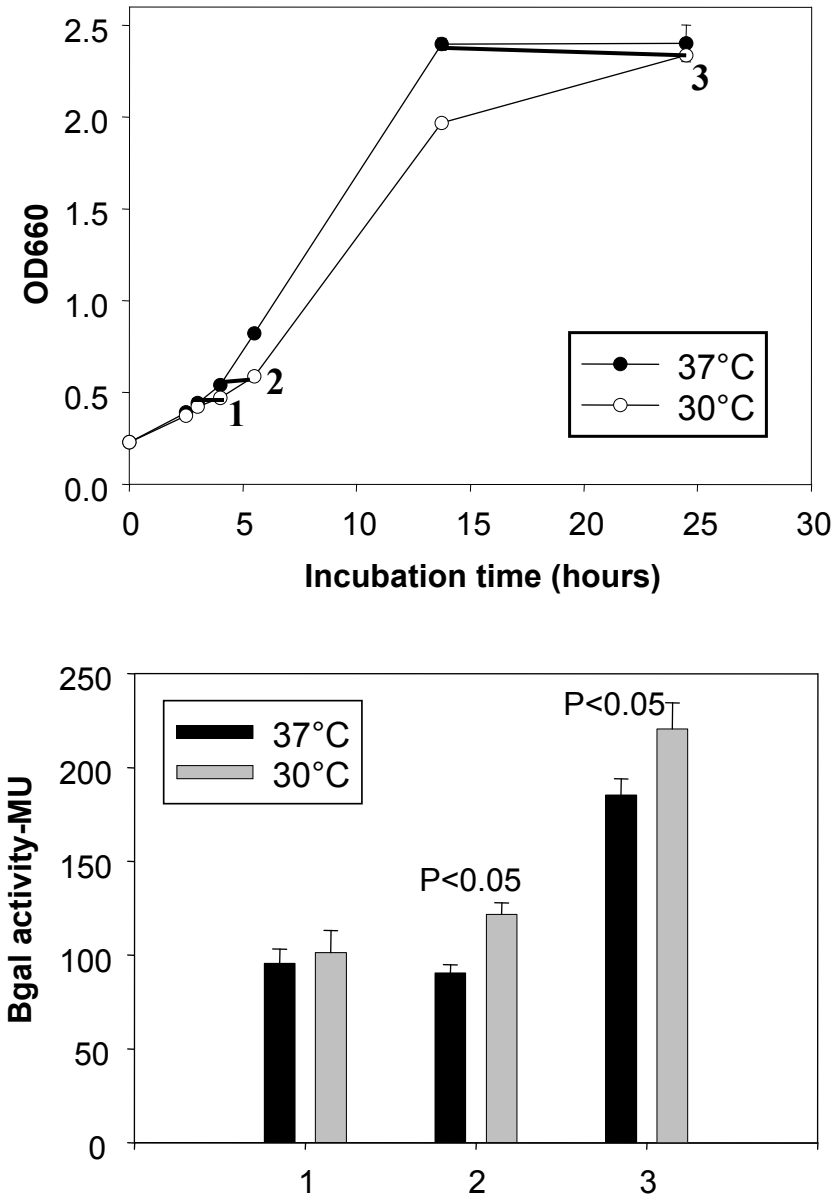

Figure 1-3. Effect of temperature shifts from 37°C to 30°C on the β-galactosidase production by NU286. Log-phase NU286 bacteria were inoculated into BYE broth at 37°C then at 2.5 hours half of the cultures were shifted to 30°C. The growth of the cultures was monitored spectrophotometrically (top panels) and, at three indicated time points (labeled as 1, 2, and 3) 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.

Next, it was hypothesized that if the growth rate at 37°C could be slowed down to the same levels seen when the bacteria were grown at 30°C, then if an increase in Bgal levels were seen in these slower growing cultures, this increase would be due to the change in growth rate. If the Bgal levels were the same then the increase seen at 30°C would be due to temperature. To test this, NU286 were grown in BYE and chemically defined medium (CDM) at 37°C (**Fig. 1-4**). The growth rate for NU286 in CDM at 37°C slowed to the same level as growth at 30°C in BYE but in contrast to growth at 30°C in BYE the maximum OD660 reached in the CDM culture was lower. Bgal assessments done did not show any consistent increase in Bgal levels between the BYE and the CDM cultures and therefore the increase in Bgal levels seen at 30°C is most likely due to a temperature regulation of *pilD*.

If *pilD* expression is temperature regulated, then expression might be lower at an even higher temperature. To test this hypothesis, NU286 was grown in BYE broth at 37°C and 42°C and Bgal levels were assessed at several time points along the growth curve (**Fig. 1- 5**). The growth rate of NU286 was equal at the two temperatures while the Bgal levels at 42°C were 10-20% lower than at 37°C. This is in agreement with a temperature regulation of *pilD*.

Next, Bgal expression was measure at lower temperatures than 30°C.; i.e., room temperature (RT). Since *Legionella* had not previously been grown in the lab at this temperature, initial growth assessments were done. These initial trials showed that NU286 grew at RT but that the growth rate slowed as the temperature went down (**Fig 1-6**). While at 37°C the bacteria needed 12 hours to reach maximum OD660 in BYE, at 30°C 20-30 hours were required and at RT 40-50 hours. NU286 also reached a slighter

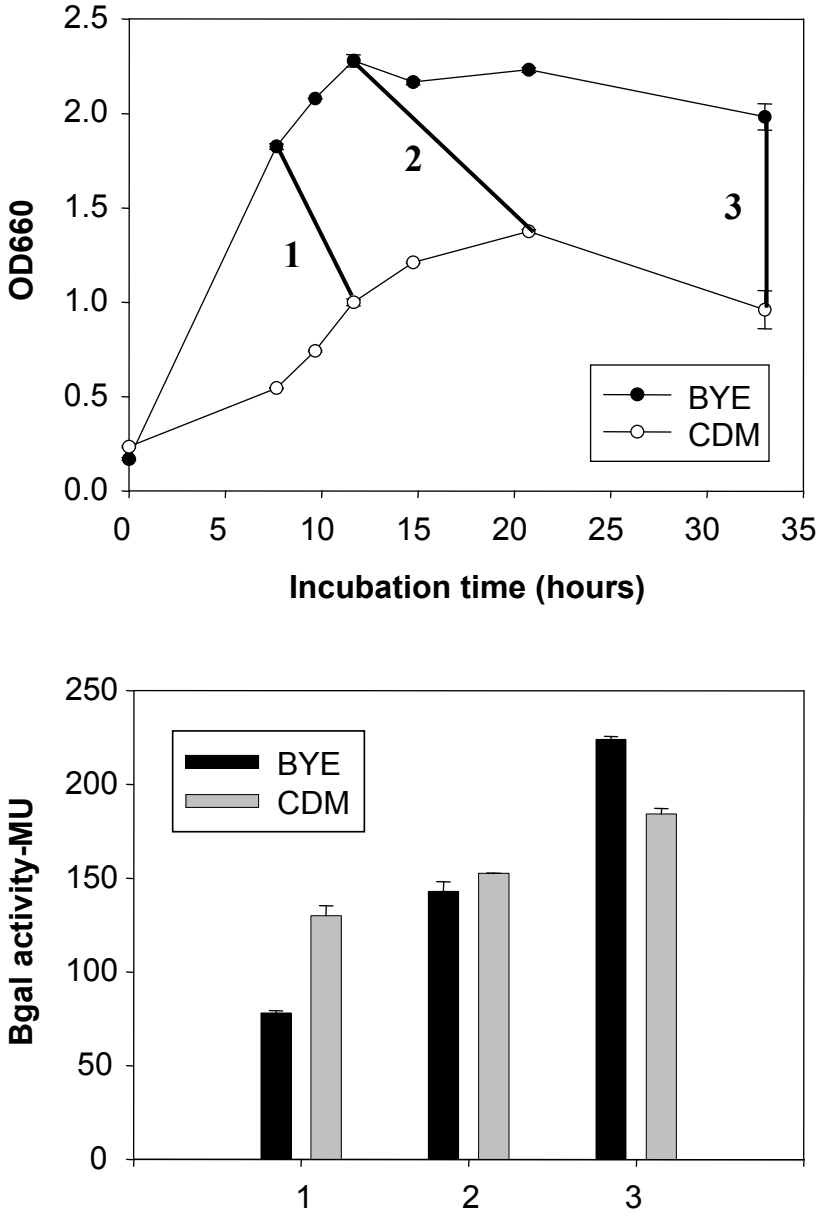

Figure 1-4. Effect of growth in CDM at 37°C on the β-galactosidase production by NU286. Log-phase NU286 bacteria were inoculated into BYE broth or CDM then incubated at 37°C. The growth of the cultures was monitored spectrophotometrically (top panels) and, at three indicated time points (labeled as 1, 2, and 3) 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 two samples.

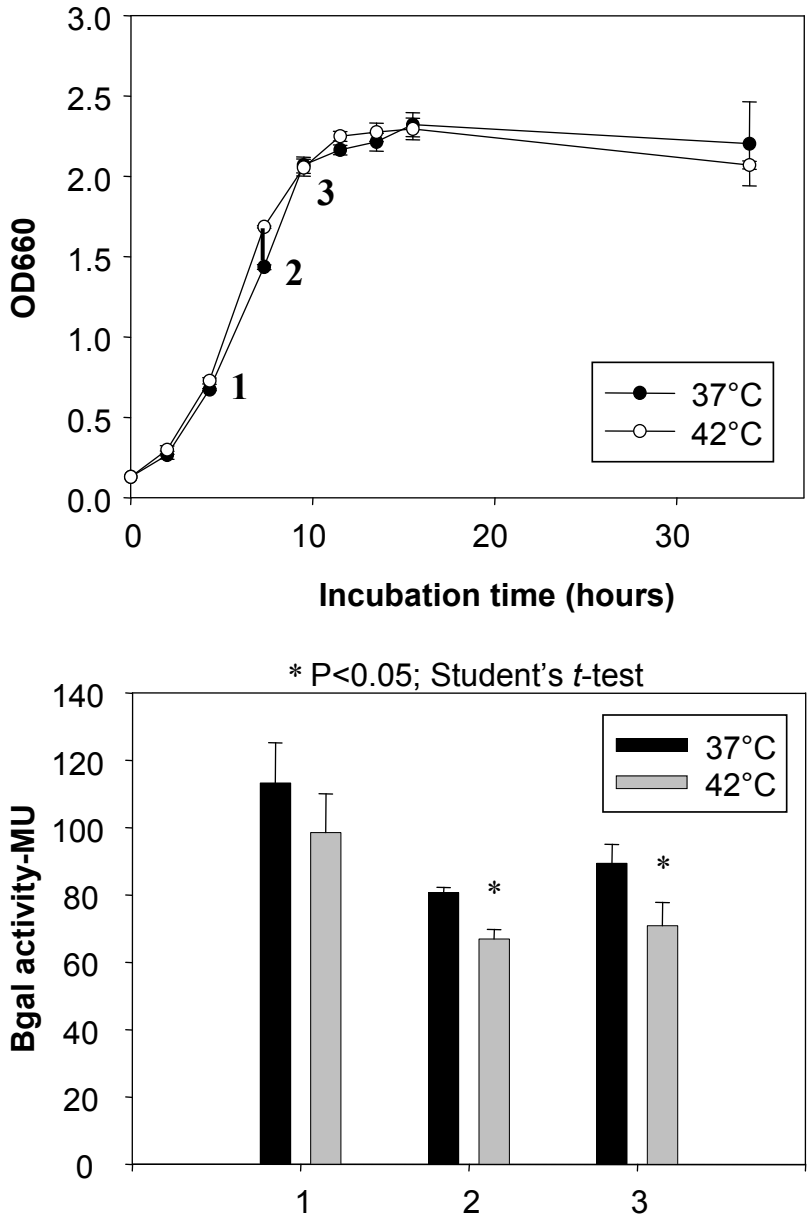

Figure 1-5. Effect of temperature on the growth of and β-galactosidase production by NU286 at 37°C and 42°C. Log-phase NU286 bacteria were inoculated into BYE broth and then incubated, as noted, at 37°C and 42°C. The growth of the cultures was monitored spectrophotometrically (top panels) and, at three indicated time points (labeled as 1, 2, and 3) 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 three samples.

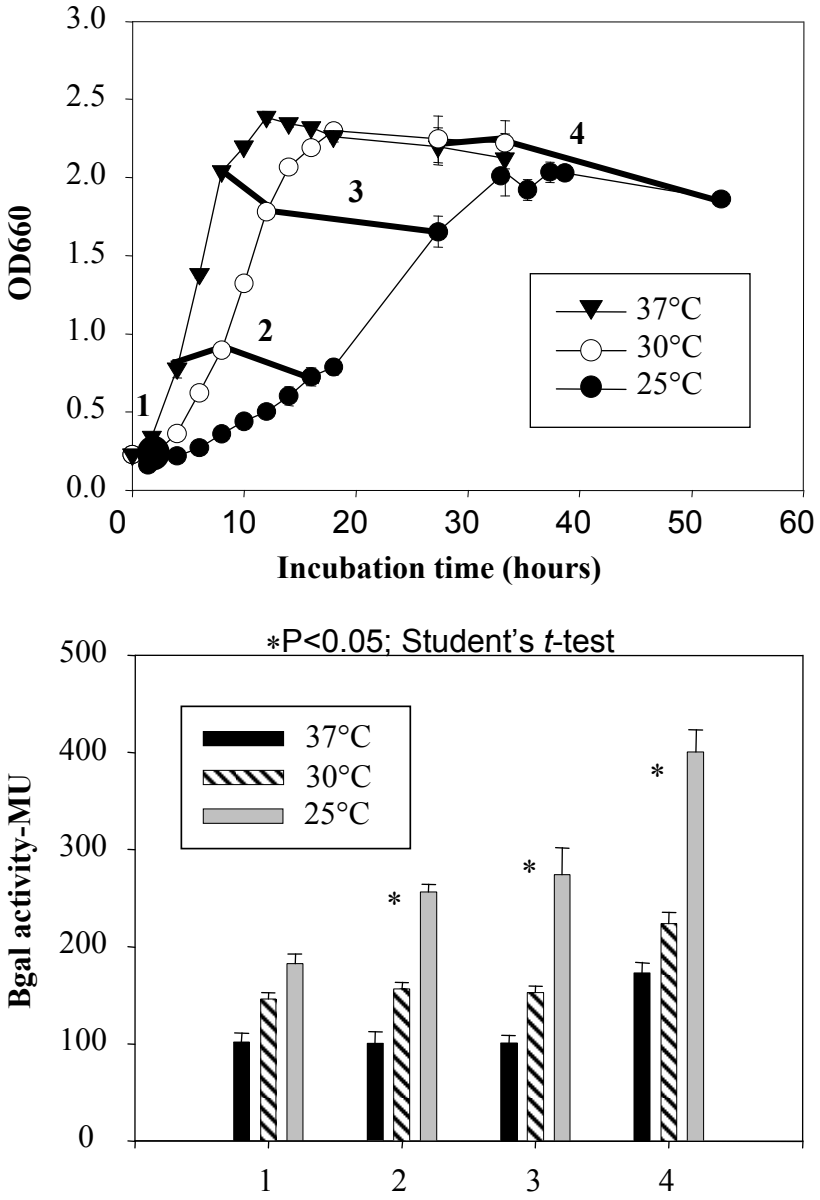

Figure 1-6. Effect of temperature on the growth of and β-galactosidase production by NU286 at 37, 30, and 25°C. Log-phase NU286 bacteria were inoculated into BYE broth and then incubated, as noted, at 37, 30, and 25°C. The growth of the cultures was monitored spectrophotometrically (top panels) and, at three 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.

lower OD660 at RT as compared to 30°C and 37°C. The Bgal levels were now much higher with a 2-3 fold increase in Bgal levels at RT as compared to 37°C. The expression at RT was again growth phase dependent with expression in stationary phase double that of expression in early log phase. These results were also seen in a repeat experiment (**Fig. 1-7**).

Finally, Bgal comparison was done for bacteria grown in BYE broth at 17°C and 37°C. Since *L*. *pneumophila*, to our knowledge, had not been grown at 17°C before, initial growth assessments were done (**Fig. 1-8**). As before the growth rate slowed as the temperature went down, and this time NU286 needed 80-100 hours to reach maximum OD660. While a reduction in maximum OD660 reached had been apparent in the room temperature cultures, this was now even more apparent at 17°C. As before the Bgal expression was growth dependent with a 2-3 times higher expression in early log phase and a 6-fold increase in late log/stationary phase when values between 17°C and 37°C cultures were compared. Similar results were seen in a repeat experiment (**Fig. 1-9**).

The Bgal activities seen for NU286 were clearly much greater at 17°C, than at 30°C or 37°C. Therefore, the Bgal level comparison between NU286 and the iron regulated *lacZ*-fusion, NU236, was redone with cultures grown at 17°C and 37°C (**Fig. 1-10**). The Bgal levels for NU236 were slightly higher in the 17°C cultures as compared to the 37°C cultures but in comparison to the increase seen for NU286 at 17°C, this increase was very small. In fact, only the values from the early and mid-log time points were statistically significant. However, this small increase could be due to a real increase in gene expression for NU236 at 17°C vs. 37°C or it could be due to a higher

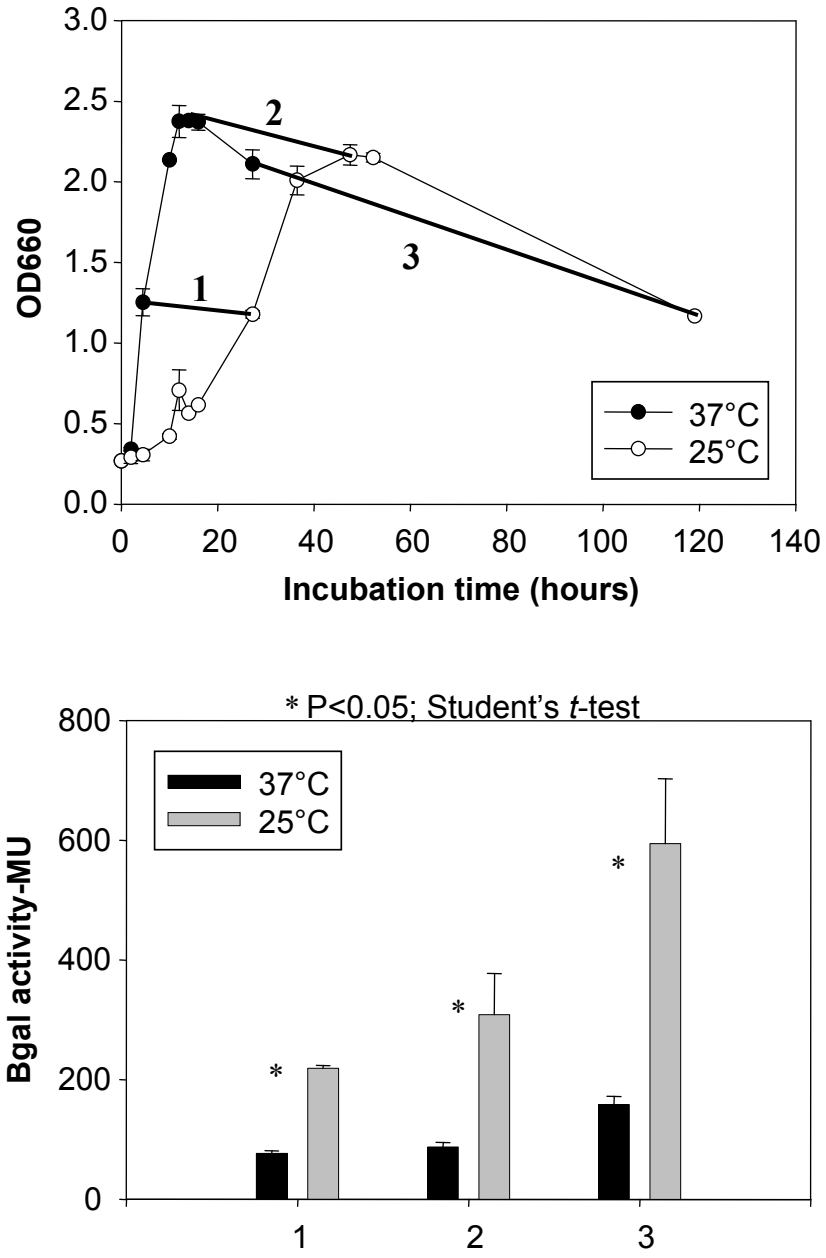

Figure 1-7. Effect of temperature on the growth of and β-galactosidase production by NU286 at 37 and 25°C. Log-phase NU286 bacteria were inoculated into BYE broth and then incubated, as noted, at 37 and 25°C. The growth of the cultures was monitored spectrophotometrically (top panels) and, at three indicated time points (labeled as 1, 2, and 3) 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 two samples.

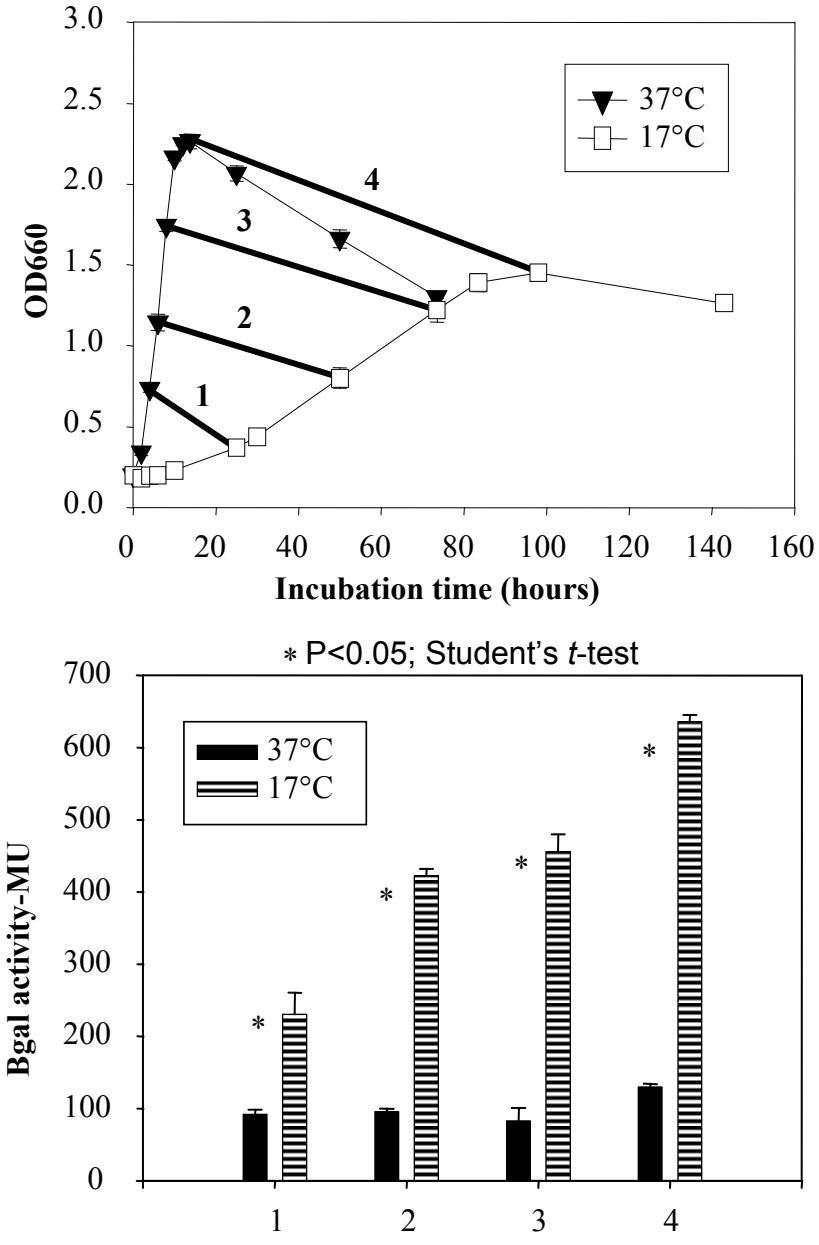

Figure 1-8. Effect of temperature on the growth of and β-galactosidase production by NU286 at 37, and 17°C. Log-phase NU286 bacteria were inoculated into BYE broth and then incubated, as noted, at 37, and 17°C. The growth of the cultures was monitored spectrophotometrically (top panels) and, at three 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.

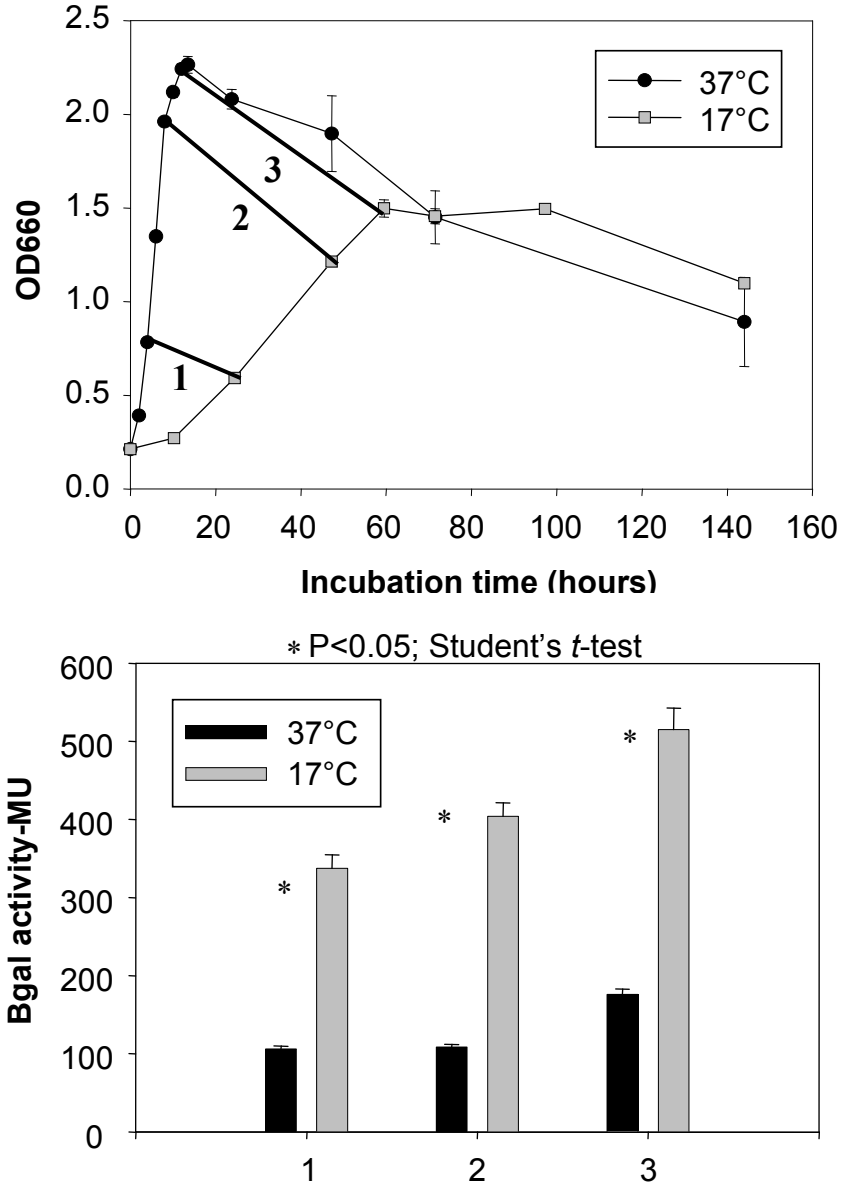

Figure 1-9. Effect of temperature on the growth of and β-galactosidase production by NU286 at 37, and 17°C. Log-phase NU286 bacteria were inoculated into BYE broth and then incubated, as noted, at 37, and 17°C. The growth of the cultures was monitored spectrophotometrically (top panels) and, at three indicated time points (labeled as 1, 2, and 3) 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 two samples.

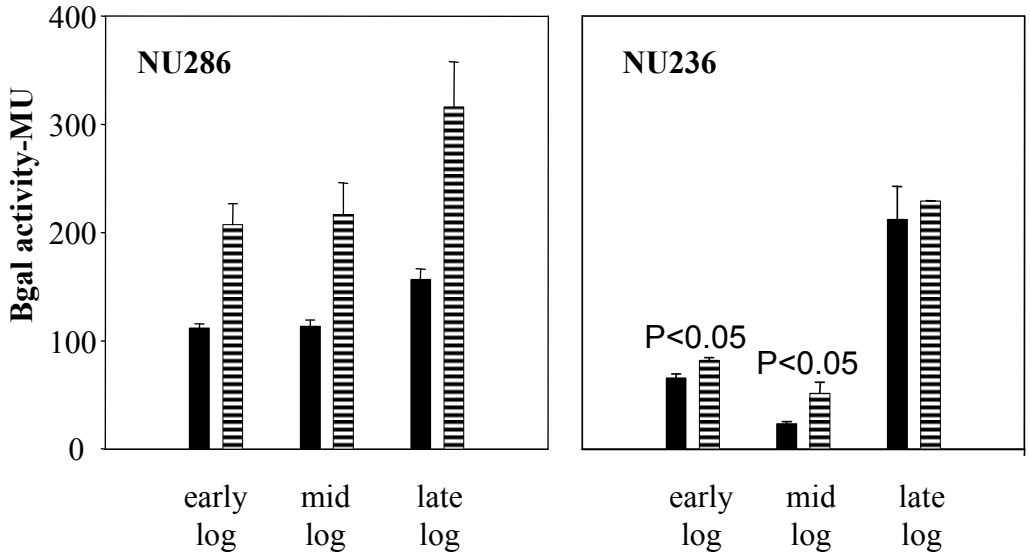

Figure 1-10. β-galactosidase production by NU286 and NU236 fusion strains grown at 37 and 17°C. Log-phase NU286 (left panel) and NU236 (right panel) bacteria were inoculated into BYE broth and then incubated at 37°C (black bars) and 17°C (lined bars). When the cultures reached early, mid-, and late log 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.

stability of the LacZ protein at the lower temperature. However, since the increase in Bgal levels seen at 17°C for NU286 is much bigger, most of this increase cannot be due to a higher stability of the LacZ protein and instead reflects a real increase in gene expression of *pilD* at low temperatures.

The Bgal growth experiments in CDM indicated that the increase in Bgal levels at lower temperatures was due to the change in temperature. However, it was possible that another trivial explanation for the Bgal increase at lower temperatures might be that a *pilD* mutant background was used. It is possible that in the *pilD* mutant background, there is a feedback regulation leading to an up-regulation of components of the type II secretion system, due to a higher concentration of type II exoproteins accumulating in the periplasm at the lower temperature. Therefore, to be sure that the increase in Bgal levels observed at the low temperature were not an artifact of assessing *lacZ* expression in a *pilD* negative strain, we repeated the 37°C vs. 17°C Bgal comparison with NU286 containing the intact *pilD* gene cloned into pMD1 (**Fig. 1-11**). The complemented NU286 showed the same increase in Bgal activity at 17°C as the original NU286 and the NU286 containing the pMMB2002 vector. Therefore the increase seen in *pilD* expression at lower temperatures was not due to making these comparisons in a mutant background.

SUMMARY

The present study showed that *pilD* expression, as measured by a *pilD*::'*lacZ* fusion, is temperature regulated both when grown on plates and in broth, since *pilD* expression goes up as the temperature goes down from 42°C to 17°C. This increase is not due to a

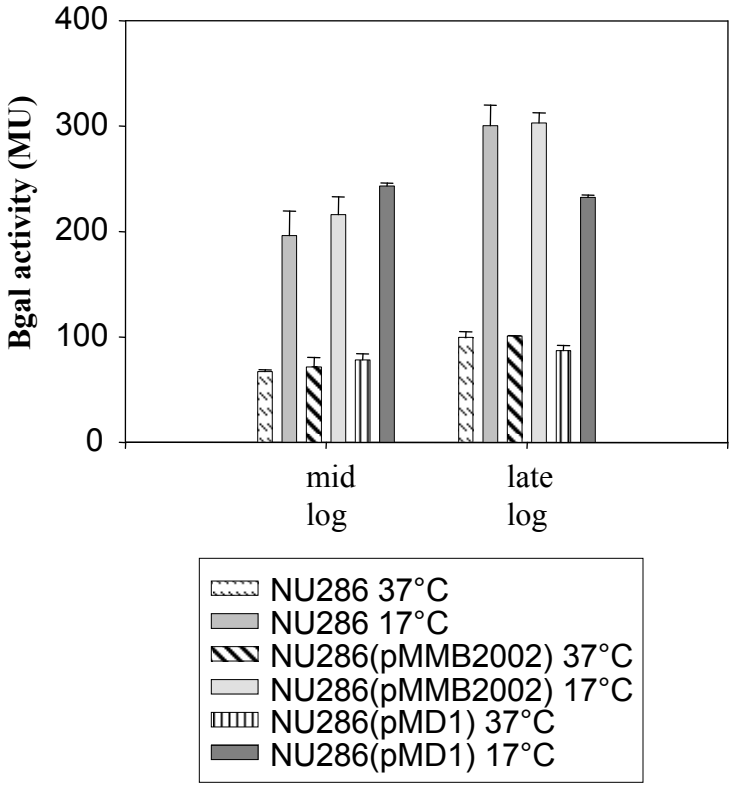

Figure 1-11. β-galactosidase production by NU286 in the presence and absence of an intact *pilD* gene at 37 and 17°C . Log-phase NU286, NU286 containing the vector pMMB2002 and NU286 containing a complementing *pilD* gene on pMD1 were inoculated into BYE broth and then incubated at 37°C and 17°C. When 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.

higher stability of the LacZ protein at lower temperatures since other *lacZ* fusions do not show the same increase. Neither, is the increase due to a slower growth rate or the fact the cultures reach a lower OD660 at the lower temperatures, since growth in CDM did not change the Bgal levels. In addition, the increase is not due to the fact the expression was measured in a *pilD* negative strain since the complemented strain did show the same kind of increase as the *pilD* mutant. Sometimes the measured *lacZ* expression from gene fusions do not reflect the real gene expression; e.g. due to changes in mRNA stabilizing or destabilizing sites located within the transcribed region where the *lacZ* gene is inserted (181). However, both Northern blot data and the *lacZ* expression experiments indicate an up-regulation of *pilD* expression at the lower temperature. Hence, this temperature regulation of *pilD* expression is probably real but RT-PCR could be used to confirm these data.

In addition, *pilD* gene expression is also growth phase regulated. This kind of regulation has been seen both for the *out* genes in *E*. *chrysanthemi* and the *xcp* genes in *P*. *aeruginosa* (33, 146). In *P*. *aeruginosa*, this growth phase dependent increase in *xcp* gene expression is due to regulation of these genes by quorom sensing (33). It is therefore possible that the *L*. *pneumophila pilD* gene is also regulated by quorum sensing. In support of this idea, the first quorum sensing genes, *lqsA*, *lqsS* and *lsqR*, were very recently identified in *L*. *pneumophila* (231).

This was the first report of temperature regulation of a type II secretion gene, but recently HofQ, a type II secretion gene in *E*. *amylovora*, has also been shown to have higher expression at 18°C vs. 28°C (84). Future studies are required to assess if

temperature regulation of type II secretion genes might be a widespread phenomenon among bacteria living in the environment.

Chapter 2

THE TYPE II SECRETION SYSTEM IN *L. PNEUMOPHILA* IS IMPORTANT FOR LOW TEMPERATURE GROWTH AND SURVIVAL

INTRODUCTION

pilD expression in *L. pneumophila* strain 130b is temperature regulated with gene expression 3-fold higher at RT and 6-fold higher at 17°C than at 37°C. This increase in expression correlates with the *pilD* mutant reaching a lower OD660 at 17°C and at RT as compared to the OD660 reached at 30-37°C. Since these experiments lacked a parental strain control, it was hard to know if this reduction in maximum OD660 reached would also be seen by strain 130b grown at these temperatures. However, since a *pilD* mutant is defective in both type IV pilus production and type II secretion, it was possible that this potential growth defect of the *pilD* mutant at lower temperatures might indicate that the type IV pili apparatus and/or the type II secretion machinery could be important for low temperature survival and/or growth (223). This was a novel idea, and I therefore started with re-growing the *pilD* mutant, together with strain 130b, at different temperatures to compare their growth and to confirm if the *pilD* mutant had a growth defect at these lower temperatures.

RESULTS

From the previous Bgal experiments (**Chapter 1)**, it was clear that the *pilD* mutant NU286 reached a lower OD660 at RT and 17°C as compared to 37°C. At RT (**Fig. 1-6**),

the difference in OD660 was only approximately 0.4 (OD660 2.4 at 37°C vs. OD660 2.0 at RT) while at 17°C (**Fig. 1-8**) the difference in OD660 was approximately 0.7 (OD660 2.2 at 37°C vs. OD660 1.5 at 17°C). These data indicated NU286 might have a growth defect at 17°C, but since these experiments lacked a parental strain control it was hard to conclude this. Therefore, growth at 37°C, 30°C, RT, and 17°C were repeated in BYE with both NU286 and strain 130b (**Fig. 2-1**). These data showed strain 130b growing well at all temperatures, but as before the bacterial growth rate slowed as the temperature went down. In addition, these data confirmed that NU286 grew like strain 130b at 37°C and 30°C as previously seen (**Fig. 1-2**). At RT, NU286 had a small reduction in OD660 of 0.3 when compared to strain 130b (OD660 2.4) values. All in all, this modest reduction in maximum OD660 reached, for NU286 at RT, was seen in three experiments. At 17°C, NU286 again grew to a maximum OD660 of 1.5, while strain 130b grew to an OD660 of 2.4. The difference in OD660 between NU286 and strain 130b was 0.9 which indicates that NU286 really has a temperature induced growth defect that at 17°C is quite severe. Similar results were obtained in another 6 experiments.

To determine if the same reduction in growth could be seen on BCYE agar at temperatures below 37°C, we plated both strain 130b and several *pilD* mutants; i.e. NU243, NU272, and NU286, at RT. Since plating experiments on BCYE plates at RT had not been done in the lab before, initial EOP (efficiency of plating) assessments were done. The 130b strain grew well at RT, albeit 5 days slower than at 37°C (7-10 days vs. 3 days), with an EOP (defined as CFU at RT on day 7-10/CFU at 37°C on day 3) at RT of 61% ± 33% (**Fig. 2-2, Table 4**). In contrast, while a *pilD* mutant had an

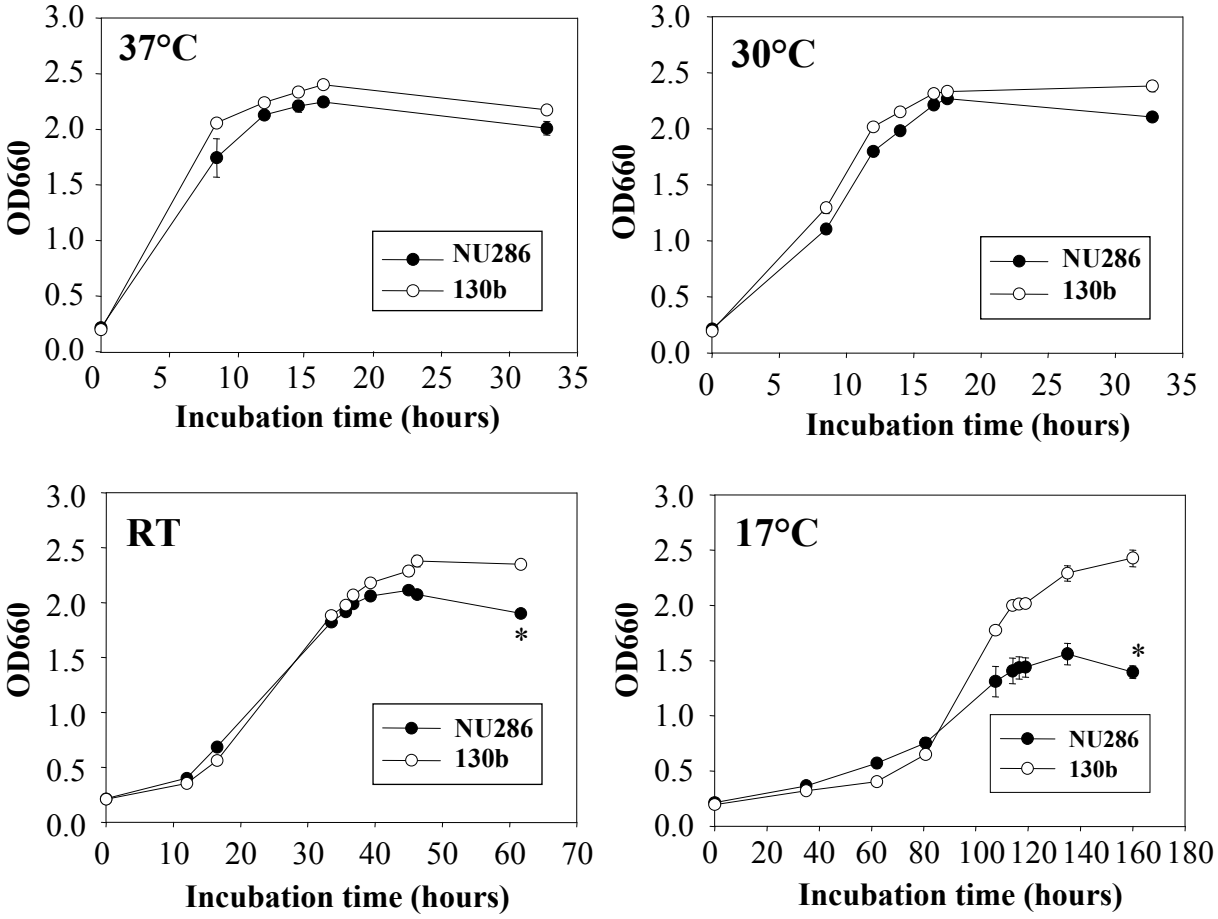

Figure 2-1. Effect of temperature on the growth of strain 130b and *pilD* mutant at 37°C, 30°C, 25°C and 17°C. Log-phase 130b and *pilD* mutant NU286 bacteria were inoculated into BYE broth and then incubated at 37°C, 30°C, 25°C and 17°C. The growth of the strains was monitored by recording the optical 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.

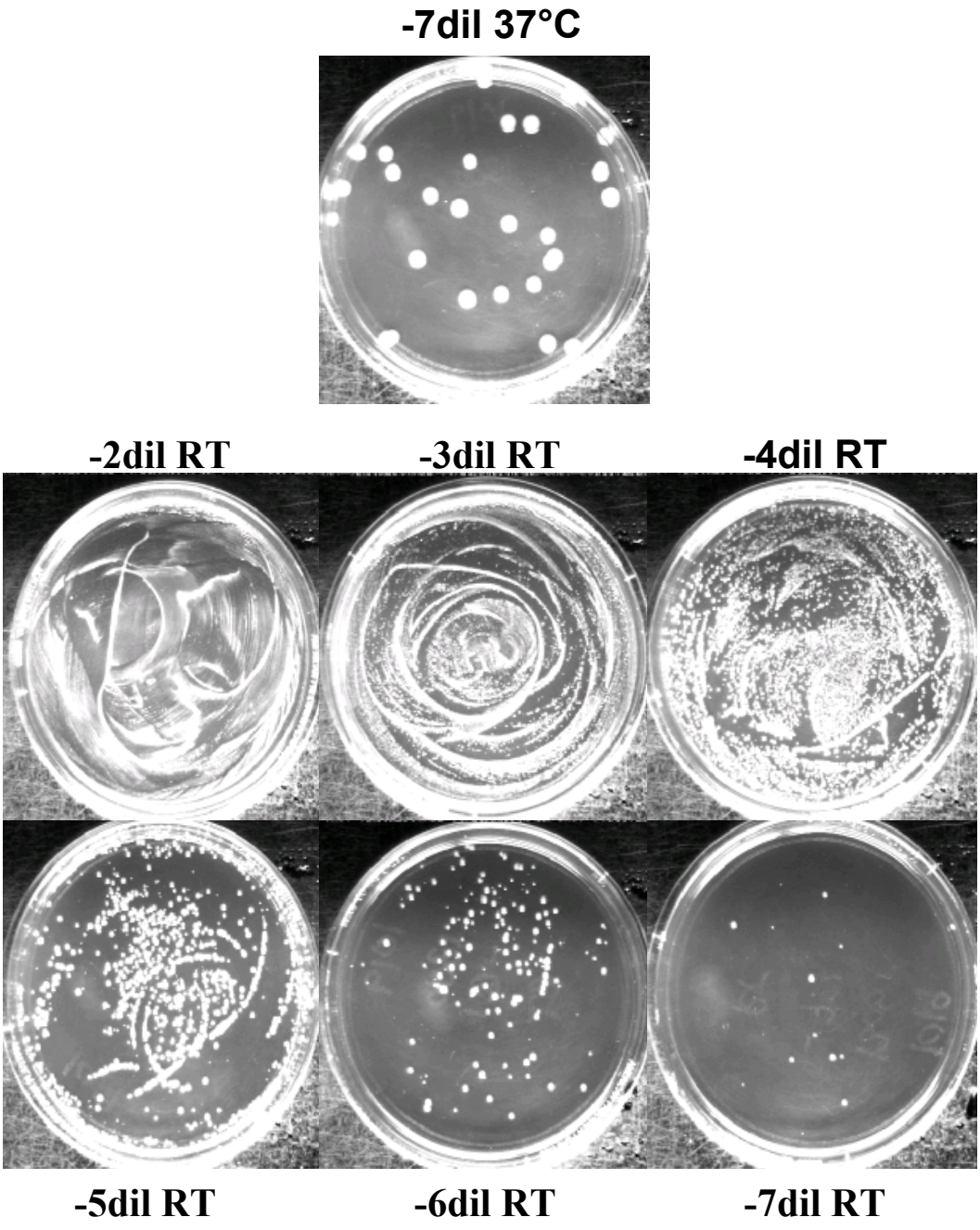

Figure 2-2. Comparison of strain 130b survival at 37°C and RT. Several dilutions (dil) of strain 130b were plated for CFU on a series of BCYE plates. These plates were incubated at 37°C for 3 days or for 8 days at RT before pictures were taken and survival comparisons were done. These results are representative of at least 2 independent experiments.

Table 4. *L*. *pneumophila* strains and their efficiency of plating (EOP) at room temperature

Strain	Relevant genotype	Deficiency	EOP at RT* (%)	Reference
130b	wild type strain	None	61 ± 33	(63)
NU286	*pilD::'lacZ*aphA***	Prepilin peptidase	0.032 ± 0.009	This study
NU243	*pilD::aphA*	Prepilin peptidase	0.036 ± 0.036	(144)
NU272	*pilD::aacC1****	Prepilin peptidase	0.003 ± 0.003	(198)
NU258	*lspDE::aphA*	Type II secretin and ATPase	0.028 ± 0.021	(196)
NU259	*lspG::aphA*	Type II pseudopilin	0.020 ± 0.019	(198)
NU275	*lspF::aphA*	Type II membrane protein	0.01 ± 0.002	(198)
BS100	$pilE_L$*::aphA*	Type IV pilus pilin	52 ± 12	(220)
NU279	*pilQ::aacC1*	Type IV pilus secretin	37 ± 5	(198)
NU283	*lspDE::aphA*	Type II secretin and ATPase	0.0005 ± 0.0001	(198)
	pilQ::aacC1	Type IV pilus secretin		
GG105	*dotA::aphA*	Type IV secretion	57 ± 21	(251)
GQ262	*dotDCB::aphA*	Type IV secretion	39 ± 27	(251)
GN142	*icmGCD::aphA*	Type IV secretion	68 ± 7	(251)
NU291	*pilD::aacC1*	Prepilin peptidase	0.006 ± 0.006	This study
	icmGCD::aphA	Type IV secretion		
AA200	*proA::aphA*	ProA metalloprotease	73 ± 42	(158)
NU254	*map::aphA*	Major acid phosphatase	74 ± 26	(10)
NU267	*lipA::aphA*	LipA and LipB lipases	109 ± 5	(12)
	lipB::aacC1			
NU268	*plcA::aphA*	PlcA phospholipase C	87 ± 18	(12)
NU270	*plaA::aphA*	PlaA lysophospholipase A	80 ± 9	(71)

*Calculated as follows: 100 x (CFU formed at room temperature in 7-10 days/CFU formed at 37C in 3 days). The results are the mean and standard deviations of at least two independent experiments. *promoter-less *lacZ* gene, ***aphA* encodes kanamycin resistance, ****aacC1* encodes gentamicin resistance

identical EOP as strain 130b at 37°C, it had a severe reduction in EOP at RT with an EOP of only 0.036% ± 0.036%, 0.003% ± 0.003%, and 0.032% ± 0.009% for NU243, NU272, and NU286, respectively (**Fig. 2-3, Table 4**). This reduction in EOP of the *pilD* mutant was due to the loss of *pilD* and not a second site mutation (**Fig. 2-4**) since the EOP at RT for the *pilD* mutant NU272 with the complementing plasmid pMD1 was 35%±23%, while the EOP for 130b with same vector was 41%±25% (**Table 5**).

Since PilD is important both for type IV pilus biogenesis and type II secretion, it was possible that the *pilD* mutant reduction in EOP at these lower temperatures was due to the loss of the type IV pilus apparatus, loss of the type II secretion, or perhaps due to the loss of both these systems (223). To further investigate the contribution of the type IV pilus apparatus for low temperature growth and survival, the type IV pilus secretin mutant, *pilQ*, and the pilin mutant, *pilE*, were plated on BCYE plates at 37°C and RT. Both of these mutants had an EOP equal to strain 130b, both at 37°C and at RT; i.e., EOP at RT of 37% ± 5% for the *pilQ* mutant and of 52% ± 12% for the *pilE* mutant (**Fig. 2-5, Table 4**). These data show that the type IV pilus apparatus is not required for low temperature survival or growth.

To test the importance of the type II secretion apparatus for low temperature survival and growth, three *lsp* mutants, *lspDE* (lacking the LspD secretin and the LspE ATPase), *lspF* (lacking the inner membrane protein LspF) and *lspG* (lacking the LspG pseudopilin) were plated at 37°C and RT. The *lsp* mutants had an EOP like strain 130b at 37°C. In contrast, at RT the *lsp* mutants had an EOP as low as the EOP for the *pilD* mutant; i.e. EOP of 0.028% ± 0.021%, 0.010% ± 0.002%, 0.020% ± 0.019% for *lspDE* mutant, *lspF* mutant and *lspG* mutant, respectively (**Fig. 2-6, Fig. 2-7, Fig. 2-8, Fig. 2-9,**

Table 5. Complementation of type II secretion mutants

Strain	Relevant genotype	Plasmid gene	EOP* at RT
130b(pMMB2002)	wild type strain	Cm^r vector	59 ± 9
130b(pMD1)	wild type strain	*pilD* in pMMB2002	41 ± 25
130b(pMF1)	wild type strain	*lspF* in pMMB2002	55 ± 16
NU272(pMMB2002)	*pilD*::*aacC1**	Cm^r vector	0.010 ± 0.014
NU272(pMD1)	*pilD*::*aacC1*	*pilD* in pMMB2002	35 ± 23
NU275(pMMB2002)	*lspF*::*aphA***	Cm^r vector	0.056 ± 0.071
NU275(pMF1)	*lspF*::*aphA*	*lspF* in pMMB2002	51 ± 6

*Efficiency of plating, calculated as 100 x (CFU formed at RT 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. **aacC1* encodes gentamicin resistance, ***aphA* encodes kanamycin resistance

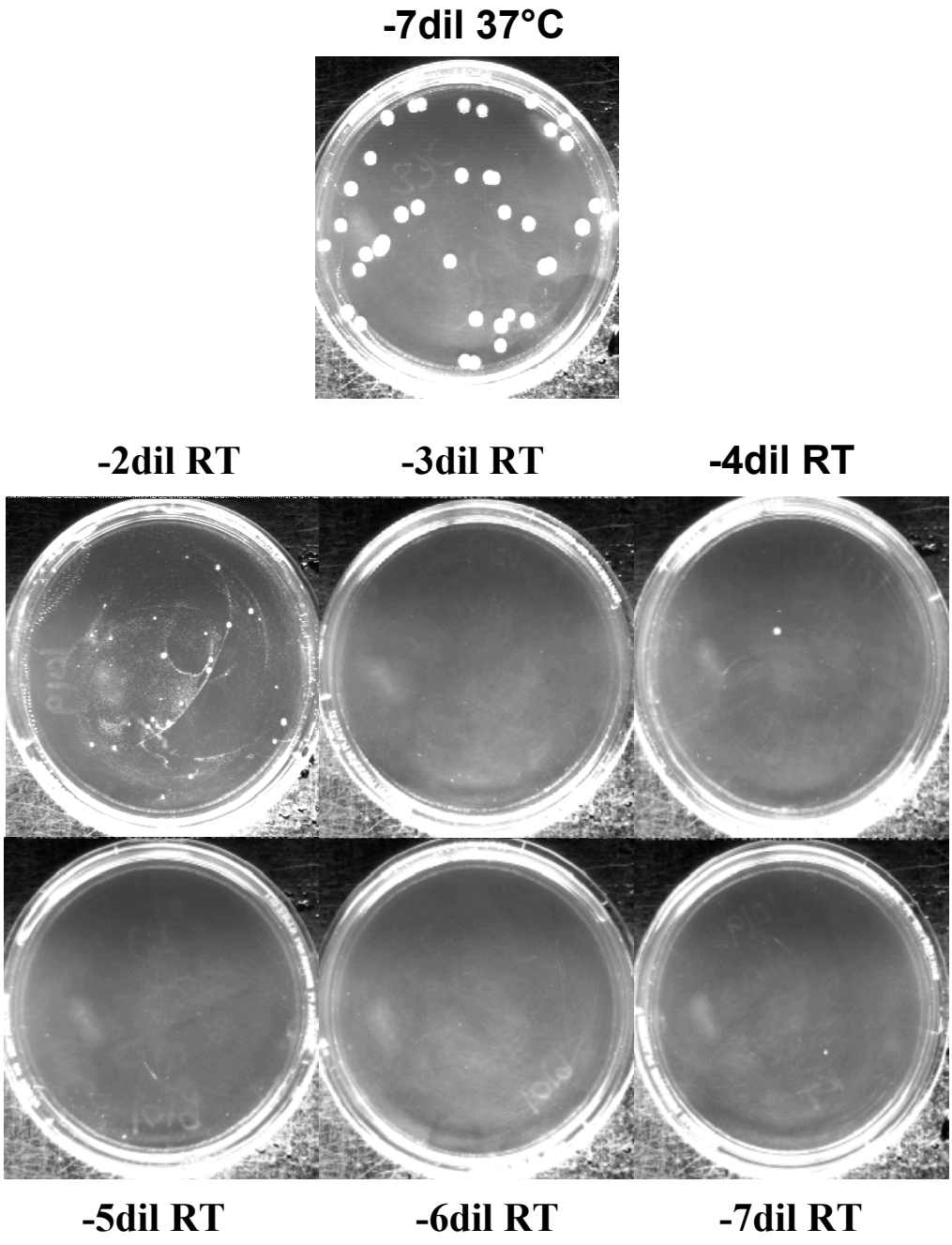

Figure 2-3. Comparison of *pilD* mutant survival at 37°C and RT. Several dilutions (dil) of the *pilD* mutant NU272 were plated for CFU on a series of BCYE plates. These plates were incubated at 37°C for 3 days or for 8 days at RT before pictures were taken and survival comparisons were done. These results are representative of at least 2 independent experiments.

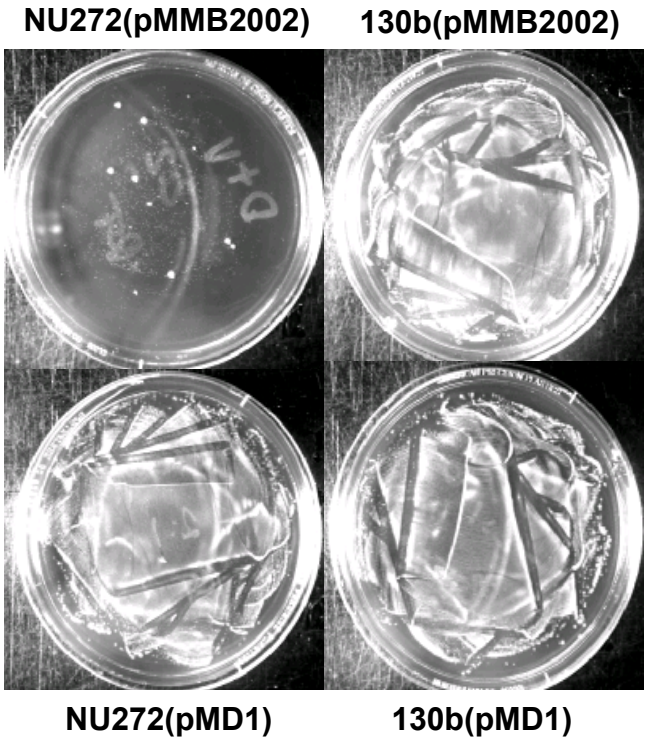

Figure 2-4. Complementation of the *pilD* mutant NU272 on BCYE plates at RT. NU272 and strain 130b by themselves or containing the vector pMMB2002 or the complementing *pilD* gene on pMD1 were plated at a concentration of 10^6 CFU/ml. The plates were incubated at RT for 8 days before pictures were taken. These results are representative of at least 2 independent experiments.

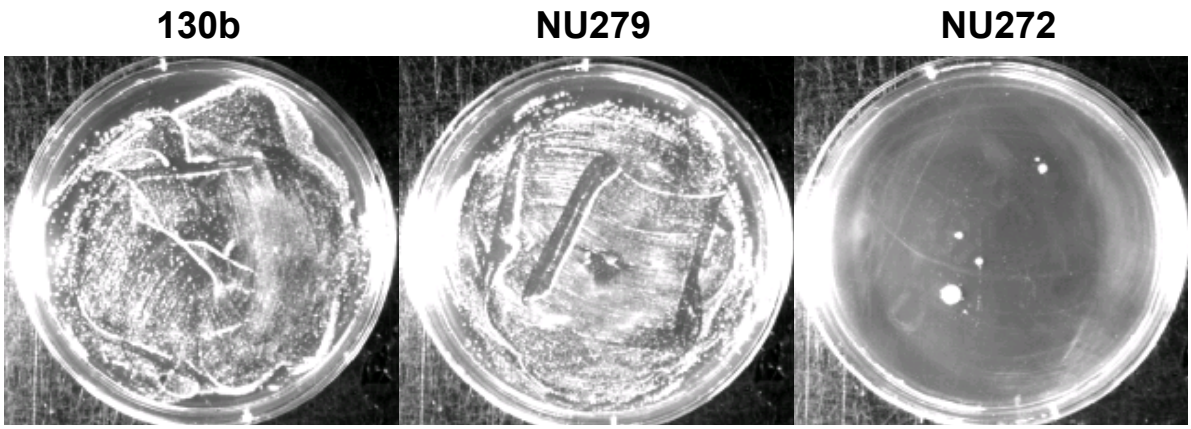

Figure 2-5. Survival comparison of strain 130b and *pilQ* and *pilD* mutants at RT. Several dilutions of strain 130b, the *pilQ* mutant NU279 and the *pilD* mutant NU272 were plated for CFU at a concentration of 10^6 CFU/ml. These plates were incubated at 8 days at RT before pictures were taken and survival comparisons were done. These results are representative of at least 2 independent experiments.

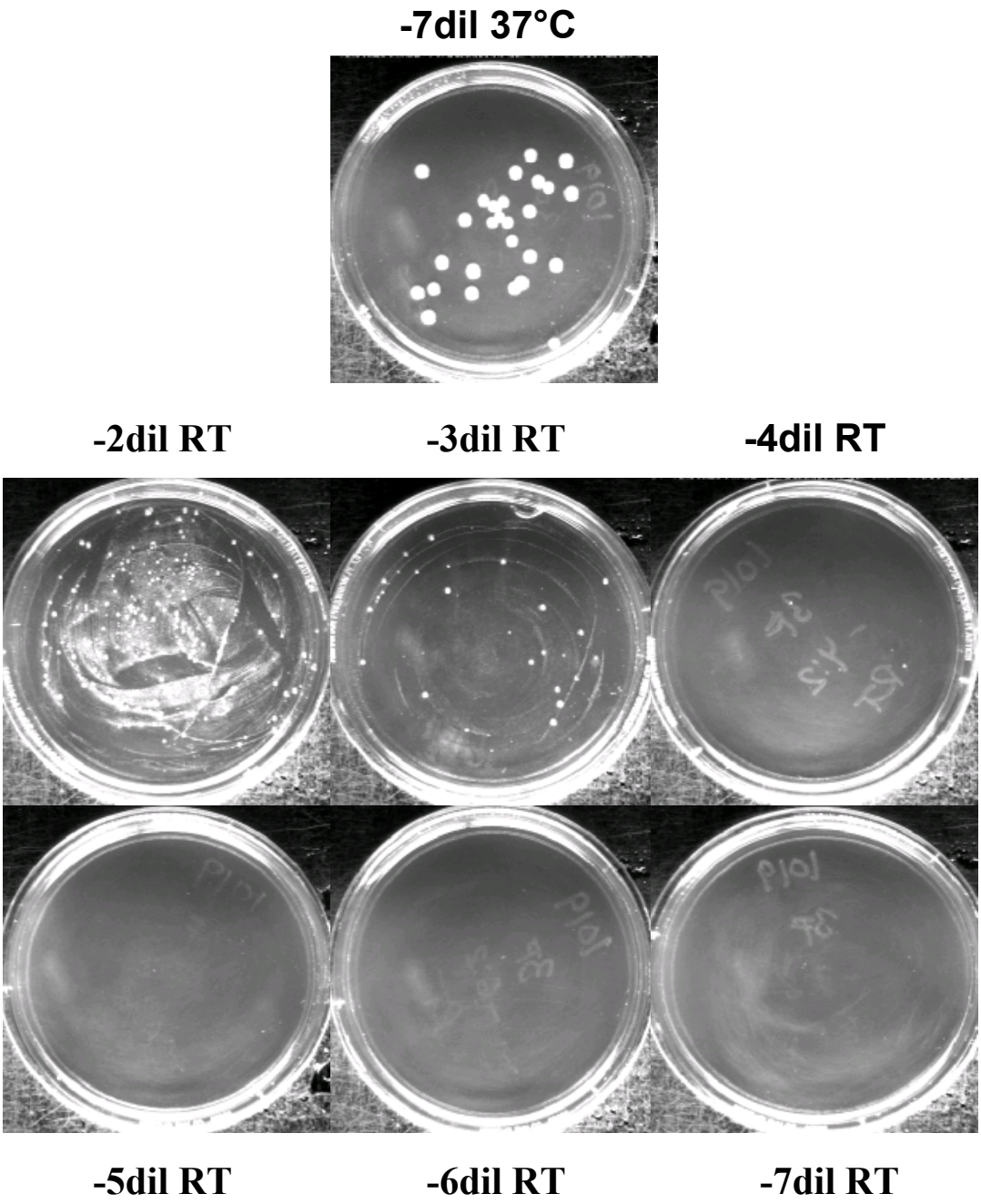

Figure 2-6. Comparison of *lspDE* mutant survival at 37°C and RT. Several dilutions (dil) of the *lspDE* mutant NU258 were plated for CFU on a series of BCYE plates. These plates were incubated at 37°C for 3 days or for 8 days at RT before pictures were taken and survival comparisons were done. These results are representative of at least 2 independent experiments.

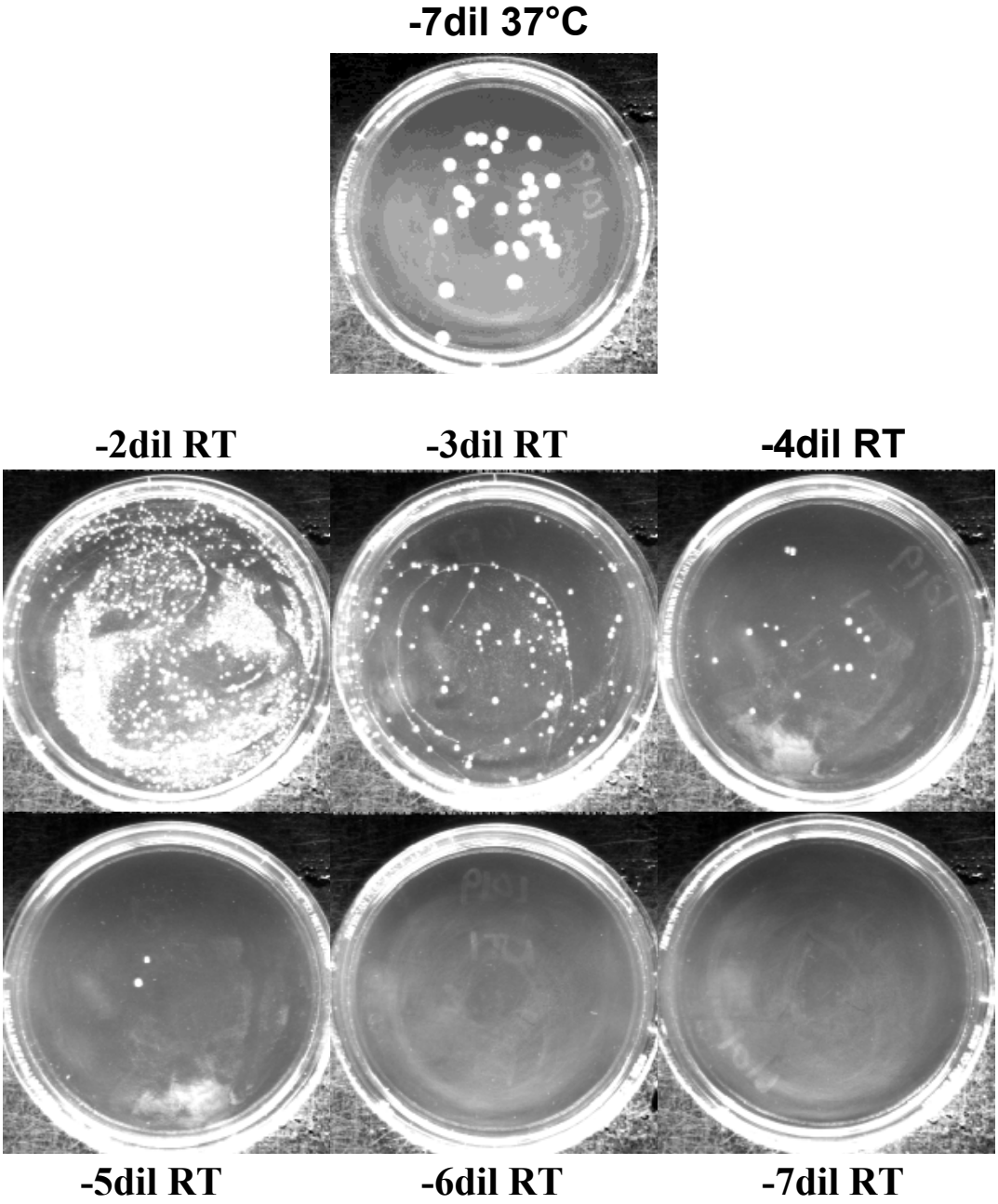

Figure 2-7. Comparison of *lspG* mutant survival at 37°C and RT. Several dilutions (dil) of the *lspG* mutant NU259 were plated for CFU on a series of BCYE plates. These plates were incubated at 37°C for 3 days or for 8 days at RT before pictures were taken and survival comparisons were done. These results are representative of at least 2 independent experiments.

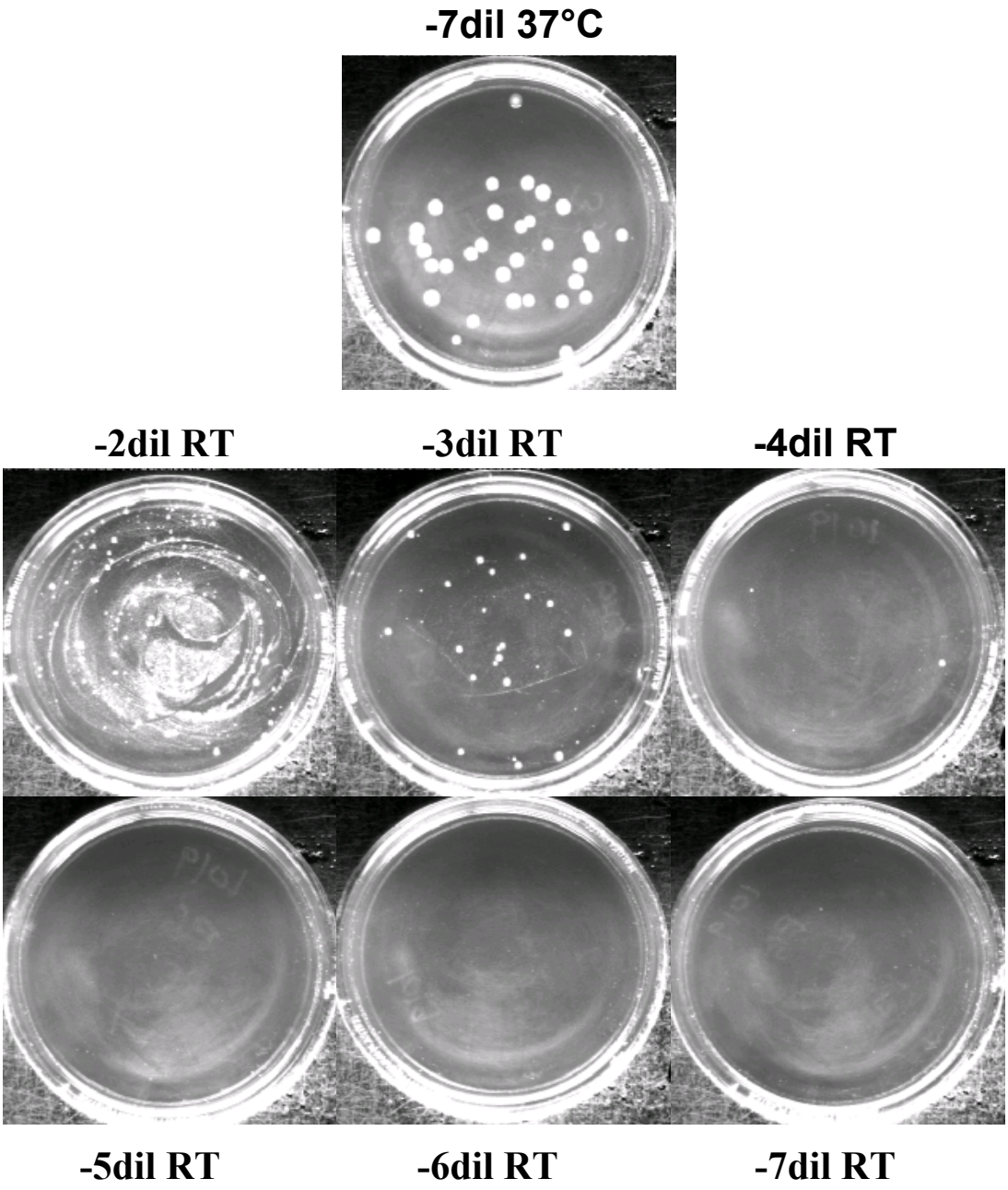

Figure 2-8. Comparison of *lspF* mutant survival at 37°C and RT. Several dilutions of the *lspF* mutant NU275 were plated for CFU on a series of BCYE plates. These plates were incubated at 37°C for 3 days or for 8 days at RT before pictures were taken and survival comparisons were done. These results are representative of at least 2 independent experiments.

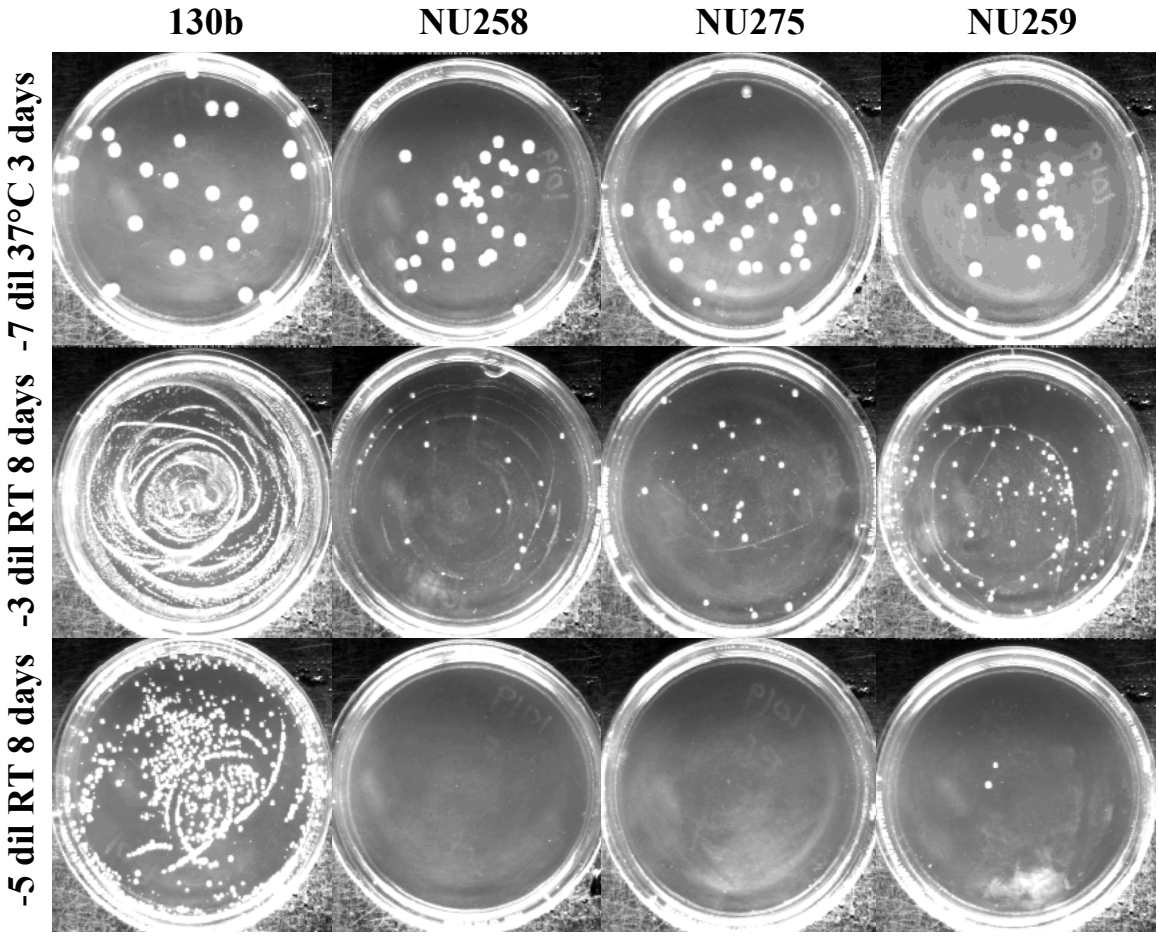

Figure 2-9. Survival comparison between strain 130b and several *lsp* mutants at 37°C and RT. Several dilutions of strain 130b, *lspDE* mutant NU258, *lspF* mutant NU275 and *lspG* mutant NU259 were plated for CFU on a series of BCYE plates. These plates were incubated at 37°C for 3 days or for 8 days at RT before pictures were taken and survival comparisons were done. These results are representative of at least 2 independent experiments.

Table 4). The reduction in EOP of the *lsp* mutants was not due to a second site mutation since the *lspF* mutant with the complementing plasmid pMF1 had an EOP of 51% ± 6% while strain 130b with the same plasmid had an EOP of 55% ± 16% (**Fig. 2-10, Table 5**). Therefore, the type II secretion system is clearly important for growth and survival at low temperatures. A *lspDE*:*pilQ* double mutant was also tested for EOP at RT and 37°C. This mutant had an EOP of 0.0005% ± 0.0001% (**Table 4**) which was lower than the EOP of 0.036% ± 0.036% for the *pilD* mutant. This might mean that the type IV pilus apparatus has a small but dispensable role in low temperature growth and survival.

Both *pilD* and *lsp* mutants had two populations of colonies at RT. A few colonies grew as fast as and were mostly as big as the 130b strain colonies at 7-10 days. These colonies occurred with a frequency of 10^{-5}. The other population consisted of slow growing colonies that sometimes could be seen at 7 days but sometimes did not become apparent until later (**Fig. 2-11**). The number of colonies continued to increase up to 2 weeks after plating but after that not much change was seen, possibly because the plates became too dry (**Fig. 2-11**). The EOP for a *pilD* mutant was 0.03% ± 0.04% at 1 week and 3.7% ± 7.3% at 2 weeks while a *lspDE* mutant had an EOP of 0.03% ± 0.03% at 1 week and 3.4% ± 3.5% at 2 weeks. The number of colonies of strain 130b also increased over time, with an EOP of 71% ± 24% and 87% ± 12% at 1 and 2 weeks, respectively. In summary, the number of colonies for both the mutants and the 130b strain increase with time but the EOP of the *pilD*/*lspDE* mutants never reached the levels of the 130b strain.

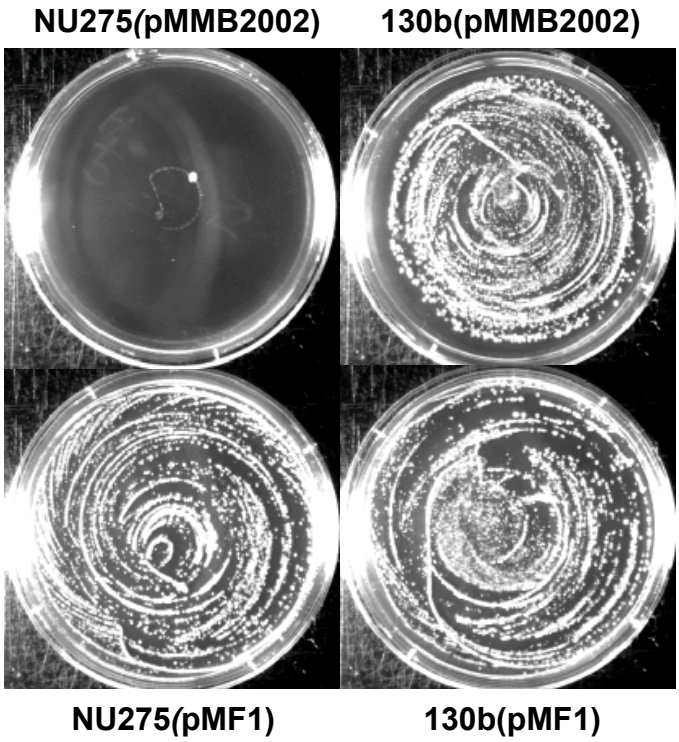

Figure 2-10. Complementation of the *lspF* mutant on BCYE plates at RT. The *lspF* mutant NU275 and strain 130b by themselves or containing the vector pMMB2002 or the complementing *lspF* gene on pMF1 were plated at a concentration of 10^5 CFU/ml. The plates were incubated at RT for 8 days before pictures were taken. These results are representative of at least 2 independent experiments.

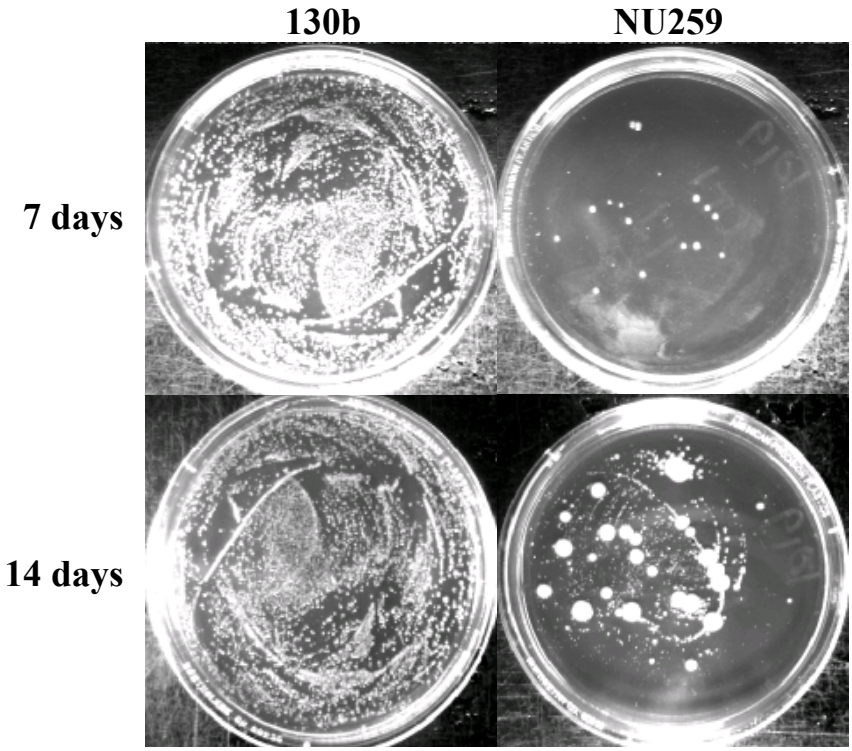

Figure 2-11. Strain 130b and *lsp* mutant survival at RT improve with time. Strain 130b and *lspG* mutant NU259 were plated at a concentration of 10^5 CFU/ml on a series of BCYE plates. These plates were incubated at RT and survival comparisons were done at 7 and day 14. These results are representative of at least 2 independent experiments.

These data suggested this fast growing mutant population could survive and grow as well as strain 130b at RT. To test this, some of these early fast growing colonies were replated onto BCYE plates at RT and 37°C. A big *lspDE* mutant variety had an EOP of 20% while a big *pilD* mutant variety had an EOP equal to strain 130b. These colonies were not contaminants since they did not grow on LB plates and they were still true mutants since they still grew on BCYE, Km^R plates. In addition, they still lacked the lipolytic and proteolytic type II dependent enzymatic activities seen by the 130b strain when patched onto egg yolk plates (**data not shown**). Sometimes these colonies had, instead of the pale color associated with type II mutants, a more yellowish color like strain 130b (198). It is not currently understood why these suppressor mutants can grow much faster than the rest of the population especially since they still lack the type II secretion associated activities of strain 130b, at least as determined by assessment on egg yolk plates. Since the EOP between different big colonies varied it is possible that more than one change had to occur for these colonies to survive and grow better than the population in general. These changes could e.g. be due to point mutations in the promoter region of a protein that is secreted through another secretion system than type II secretion. It is possible that this protein normally is not expressed and/or secreted at low temperatures, but that these mutations now allows expression and secretion at these lower temperatures. If this protein then has a similar function as a type II secreted protein important for low temperature growth and survival, this could explain why these suppressor mutations now are able to survive and grow better.

The *pilD* mutant had a severe growth defect at 17°C in BYE broth. To see if the same was true for an *lsp* mutant, an *lspF* mutant and strain 130b was grown in BYE

broth at 37°C and 17°C. The *lspF* mutant did show the same growth defect as the *pilD* mutant, only reaching an OD660 of 1.3 while strain 130b in this experiment grew to an OD660 of 2.7 (**Fig. 2-12**). The apparent differences between strain 130b and mutant cultures at 37 and 17°C were statistically significant ($P < 0.05$; Student's t test). Similar results were seen in another 10 experiments.

The reduction in EOP on BCYE plates at RT for the *lsp* mutants was severe but it was possible that some of the *lsp* mutant bacteria would still be able to survive and grow at 17°C. Since *L. pneumophila* had never been plated at this temperature before, initial plating assessments were done. Strain 130b grew at 17°C , albeit 18 days slower than at 37°C, with an EOP of 18% ± 14% (defined as CFU at 17°C on day 20-21/CFU at 37°C on day 3) while very few *lspF* mutant bacteria survived and grew ; i.e. EOP of only 0.0004% ± 0.0004%. Again, this reduction in EOP could be fully complemented since *lspF* with the complementing plasmid pMF1 had an EOP of 13%, while 130b with the same plasmid had an EOP of 10%.

Next, growth comparisons were done in BYE broth at 37°C and 12°C for 130b and the *lspF* mutant. These data showed that the *lspF* mutant failed to grow at 12°C. In fact, it suggested the mutant was dying since the OD660 went down with time. The 130b strain still grew at this temperature but only reached an OD660 of 1.2 and it now took 13 days to reach maximum OD660 (**Fig. 2-13**). This was followed by plating assessments at 12°C. At this temperature 130b formed colonies after 46 days, and by day 72 achieved 0.2% of the number of CFU seen on 37°C plates. The *lspDE*, *lspG* and *pilD* mutants on the other hand failed to survive and grow at 12°C.

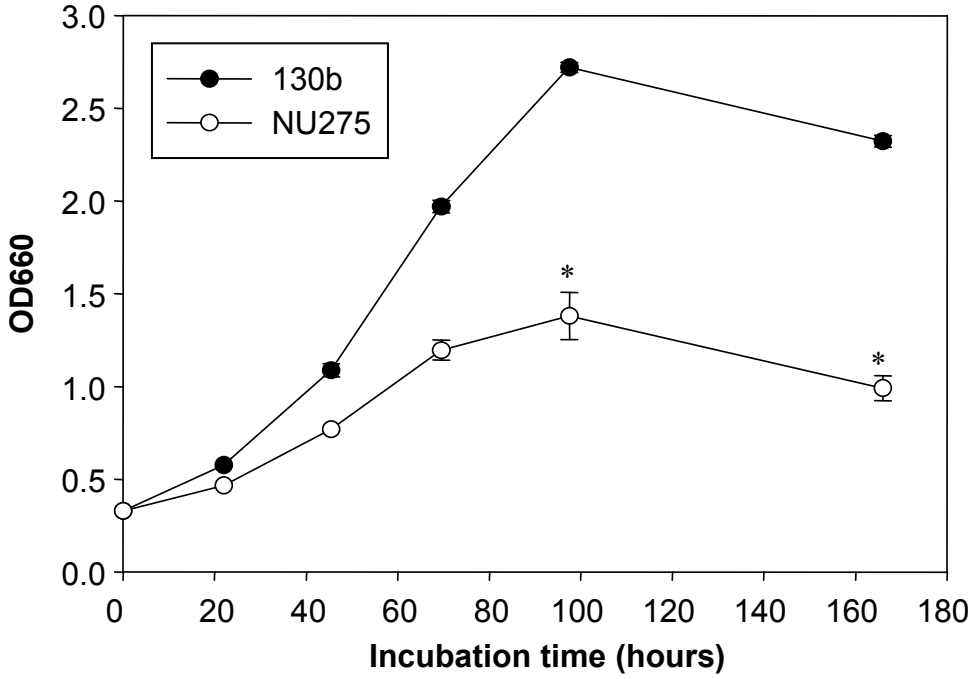

Figure 2-12. Growth comparison of strain 130b and *lspF* mutant in BYE broth at 17°C. Log-phase strain 130b and *lspF* mutant NU275 were inoculated into BYE broth and then incubated at 17°C. The growth of the cultures was monitored spectrophotometrically. The apparent differences between strain 130b and mutant cultures at 17°C were statistically significant (∗ $P < 0.05$; Student's t test). The results are the means and standard deviations from duplicate samples and are representative of at least 10 independent experiments.

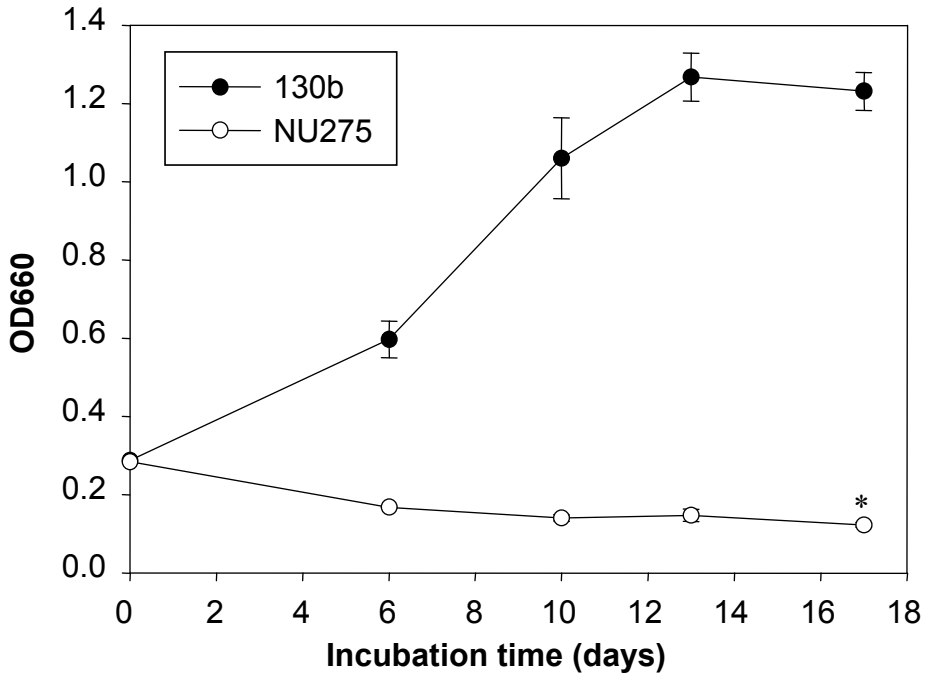

Figure 2-13. Growth comparison of strain 130b and *lspF* mutant in BYE broth at 12°C. Log-phase strain 130b and *lspF* mutant NU275 were inoculated into BYE broth and then incubated at 12°C. The growth of the cultures was monitored spectrophotometrically. The apparent differences between the strain 130b and mutant cultures at 12°C were statistically significant (* $P < 0.05$; Student's t test). The results are the means and standard deviations from duplicate samples

To test if 130b could also survive and grow at 4°C, roughly the same amount of 130b, *lspDE* and *pilD* mutants were swabbed onto plates in an amount enough to create a lawn if the plates had been placed at 37°C. These plates were placed in the cold room inside a closed bag. No growth occurred at this temperature for either 130b or the *lsp*/*pilD* mutants and therefore, after 18 months, these plates were placed in the 37°C incubator to check for viability**.** After 13 days at 37°C, the plate with 130b had 1000's of colonies and the plate with *lspDE* mutant had 36 colonies while the plate with *pilD* mutants had no colonies. Surprisingly, these data show *L*. *pneumophila* can stay viable over very long periods of time in the cold. It was also evident that the viability of the *lsp*/*pilD* mutants dropped more than the viability for 130b even when no colonial growth occurred. In summary, while at 37°C and 30°C the *lsp* mutants grew like the 130b strain, as the temperature went down their defect in survival and growth became more and more apparent. Thus, the *L*. *pneumophila* type II secretion becomes increasingly more important as temperature decreases.

The low EOP of the *lsp* mutants at low temperatures might be due to a slow growth rate or the poor growth might be a result of a large portion of the mutant population not surviving. To try to distinguish between these possibilities 130b and *lspF* were plated at RT, and at 0, 4, and 8 days plates were moved from RT to 37°C. When the CFUs, from the plates previously at RT, were counted after 6 day incubation at 37°C, the *lspF* mutant had an EOP of 11%, and 6% after 4, and 8 days at RT, respectively. In comparison, the EOP for *lspF* plates left at RT for 4 days was 0% and for plates left for 8 days 0.004%. In contrast, the 130b strain plates moved from RT at 4 and 8 days had an EOP of 106% and 79% after 6 days incubation at 37°C, respectively

while the EOP for the plates left at RT for 4 days were 0% and those left for 8 days were 49%. Thus, the viability of the *lspF* mutants dropped faster (reduction of almost 90% of the CFUs after 4 days at RT) than for the 130b strain (no reduction in CFUs after 4 days at RT) indicating that the reason *lspF* has such low EOP at low temperatures is at least in part dependent on a loss of viability over time**.**

To determine if other bacterial PilD proteins promote survival and growth at low temperatures, we compared a *pilD* mutant of *P*. *aeruginosa* and its complemented derivative (222) for their ability to survive and grow on BCYE agar at 37, RT and 12°C. After 3 days at RT, the strain containing an intact *pilD* gene yielded 92% ± 23% of the number of CFU obtained at 37°C in 1 day. In contrast to the behavior of the *L*. *pneumophila lsp* mutants, the *P*. *aeruginosa pilD* mutant displayed a normal EOP at RT; i.e., 100% ± 21%. Both PilD$^-$ and PilD$^+$ *P*. *aeruginosa* strains showed an 86-91% EOP when incubated at 12°C for 9 days. In summary, a *P*. *aeruginosa pilD* mutant does not have a survival or growth defect at low temperatures. Since the lab does not have *pilD*/*lsp* mutants from any other species, it will have to be a future question to answer if the type II secretion system in *L*. *pneumophila* is the only one that is important for low temperature survival and growth.

The type II secretion system of *L*. *pneumophila* is very important for survival and growth at low temperatures. To test if this survival and growth defect was specific to the loss of type II secretion or if loss of any secretion system would lead to the same defect, mutants in one of the type IV secretion systems in *L*. *pneumophila*, the *icm*/*dot*, were plated for growth at 37°C and RT. The EOP at RT for a *dotA*, a *dotDCB*, and an *icmGCD* mutant were 57% ± 21%, 39% ± 27%, 68% ± 7%, respectively while the EOP

for the 130b strain was 61% ± 33% (**Table 4**). In addition, an *icmGCD*:*pilD* double mutant had an EOP like the *pilD* mutant (**Table 4**). To be able to compare EOP at 17°C, 130b, the *dot*/*icm* mutants and a mutant in *L*. *pneumophila*'s second type IV secretion system, *lvh*, were spot diluted onto plates. These plates containing 6 dilutions spots per strain were placed at 37°C for 3 days (**data not shown**) or at 17°C for 20 days before EOP comparisons were done (**Fig. 2-14**). These data from both RT and 17°C show that the type IV secretion systems are not required for survival and growth at 37°C, RT or at 17°C. In addition, it suggests that the reduction in EOP caused by the lack of the type II secretion apparatus is probably not due to membrane sensitivity, caused by an altered protein compositions in the inner and outer membrane due to the lack of a membrane spanning protein complex, but instead due to the loss of a type II secreted factor/s.

One clue to why the *lsp* mutants were defective for survival and growth at the low temperatures came when the *lspDE* and *pilD* mutants were plated on BCYE plates lacking the 0.25g/L ferric pyrophosphate iron supplement (**Fig. 2-15**). On these plates the *lspDE* and the *pilD* mutant had a much better EOP than on the normal BCYE plates. The EOP for the *lspDE* mutant improved 100 fold from 0.05% on plates with ferric pyrophosphate to 5% on plates without the iron supplement. The corresponding values for the *pilD* mutant were 0.001% with ferric pyrophosphate and 2% without. The EOP for strain 130b and a *pilQ* mutant at RT on plates with and without the iron supplement did not change. No differences in EOP could be seen at 37°C for either strain 130b or the mutants on either kind of plates. In addition, the improved EOP was due to the lack of iron and not the lack of pyrophosphate since the *lspDE* and *pilD* mutants had the same

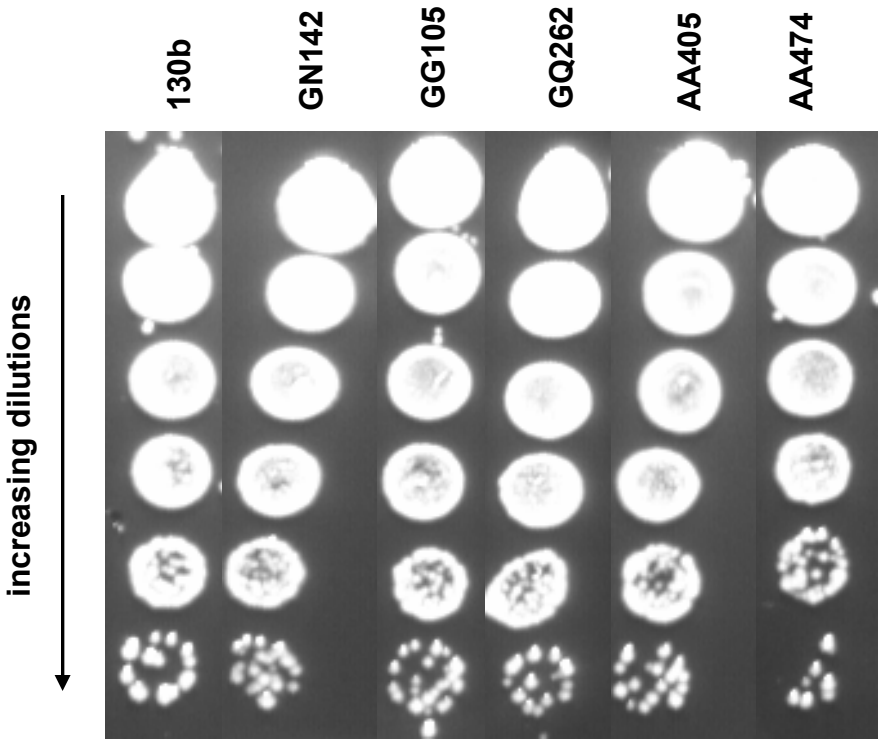

Figure 2-14. EOP comparison of strain 130b, *dot*/*icm* and *lvh* mutants at 17°C. Several dilutions of strain 130b, *icmGCD* mutant GN142, *dotA* mutant GG105, *dotDCB* mutant GQ262 *dotG* mutant AA405 and *lvh* mutant AA474 were spot diluted onto BCYE plates. These plates were incubated at 17°C for 21 days before pictures were taken and EOP comparisons were done. These results are representative of at least 2 independent experiments

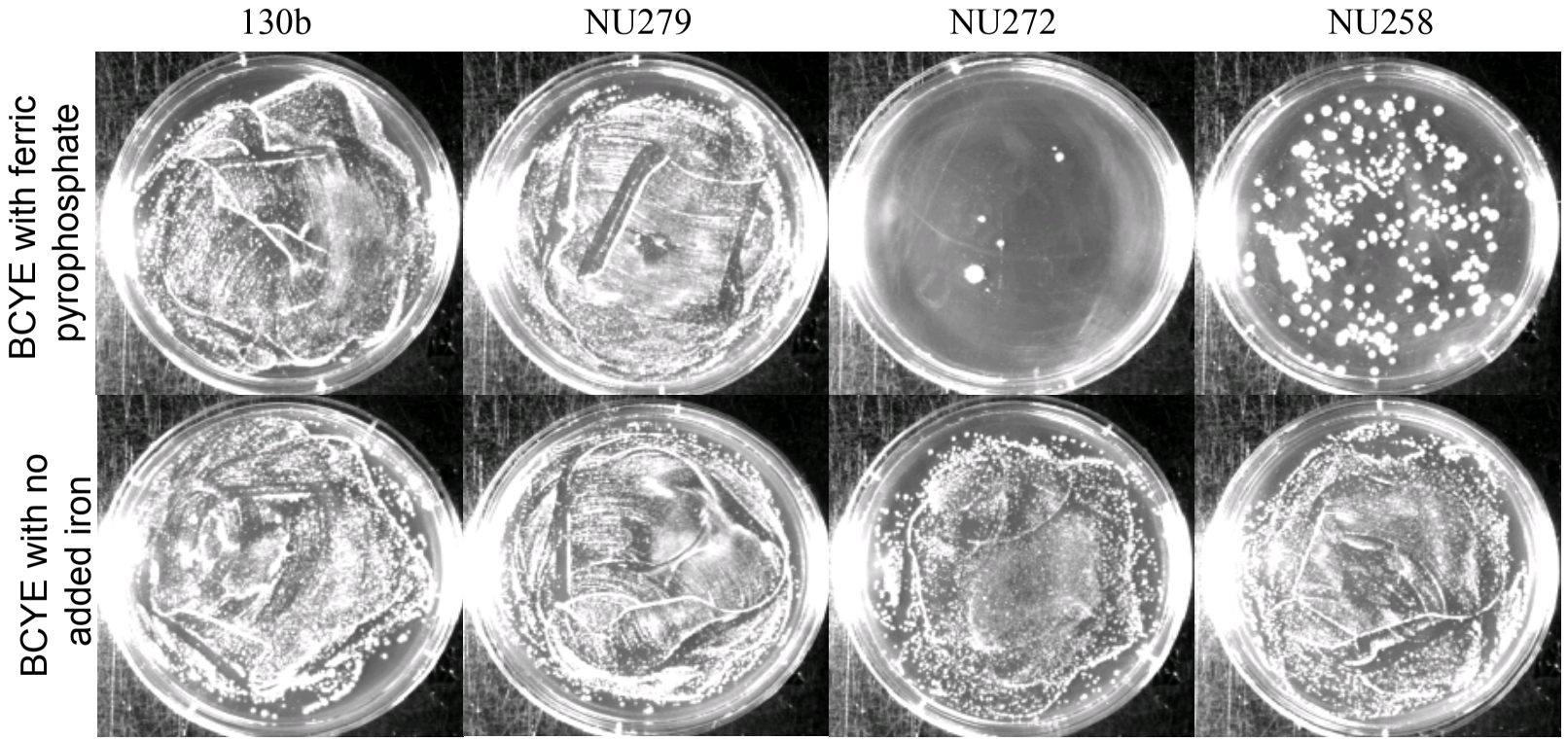

Figure 2-15. Improved survival at RT of type II secretion mutants plated on BCYE plates lacking the ferric pyrophosphate iron supplement. Strain 130b, *pilQ* mutant NU279, *pilD* mutant NU272 and *lspDE* mutant NU258 were plated at a concentration of 10^6 CFU/ml on BCYE plates with and without the 0.25g/L ferric pyrophosphate iron supplement. These plates were incubated for 8 days at RT before pictures were taken and survival comparisons were done. These results are representative of at least 2 independent experiments.

reduction in EOP when grown on plates with added ferric chloride or ferrous sulphate as on plates with added ferric pyrophosphate (**Fig. 2-16**).

The reason the type II secretion mutants are defective for survival and growth at low temperatures is most likely due to the lack of a secreted factor, though it is also possible that a diffusible factor produced by the action of an outer membrane protein could be responsible. In addition, it is also possible that the reduction in EOP is due to a cell-associated defect such as an alteration in outer membrane function. This has been seen e.g. in *V. cholerae* and *Aeromonas hydrophila* where type II secretion mutants have reduced amount of certain membrane proteins in their outer membrane (112, 204). If the reduction in EOP is due to the loss of a secreted factor, then a streak of the 130b strain should be able to help the survival and growth of the *lsp* mutants at low temperatures. To test this hypothesis, the *lspDE* mutant was plated at a concentration of 10^5 CFU/ml onto BCYE plates, and then a streak of the 130b strain or a streak of the *lspDE* mutant itself was added to one side of the plates (**Fig. 2-17**). By day 8, strong growth promotion could be seen next to the 130b strain streak. This growth promotion continued to spread with time, until the whole plate was filled with mutant growth. This growth promotion was on all days checked better than growth without a streak. The *lspDE* mutant could also promote the growth of itself but it did so in a lesser extent than strain 130b streak (**Fig. 2-17**). The reason the *lspDE* streak could help its own growth might be because of some lysis of the bacteria in the heavy streak with time or perhaps some help is provided by the fast growing big colony population. A streak of the 130b strain did not promote better growth of itself at RT. Neither did a streak of the 130b strain promote better growth of itself or of the *lspDE* mutant at 37°C. This kind of growth

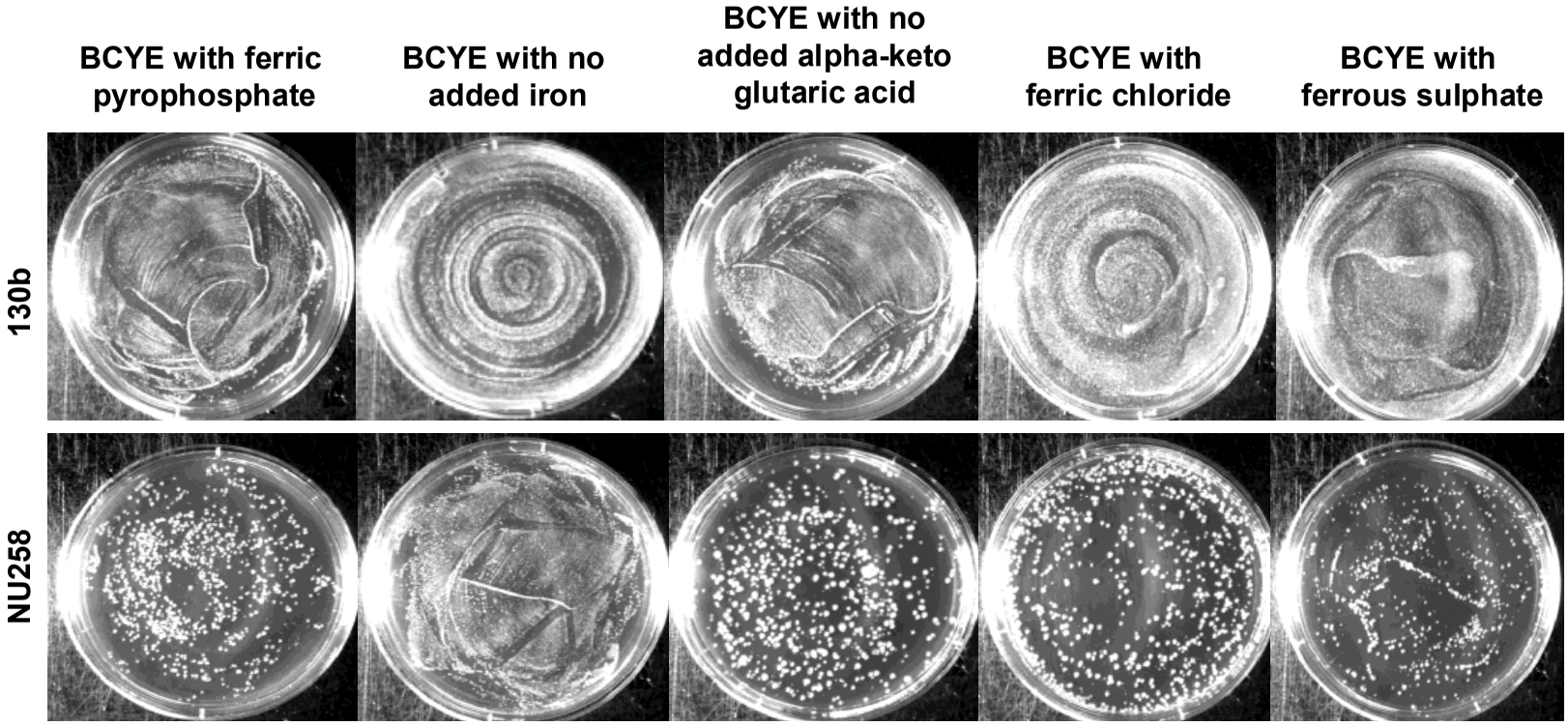

Figure 2-16. Survival at RT on BCYE plates with different iron sources. Strain 130b and *lspDE* mutant NU258 were plated at a concentration of 10^5 CFU/ml on BCYE plates without any added iron source, without alpha-ketoglutaric acid or with equal molar amounts of ferric pyrophosphate, ferric chloride or ferrous sulphate. These plates were incubated for 7 days at RT before pictures were taken and survival comparisons were done.

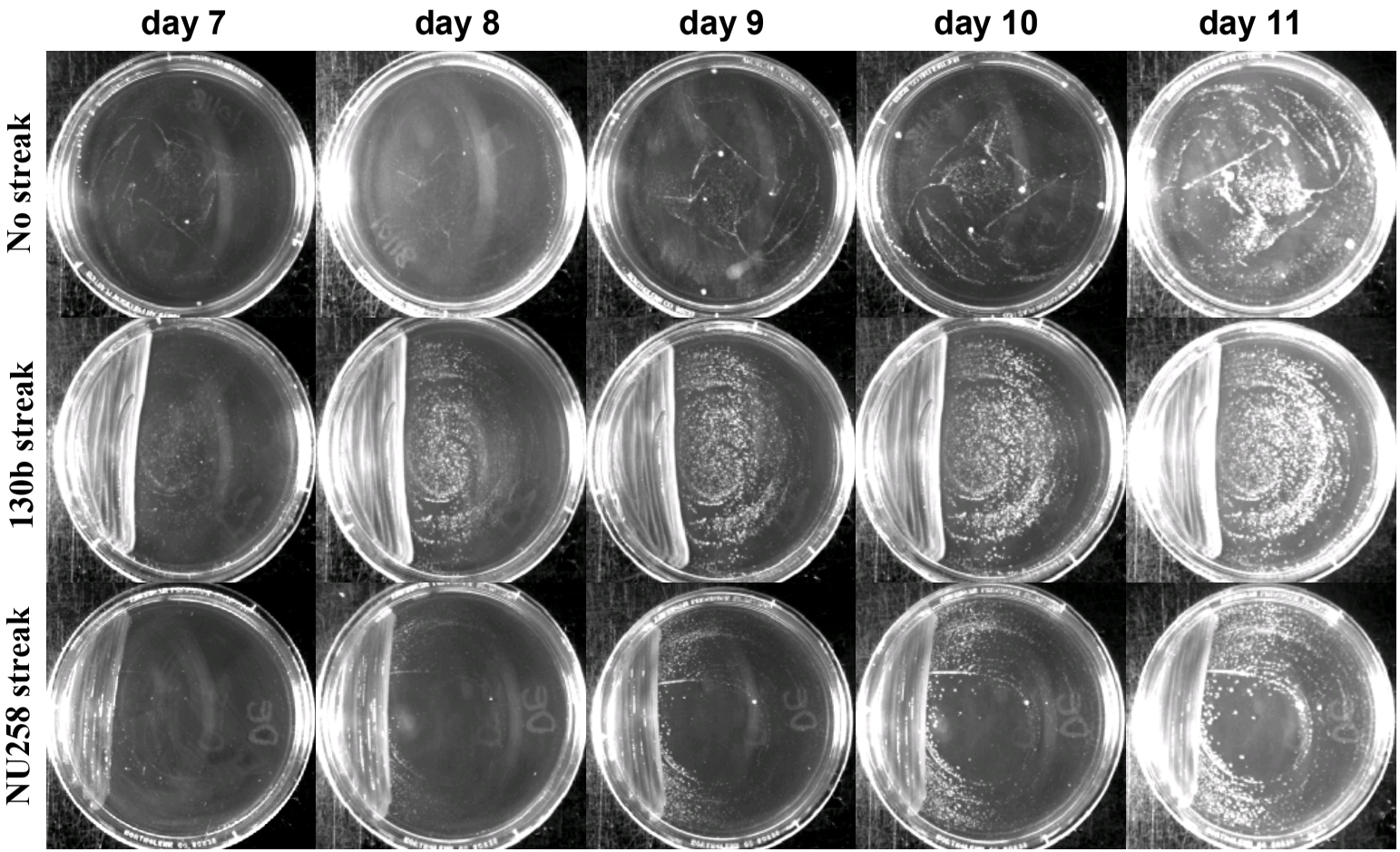

Figure 2-17. The effect of adjacent strain 130b bacteria on the low-temperature survival rate of an *L. pneumophila* type II secretion mutant at RT. Approximately 10^5 CFU of *lspDE* mutant NU258 were plated for CFU on a series of BCYE agar plates. Some plates were then, as indicated, also inoculated in one sector with streaks of strain 130b or mutant bacteria. One set of plates (shown here) was incubated at RT, and another (data not shown) was stored at 37°C. The picture depicts the RT colonial growth of the *lspDE* mutant on days 7, 8, and 9. The results presented are representative of at least two experiments.

promotion could also be seen when a 130b strain streak was added to a plate with previously plated *lspF* mutant (**Fig. 2-18**). Again a streak of the *lspF* mutant could help the growth of itself but to a lesser extent than the 130b strain streak. All in all, these data are consistent with a secreted factor that augments *lsp* mutant survival and growth at low temperatures.

To test if this growth promoting substance was also present in supernatants from the 130b strain grown at even lower temperatures, the 130b strain was grown at 17°C in BYE broth until late log phase. Then cultures were collected, centrifuged and filter sterilized before use. The greatest growth promotion was seen when supernatants were concentrated onto small sterile paper discs that were added to plates with a previously plated *lspF* mutant giving a total of approximately 2 ml of dried supernatants per plate. Good growth promotion could be seen by day 10 (**Fig. 2-19**). BYE did not promote growth (**data not shown**). The fact that supernatants from strain 130b can promote growth suggests, just like the streak assay data, that a secreted factor can support survival and growth of the *lsp* mutants at low temperatures.

Next, a coculture experiment was done with mixed cultures of *lspF* mutant and strain 130b in a ratio of 1:5, 1:1, and 5:1 in addition to cultures with only strain 130b or only *lspF* mutant. At 0 and 2 days, a sample was removed for CFU determination. The CFUs for 130b and mutant in each flask was distinguished by plating on BCYE agar and on BCYE agar containing kanamycin. To determine if the *lspF* mutant was growing better in the mixed cultures, the CFUs obtained for the mutant in each coculture was divided with the CFUs for *lspF* growing by itself. On day 0 the *lspF* mutant in the cocultures with 130b:*lspF* in a ratio of 5:1, 1:1 and 1:5 had 18%, 48% and 90% of the

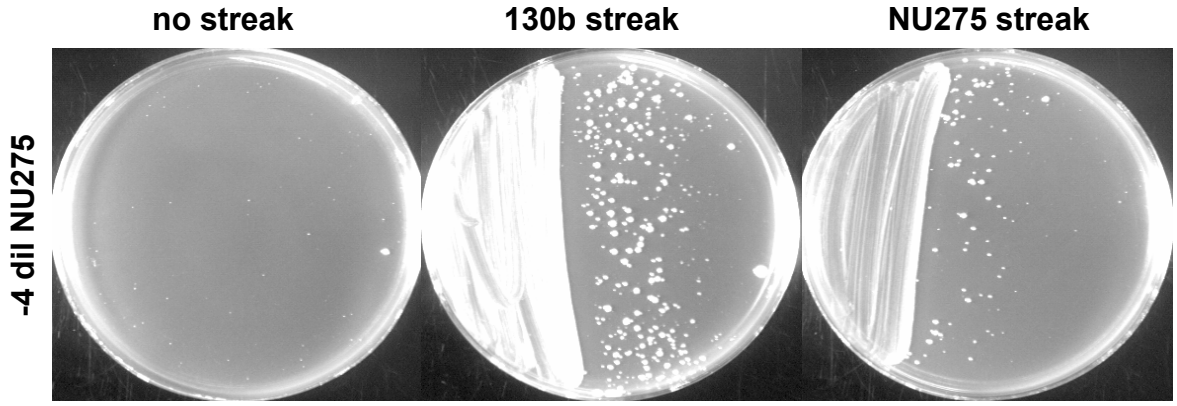

Figure 2-18. The effect of adjacent strain 130b bacteria on low-temperature survival of an *lspF* mutant at RT. Approximately 10^4 CFU of *lspF* mutant NU275 were plated for CFU on a series of BCYE agar plates. Some plates were then, as indicated, also inoculated in one sector with streaks of strain 130b or mutant bacteria. One set of plates (shown here) was incubated at RT, and another (data not shown) was stored at 37°C. The picture depicts the RT colonial growth of the *lspF* mutant on day 11. The results presented are representative of at least two experiments

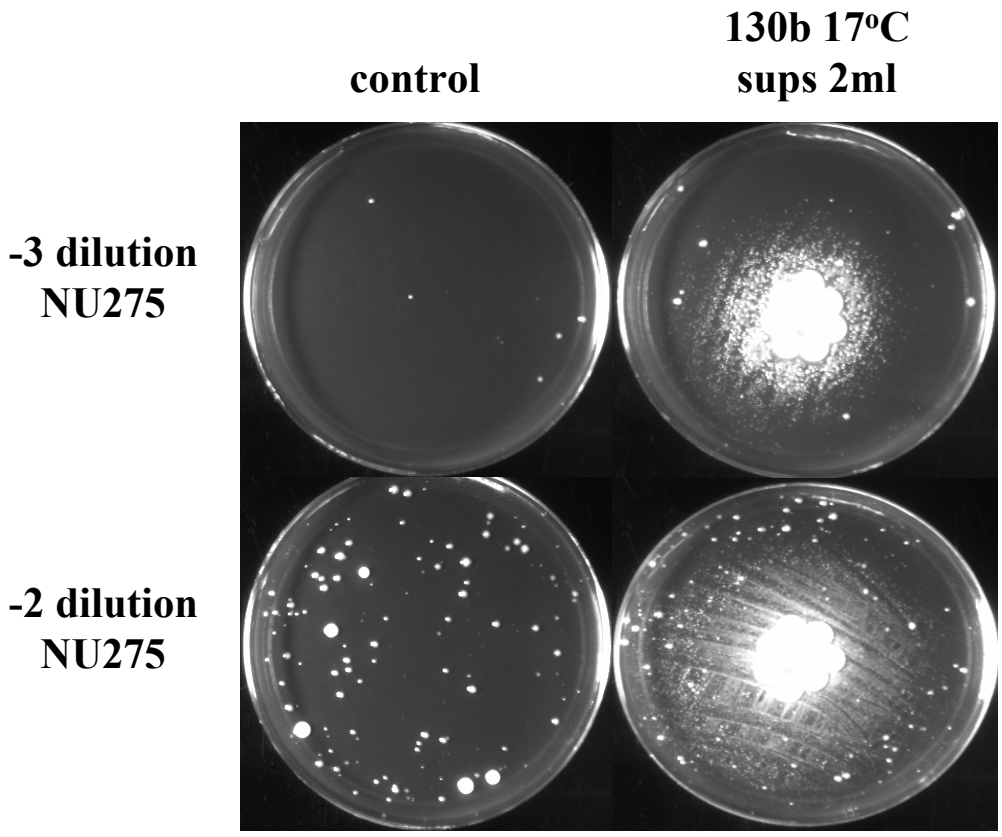

Figure 2-19. The effect of addition of strain 130b low temperature supernatants on the survival of an *lspF* mutant at RT. Strain 130b supernatants from late log BYE cultures at 17°C were concentrated onto discs by repeated cycles of adding and drying the supernatants. Discs with a total of 2 ml of dried in wild type supernatants were then added to plates with 10^5-10^6 CFU/ml plated *lspF* mutant NU275. The plates were incubated at 22°C and pictures of the colonial growth were taken on day 10 day. Similar results were seen in one other experiment.

CFUs of *lspF* growing by itself. Those numbers grew to 29%, 79% and 100% by day 2. The percentage growth for the 130b strain in coculture compared to growth by itself was 113%, 60% and 16% on day 0 and 118%, 52% and 12% on day 2 looking at the 130b:*lspF* in a ratio of 5:1, 1:1 and 1:5, respectively. The overall growth from day 0 to day 2 for *lspF* in all coculture flasks was better in cultures with 130b than in cultures by itself. This same overall increase was not seen in the 130b strain cocultures. Therefore, the presence of 130b improved the growth of the *lspF* while the growth of 130b did not improve in the presence of *lspF*. This growth improvement is most likely due to a type II dependent secreted factor. In summary, the 130b strain streaks, the 130b strain supernatants and the coculture experiments all indicate a type II dependent secreted factor/s is able to improve the growth of the *lsp* mutants.

Several mutants lacking specific type II dependent enzymes already existed in the laboratory, and it was possible that one or more of these enzyme activities could be important for low temperature survival and growth. Therefore, the *proA* mutant AA200 (protease), *plaA* mutant NU270 (lysophospholipase A), *map* mutant NU254 (tartrate sensitive acid phosphatase), *plcA* mutant NU268 (phospholipase C) and *lipAlipB* double mutant NU267 (lipases) mutants were plated at RT. Their EOP was 73% ± 42%, 74% ± 26%, 87% ± 18%, 80% ± 9% and 109 %± 5%, respectively (**Table 4**). Since all of these mutants had an EOP like strain 130b (EOP 61% ± 33%), these enzymes are not important for low temperature growth. However, in many cases, the enzymatic activity seen in supernatants on a certain substrate is the combined action from more than one enzyme. Therefore, supernatants from strain 130b grown at 37°C, RT, and 17°C were tested for phosphatase and protease activities to see if there were some differences in

the amount of these secreted activities at the different temperatures (**Fig 2-20**). There were no differences in protease activity using azocasein as a substrate or for the tartate sensitive acid phosphatase activity using *p*-nitrophenyl phosphate (pNPP) as a substrate. However, the tartrate resistant acid phosphatase activity using pNPP as a substrate was down in the low temperature supernatants indicating that 130b does secrete higher levels of some proteins at one temperature but not at another. These data indicate that there might be yet to be defined secreted effectors that have important roles in cold adaptation.

Our wild type strain, 130b, grew well at lower temperatures but how was its EOP in comparison to other *Legionella* species? To make a comparison, 20 species covering a total of 28 different strains of *Legionella* were plated on BCYE plates at 37°C and 17°C and CFUs were counted on day 3 and day 20, respectively (**Table 6**). Some species had higher EOP than *L*. *pneumophila* strain 130b (EOP in this experiment of 3%) e.g. *L*. *cincinnatiensis* and *L*. *feeleii* with an EOP of 86% and 75%, respectively. Other species had an EOP at 17°C as low as the EOP for the *lspF* mutant e.g. *L*. *brunensis*, *L*. *erythra*, *L*. *hackliae*, *L*. *londinensis*, some species of *L*. *micdadei*, and *L*. *oakridgensis* which all had an EOP of less than 0.01%. In addition, there were differences in EOP between different strains within one species; e.g. the EOP for *L*. *pneumophila*, strain Philadelphia-1 was as poor as for the *lsp* mutants while another 5 *L*. *pneumophila* strains had a higher EOP than strain 130b (**Table 6**). In addition, there were also differences in different strains of *L*. *micdadei* where strain 31B had a higher EOP (EOP 1%) than the other 3 strains tested (EOP<0.01%). Overall, there were not any correlation between the ability to grow at low temperatures and human

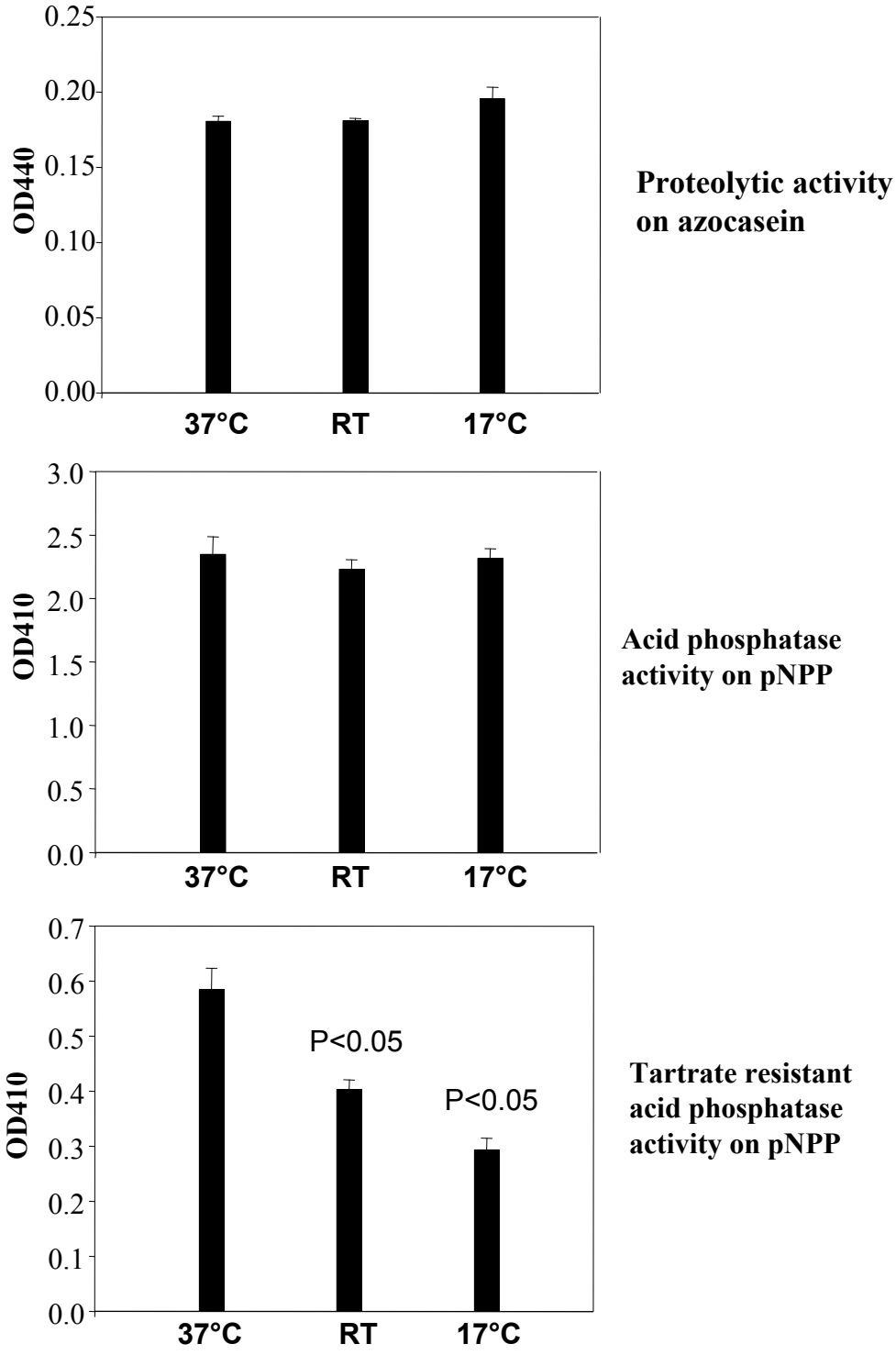

Figure 2-20. Comparison of enzymatic activities in strain 130b supernatants from 37°C, RT and 17°C. Supernatants from strain 130b grown at 37°C, RT and 17°C were tested for proteolytic,and tartrate resistant/sensitive acid phosphatase activities using azocasein or pNPP as a substrate, respectively. These data represent the mean and standard deviation from duplicate cultures.

Table 6. Growth comparison between different *Legionella* species at 17°C

Species	EOP*	Strain designation	Pathogen
L. pneumophila 130b	3%	BAA-74	Yes
L. pneumophila 130b-*lspF* mutant NU275	0.0003%		
L. pneumophila Philadelphia-1	0.005%	33152	Yes
L .pneumophila Oxford	17%	43110	Yes
L. pneumophila Concord	50%	35096	Yes
L. pneumophila 82A3105	73%	43736	Yes
L. pneumophila 1169-MN-H	56%	43703	Yes
L. anisa	33%	35292	Yes
L. birminghamensis	3%	43702	Yes
L. bozemanae	13%	33217	Yes
L. brunensis	0.004%	43878	No
L. cherrii	0.06%	35252	No
L. cincinnatiensis	86%	43753	Yes
L. erythra	0.0006%	35303	Yes
L. feeleii	75%	35072	Yes
L. gratiana	0.04%	49413	No
L .hackliae	0.002	35250	Yes
L. israelensis	0.2%	43119	No
L. jamestowniensis	2.0%	35298	No
L. jordanis	0.03%	33623	Yes
L. londinensis	0.001%	49505	No
L. longbeachae	23%	33462	Yes
L. micdadei 31B	1%	see ref.	Yes
L. micdadei	0.0008%	33218	Yes
L. micdadei Detroit I	0.009%	see ref.	Yes
L. micdadei Stanford-R	0.002%	see ref.	Yes
L. moravica	38%	43877	No
L. oakridgensis	0.001%	33761	Yes
L. parisiensis	0.5%	35299	Yes

*EOP defined as 100x(CFU at 17°C at 20-21 days/ CFU at 37°C at 3 days)

The strain designations represent American Type Culture Collection (ATCC) strain numbers. For the source of the *L. micdadei* strains not from ATCC and a reference for all strains listed above see references (5, 171, 217).

pathogenicity. In contrast, the substantial reduction in EOP for *L*. *micdadei* strain Standford-R correlated with that strain lacking type II dependent proteolytic and lipolytic activities as assessed by patching on egg yolk plates (198). Conversely, *L*. *parisiensis* and *L*. *longbeachae*, which both have an EOP in a comparable range to strain 130b, have both of these activities (198). Thus, a correlation might exist between having a functional type II secretion system and the ability to survive and grow at low temperatures.

SUMMARY

A *pilD* mutant has a severe reduction in survival and growth in broth and on plates at 17°C-RT. This reduction in survival and growth was also seen with mutants in the type II secretion system but not with mutants in the type IV pilus apparatus. This reduction in survival and growth could be fully complemented at both 17°C and RT. In addition, the 130b strain survived and grew at 12°C in broth and on plates, while the *lsp* mutants did not. Thus, the type II secretion system becomes more important for growth and survival as the temperature decreases. These data show for the first time that a type II secretion system is important for low temperature survival and growth.

Mutants lacking the type IV pilus apparatus, the *dot*/*icm* and the *lvh* type IV secretion systems did not have a reduction in EOP at lower temperatures. In addition, a *L*. *pneumophila tat* mutant did not have a reduction in EOP (195). It is therefore highly unlikely that the severe reduction in EOP seen for the *lsp* mutants would be caused by cell associated changes due to the lack of a membrane spanning protein complex. In addition, streaks, supernatants, and coculture of the 130b strain can increase the

survival rate of the *lsp* mutants. These data support the idea that a secreted factor/s, instead of a cell associated factor, is important for low temperature survival and growth.

This reduction in EOP is partly due to the presence of iron in the media since a *pilD*/*lsp* mutant had an increased EOP on plates without the ferric pyrophosphate iron supplement. How can this growth defect be explained? Iron is known to react with hydrogen peroxide in the medium through the Fenton reaction: $H_2O_2 + Fe^{2+} = Fe^{3+} + OH^- + OH^{\cdot}$, creating free radicals (234). Since the amount of dissolved oxygen in solution will increase when the temperature goes down, there might be more radicals formed in the agar plates at low temperatures (47). It is therefore possible that a factor needed for oxidative defense is secreted through the type II secretion system. An alternative explanation is that this factor is needed for binding the iron, which then would prevent radicals from forming. The reduction in EOP of the *lsp* mutants at lower temperatures would then be explained by an inability to grow in presence of or killing by radicals. It is possible that this reduction in EOP is only seen at lower temperatures because another factor is secreted at higher temperatures with the same function by pathways other than type II secretion or because there are more radicals formed at lower temperatures making this factor important only at lower temperatures. However, it has to be pointed out that even though the EOP for the type II secretion mutants did improve radically, from 0.05 to 5% for *lspDE*, their EOP did not reach the levels of the 130b strain (EOP 61%±33%). Therefore, there is probably more than one secreted factor important for low temperature survival and growth.

In addition, several type II dependent mutants, *proA*, *map*, *plaA*, *plcA* and *lipAlipB*, were assessed for EOP at lower temperatures but none of these mutants were

defective. However, a decrease in tartrate resistant acid phosphatase activity was seen at 17°C and RT vs. 37°C indicating that *L*. *pneumophila* does secrete different proteins at different temperatures. Since, there is no evidence in the literature of a secreted factor being important for growth at low temperatures, future efforts were concentrated on finding this factor.

Chapter 3

THE TYPE II SECRETION SYSTEM IN *L. PNEUMOPHILA* IS IMPORTANT FOR LONG TERM SURVIVAL IN TAP WATER

INTRODUCTION

L. pneumophila has been found in almost all freshwater habitats examined, including lakes, ponds, rivers, creeks, swamps, wet soil, marine and estuarine environments (72, 73, 95, 172, 218). *L. pneumophila* is similarly widespread in artificial water systems, in some cases reported in 60% of the plumbing systems in large and small public buildings, as well as in private residences (4, 141). In these environments *L. pneumophila* can survive as planktonic bacteria, within biofilms, as a viable but nonculturable form or replicate as an intracellular parasite of protozoa (18, 68, 114, 141, 211, 214, 219, 242). This broad distribution of *L. pneumophila* is at least in part due to its ability to survive at temperatures between 4°C to 63°C (72, 73). However, since the bacteria grows best in the laboratory at 32-37°C, there are few data on what is important for low temperature growth/survival (133, 136, 249).

Earlier studies (**Chapter 2**) have shown that the type II secretion system in *L. pneumophila* is very important for growth at low temperatures in rich bacteriological media. However, in the environment, *L. pneumophila* is often found in aquatic habitats where access to nutrients is limited. Therefore, to establish a role for the type II secretion system for survival in more biological relevant nutrient poor water several type II secretion mutants were tested for their ability to survive in tap water at different temperatures.

RESULTS

To test if type II secretion is important for survival in tap water at lower temperatures, the 130b strain and *lspDE*, *lspF* and *pilD* mutants from log-phase BYE cultures were centrifuged, rinsed twice with sterile tap water and then inoculated into 50 ml of sterile tap water. Then, these flasks were incubated at 37°C, RT or 17°C while shaking at 225 rpm. At indicated days, a sample was taken out and the CFU were determined by plating on BCYE plates (**Fig. 3-1**). At 37°C, the *lsp*/*pilD* mutants had more CFUs than the 130b strain starting on day 7. At 37 days, CFUs could no longer be detected for either strain 130b or *lsp* and *pilD* mutants. In a similar vein, at RT, the mutants were recovered slightly more than the 130b strain between days 126 and 141. In contrast, at 17°C the 130b strain had more CFUs than the mutants from 49 days and on and this difference grew to two logs after extended incubation at 17°C. When compared to the 130b strain, the *lsp*/*pilD* mutants clearly had a reduced ability to survive at the lower temperature.

Supernatants collected from all the water cultures on day 97 were tested for proteolytic, phosphatase and lipolytic activites (**data not shown**). No activities could be detected under the standard enzymatic conditions routinely used in the lab to detect enzymatic activities from BYE culture supernatants. However, it is not very likely that the bacteria could survive for months in the water cultures without nutrient acquisition from the surrounding water. Therefore, the reason these assays did not work is probably because the amount of enzyme secreted in the water cultures is too low to detect in these assays.

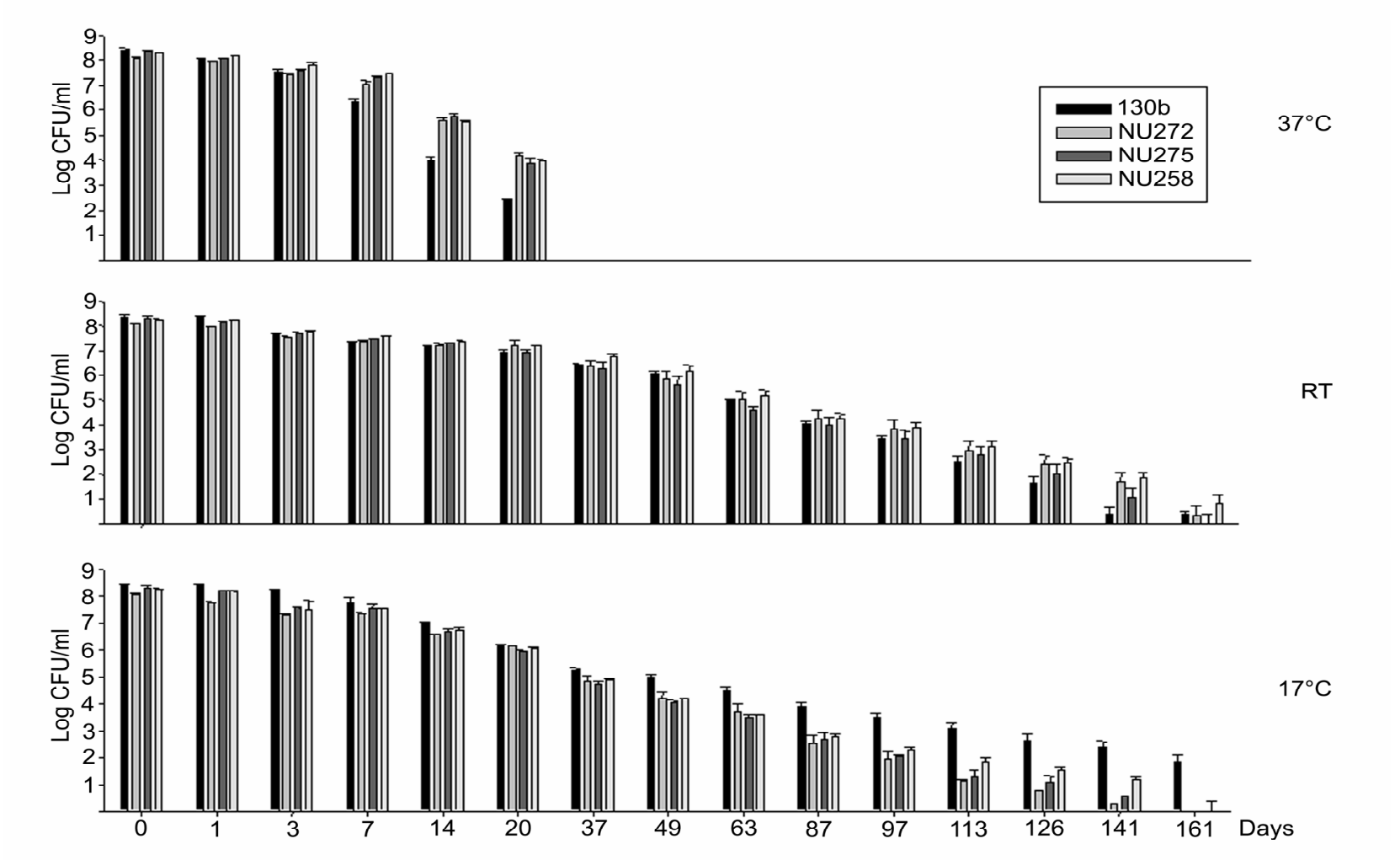

Figure 3-1. Survival of 130b, *pilD* mutant NU272, *lspF* mutant NU275 and *lspDE* mutant NU258 in sterile tap water at 37°C, RT and 17°C. Log-phase bacteria from BYE cultures were spun down, rinsed twice with sterile tap water and then inoculated into 50 ml of sterile tap water before incubation at 37°C, RT or 17°C. At indicated days a sample was taken out and the CFU count was determined. The results presented are the mean and standard deviation of duplicate samples.

To confirm that the type II secretion mutants of *L. pneumophila* have reduced survival in tap water at low temperatures, we retested the *lspF* mutant, but this time compared the 130b strain and the mutant in samples incubated at 37°C, 17°C, 12°C and 4°C. Also, we allowed twenty months to pass before performing the experiment in order to collect a tap water sample, from the same tap, that would be slightly different from the first water sample tested; i.e. to see that the difference we had seen between strain 130b and the secretion mutant were not an artifact of the particular water sample used (**Fig. 3-2**). At 37°C, the *lspF* mutant had again more CFUs than the 130b strain this time starting on day 38 while no CFUs for either 130b or the *lspF* mutant were detected on day 49. In an indication that the tap water had changed appreciably, the recoverability of the 130b strain was fully maintained at 17°C over a period of 318 days rather than gradually declining within a 161-day period as was seen in the first experiment. Similar to what had been seen before, at 17°C, the *lspF* mutant had a reduced recoverability this time starting at day 20. At 12°C and 4°C, the 130b strain had more CFUs than the mutants from 6 days on. At 12°C, this difference grew to more than 7 logs by day 318 and at 4°C a maximum difference of more than 4 logs could be seen by day 133. No CFUs were detectable for *lspF* on day 378 and day 203 at 12°C and 4°C, respectively. The 130b strain had almost no change in CFUs at 12°C for a period of 378 days while at 4°C the 130b strain had a drop in viability of approximately 5 logs in 318 days. In summary, the overall survival rate for both the 130b strain and *lspF* mutant was good at the lower temperatures. This was especially apparent at 17°C and 12°C where the 130b strain survived for 11 months without much reduction in CFUs. More importantly, the ability of the *lspF* mutant to survive at lower temperatures was again

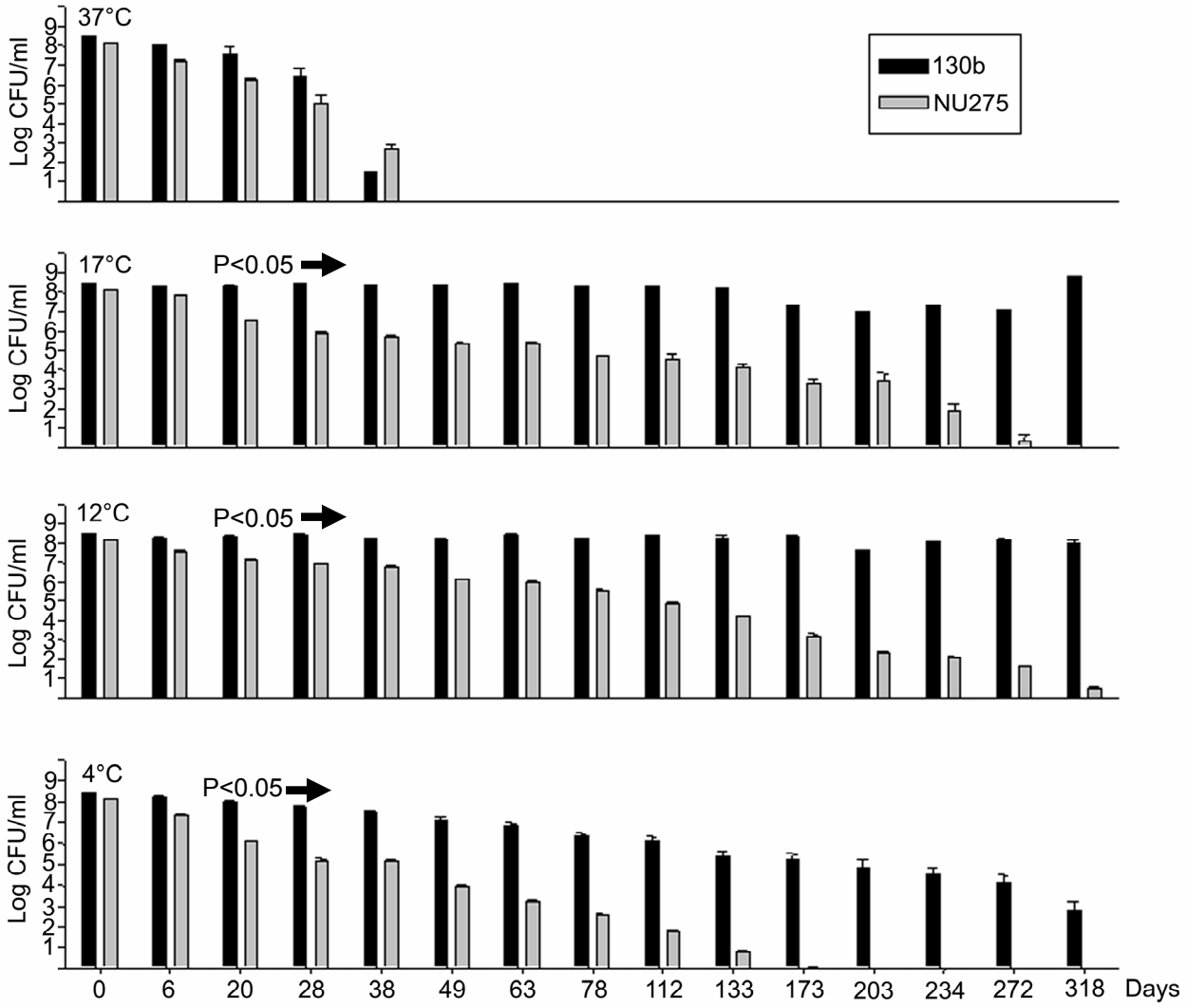

Figure 3-2. Survival of 130b and *lspF* mutant NU275 in sterile tap water at 37°C, 17°C, 12°C and 4°C. Log-phase bacteria from BYE cultures were spun down, rinsed twice with sterile tap water and then inoculated into 50 ml of sterile tap water before incubation at 37°C, 17°C, 12°C or 4°C. At indicated days a sample was taken out and the CFU count was determined. The results presented are the mean and standard deviation of duplicate samples, except for 130b at 17°C where n=1after day 28.

lower than for the 130b strain. This difference was not due to a high proportion of the *lspF* mutants being viable but non culturable as determined by live dead stain (**data not shown**). These data indicate a role for the type II secretion system in the ability of *L. pneumophila* to survive at low temperatures in nutrient poor water cultures.

Another environmentally interesting question was if *L. pneumophila* grown at lower temperatures would be as infectious as bacteria grown at 37°C. Bacteria virulence factors are often expressed at a certain temperature but not at others. For example, Yop expression in *Yersinia* is upregulated at 37°C as compared to 28°C, while the plant pathogen *P. syringae* pv. glycinea produces the phytotoxin coronatine at 18°C but not at 30°C (45, 237). Two previous papers present conflicting data about the infectiousness of *L. pneumophila* grown at RT for infection in guinea pigs in that one study saw an increased level of *in vivo* growth while the other study did not (59, 154). However, these two studies differ in the ways of growing the bacteria, the route of inoculation of the animals and the strain of *L. pneumophila* used. Since these data are not in agreement and since there are no previous reports about the infectiousness in amoebae of *L. pneumophila* grown on rich media at lower temperatures, 130b was grown on BCYE plates at 37°C for 3 days or on BCYE plates at RT for 8 days before infection with the amoeba *H. vermiformis* (**Fig. 3-3**). Briefly, 10^4 bacteria and 10^5 amoebae were added/well in a 24 well plate and the coculture was then incubated at the normal infection temperature of 35°C. Growth was assessed by CFU plating at 0, 24, 48 and 72 hours. No difference in intracellular growth was seen, and therefore *L. pneumophila* bacteria grown at RT are as infectious for amoeba as bacteria grown at 37°C.

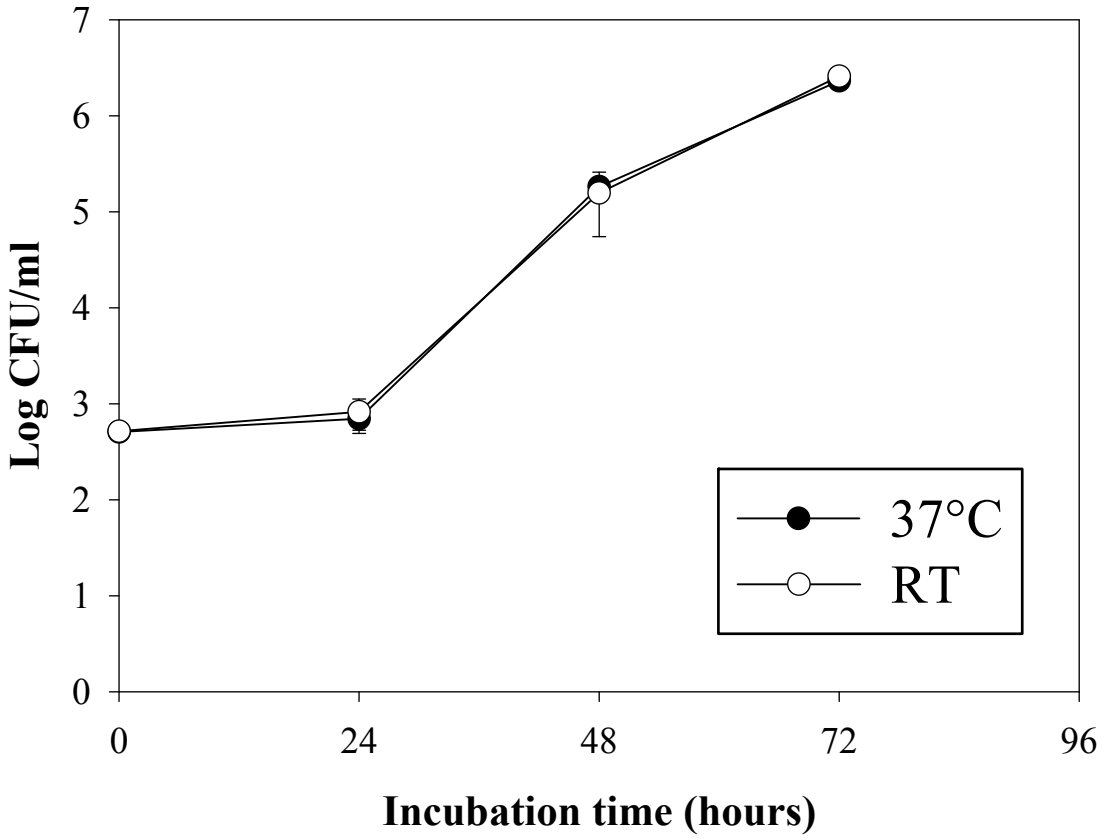

Figure 3-3. *H. vermiformis* infection with 130b grown at 37°C and RT. *H. vermiformis* was infected at an MOI of 0.1 with 130b grown at 37°C for 3 days or at RT for 8 days. At 0, 24, 48 and 72 hours samples were plated for CFUs. The results presented are the mean and standard deviation of triplicate samples.

SUMMARY

The *lsp* and *pilD* mutants' reduced survivability at 4-17°C in tap water shows the type II secretion system is important for low temperature survival not only on rich media but also in water. More importantly, these data also indicate that the type II secretion system might have an important role for the ability of *L*. *pneumophila* to persist for long periods of time in those water sources responsible for spread of disease. Interestingly, the ability of the *lsp* and *pilD* mutants to survive at RT, was at most time points equal to that of the 130b strain. This is in sharp contrast to the severe growth defect seen for both the *pilD* and *lsp* mutants when grown in bacteriological media at RT. This could be due to the fact that the *lsp* growth defect is manifested earlier under conditions of bacterial growth than when the bacteria are not replicating. Another possibility is that higher iron content in media than water allows for production of more radicals in bacteriological media at RT. Bacteriological media would therefore be more toxic manifesting differences between the 130b strain and *lsp* mutants at a higher temperature.

The fact that both the 130b strain and the *lsp* mutant persisted in water for a longer period of time in the second experiment is probably due to differences in the water. This difference is most likely due to a change in iron levels, due to different biocide levels or due to different biocides used in the water at the time of collection. Unfortunately, the details on the water treatment regimens used in our local environment over these time periods were not available to us making definite conclusions difficult. However, it is also possible that different subpopulations of bacteria, which had accumulated due to different chance events, existed in the two

experiments. The increased viability over time in the second experiment would then simply be due to a higher concentration of a subpopulation capable of surviving better in tap water cultures at lower temperatures. Additional experiments are needed to distinguish between these possibilities.

In addition, this data showed the ability of 130b to infect *H. vermiformis* was not dependent on the growth temperature of the bacteria before infection (RT vs. 37°C) indicating that bacteria grown in the environment could be as infectious to humans as bacteria grown at 37°C. As a future experiment it would be interesting to see if this would also be true for bacteria grown at 17°C and 12°C, both for infections done in amoebae and macrophages.

Chapter 4

GENETIC SCREEN TO FIND TYPE II DEPENDENT FACTOR/S IMPORTANT FOR LOW TEMPERATURE GROWTH

INTRODUCTION

Previous trans-complementation experiments with strain 130b streaks and low temperature supernatants indicated that a type II dependent secreted factor/s was needed for low temperature survival and growth. This was an interesting result, since such a factor had not been isolated before. Therefore, to isolate such secreted factor or factors, a genetic approach was devised in which mutants from a transposon library with a reduced EOP at 17°C, but not 37°C, were isolated. Two different transposons were used for this study. One transposon, on plasmid pEH40, contains a promoterless *lacZ* gene and would allow for the direct isolation of genes important for survival and growth at low temperatures but would not distinguish between insertions in genes encoding cell associated vs. secreted proteins (101). The other transposon, on plasmid pMM237, has a truncated *phoA* gene and could be used to screen for insertions in genes that actually encode proteins that are secreted by measuring the alkaline phosphatase activity in the supernatants (155).

RESULTS

To isolate genes important for low temperature growth, the transposon containing plasmids pEH40 (*lacZ*, Km^R) and pMM237 (*phoA,* Km^R) were used to mutagenize strain

130b genomic DNA. In total, 7400 transposon-derived colonies were picked and replica patched onto two plain BCYE agar plates. One set of plates was incubated at 37°C for 3 days, while the other set of plates was incubated at 17°C for 6 days. In total, 7400 colonies were patched, with 99% of those colonies coming from using the plasmid pEH40 and 1% from using the plasmid pMM237. The low number of colonies obtained using the transposon containing plasmid pMM237 was due to unexplained technical difficulties. This screen was not yet saturated, but from the 7400 colonies screened, 104 mutants were identified with a reduced EOP at 17°C; i.e. mutants that survived and grew but did so to a lesser degree than the 130b strain. A second round of screening was done to confirm these reductions in EOP. To do so, the corresponding colony was picked from the 37°C plate, which had been stored at RT and not 4°C after 3 days of growth at 37°C to prevent potential compensatory mutations from occurring in potential mutants defective for growth at lower temperatures. 10 µl spots of several dilutions were made from these mutants on two sets of plates that were then incubated at 37°C for 3 days or 17°C for 20 days. From this second round of screening, 21 mutants with confirmed reduction in EOP at 17°C were isolated (for additional EOP data see Table 8 and text below).

All 21 mutants were patched onto casein and egg yolk plates to see if any mutant was lacking type II dependent secreted proteolytic and/or lipolytic activities (15, 16, 36, 71) (**data not shown**). All mutants except two behaved like the parental strain. These two mutant strains, NU359 and NU362, behaved like the *lspF* mutant and were identified as having insertions somewhere in *lspFGH* and in *lspE*, respectively. This

showed that the screen isolated mutants with reduced ability to survive and grow at low temperatures.

The mutated genes in 16 other mutants (**Table 7a**) were also identified using either inverse PCR or cloning of the genes into pUC18 using the Km^R marker in the transposon. Of those, 6 mutants had insertions in genes encoding for proteins involved in fatty acid and amino acid metabolism. Mutant strains NU351 and NU355, had insertions in the genes *lpg1352* and *lpg0870*, which are annotated as encoding proteins 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, respectively. These two proteins catalyze the second and third step in the β-oxidation pathway of fatty acid metabolism (3). Mutant strains NU350 and NU365, both had insertions in the gene *lpg0363*, annotated as encoding a lipid A biosynthesis acyltransferase protein responsible for the attachment of laurate to the 2' (R)-3-hydroxy myristoyl chain on the lipid A molecule (240). Mutant strain NU360 had an insertion in the gene *lpg1530*, annotated as encoding a methylcitrate synthase protein involved in metabolism of the odd-chain fatty acid propionyl-CoA (164). Finally, mutant strain NU357 had an insertion in the gene *lpg1459*, annotated as encoding a aspartate aminotransferase protein responsible for the reversible conversion of aspartate and alpha-ketoglutaric acid to oxaloacetate and glutamate (94).

Another 4 mutants (**Table 7a**) had insertions in genes encoding for proteins involved with DNA/RNA/chaperone functions, some of which are known to be needed for the growth of other bacteria in the cold. These mutants included mutant strain NU352, which had an insertion in the gene *lpg2345*, annotated as encoding a ribosome associated ATP helicase protein (230). Mutant strain NU367 had an insertion in the

TABLE 7a. Mutated genes from transposon screen

Mutant	Gene*	Annotation*** Protein	Gene	Lowest e-value+	Organism with lowest e- value	In operon	Signal peptide
NU350	*lpg0363*	lipid A biosynthesis acyltransferase	*waaM/htrB*	1e-30	*Syntrophobacter fumaroxidans*	yes++	no
NU351	*lpg1352*	3-hydroxyl-CoA dehydrogenase	*fadB*	0	*Stenotrophomonas maltophilia*	yes	yes
NU352	*lpg2345*	ATP-dependent RNA helicase	*csdA/deaD*	3e-150	*Xanthomonas campestris*	no	no
NU353	*lpg0858*	heme export protein	*ccmC*	8e-66	*Pseudomonas syringae*	yes	no
NU354	*lpg1755*	transmembrane protein	-	5e-69	*Chromobacterium violaceum*	yes++	yes
NU355	*lpg0870*	enoyl- CoA hydratase	-	1e-84	*Coxiella burnetii*	yes++	no
NU356	*lpg1846*	glutathione synthetase	*gshB*	3e-94	*Alcanivorax borkumensis*	yes++	no
NU357	*lpg1459*	aspartate aminotransferase	*yfdZ*	6e-166	*Nitrococcus mobilis*	yes	no
NU358	*lpg1681*	hypothetical protein	-	3e-34	*Legionella pneumophila* Paris	no	no
NU359	*lpg1361*****	type II secretion protein LspF	*lspF*****	1e-85	*Shewanella amazonensis*	yes	no
	*lpg1362*****	type II secretion protein LspG	*lspG*****	2e-52	*Alkalilimnicola ehrlichei*	yes	no
	*lpg1363*****	type II secretion protein LspH	*lspH*****	5e-5	*Marinobacter aquaeolei*	yes	no
NU360	*lpg1530*	2-methylcitrate synthase	*prpC*	3e-133	*Pseudomonas fluorescens*	yes	yes
NU361	*lpg1415*	citrate synthase	*gltA*	0	*Coxiella burnetii*	no	no
NU362	*lpg1319*	type II secretion protein LspE	*lspE*	9e-160	*Psychromonas ingrahamii*	yes++	no
NU363	*lpc2788***	putative type I restriction enzyme	*hsdR*	0	*Bacillus* sp.	yes	no
NU364	*lpg1159*	putative metabolite permease	-	3e-34	*Crocosphaera watsonii*	no	yes
NU365	*lpg0363*	lipid A biosynthesis acyltransferase	*waaM/htrB*	1e-30	*Syntrophobacter fumaroxidans*	yes++	no
NU366	*lpg0092*	exoribonuclease R	*vacB*	0	*Coxiella burnetii*	yes	no
NU367	*lpg1862*	trigger factor	*tig*	1e-106	*Marinobacter aquaeolei*	yes	no

Temperature mutants NU368, NU369 and NU370 were never successfully sequenced

*as defined in *L. pneumophila* Philadelphia-1 genome at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
**as defined in *L. pneumophila* Corby genome at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
***as annotated in *L. pneumophila* Philadelphia-1 or Corby genome at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
****exact insertion site not known
+ all proteins annotated to be the same protein as in the *L. pneumophila* Philadelphia-1 genome database
++ last gene in operon

gene *lpg1862*, annotated as encoding a ribosome associated trigger factor. In *E. coli*, this protein is important for viability after cold shock (124). In addition, mutant strain NU366, had an insertion in the gene *lpg0092*, annotated as encoding a cold shock induced exoribonuclease R protein (29). Lastly, mutant strain NU363 had an insertion in the gene *lpc2788*, annotated as encoding a putative type I restriction protein that is thought to protect the host bacteria from the uptake of foreign DNA (252). In *L. pneumophila*, this protein only had homology to a protein in strain Corby.

One mutant strain, NU358, had an insertion in the gene *lpg1681*, annotated as encoding a short hypothetical protein only present in *L. pneumophila* (**Table 7a**). Mutant strain NU356 had an insertion in the gene *lpg1846*, annotated as encoding a glutathione synthetase protein that catalyzes the second step in the glutathione biosynthesis pathway and has some function in protection against oxidative stress (30). Mutant strain NU361 had an insertion in the gene *lpg1415*, annotated as encoding a citrate synthase. This enzyme catalyzes the first step in the citric acid cycle (164). Mutant strain NU353 had an insertion in the gene *lpg0858*, annotated as encoding a heme export protein important for cytochrome *c* maturation and growth under iron limiting conditions (39). Mutant strain NU364 had an insertion in the gene *lpg1159*, annotated as encoding a permease of the metabolite/drug transport family. Mutant strain NU354 had an insertion in the gene *lpg1755*, annotated as encoding an inner membrane protein with unknown function. Three mutant strains, NU368, NU369 and NU370, were never successfully sequenced, even though both inverse PCR and cloning strategies were tried. However, these three mutants did not belong to the group with the biggest reduction in EOP at 17°C; i.e., EOP < 0.1% (**Table 8**).

Of these 18 mutants with known insertions, 14 might be in an operon (**Table 7a and 7b**). However, 6 of those have insertions in the last gene of these operons (**Table 7a**). In another 5 cases (NU351, NU353, NU359, NU360, NU366), the proteins encoded by the genes downstream of the insertions are predicted to work in the same pathway; e.g., the genes in the *ccm* operon are all involved in cytochrome *c* maturation, the gene downstream of 3-hydroxyl-CoA dehydrogenase is involved in fatty acid metabolism etc (**Table 7b**). However, the gene (*lpg1459*), encoding for an aminotransferase, is upstream of a gene encoding for a hypothetical protein and a gene encoding for a DNA specific exonuclease. In addition, the gene (*lpc2788*) encoding for a type I restriction protein is upstream of two genes encoding for proteins with unknown functions. Finally, the gene (*lpg1862*) encoding for trigger factor is upstream of a gene encoding for an ATP-dependent protease. It is therefore possible that the insertion in these three genes might have a polar effect on downstream genes.

A few of the proteins isolated in the screen are predicted to have the classical Sec pathway signal peptide used by most type II secreted exoproteins to cross the inner membrane (**Table 7a**). However, not only type II secreted proteins, but also most periplasmic and membrane proteins have this kind of signal peptides. Therefore, by itself, this is not a good indication that a protein is secreted to the extracellular environment or not. Instead, other programs; e.g. PSORTb, can be used to better determine the localization of proteins. In this screen, only mutant strains NU351 and NU360, with insertions in genes encoding for the proteins 3-hydroxyacyl-CoA dehydrogenase and 2-methylcitrate dehydratase, respectively, were predicted to be periplasmic by PSORTb. Mutant strains NU353, NU354, NU358, NU361 and NU364,

TABLE 7b. Additional information for those mutants containing insertions in an operon

	Annotation*		Downstream genes	
Mutant	Gene	Protein	Protein annotation	Signal peptide
NU351	*lpg1352*	3-hydroxyl-CoA dehydrogenase	acetyl-CoA acetyltransferase	no
NU353	*lpg0858*	heme export protein	cytochrome c-type biogenesis protein CcmD	no
			cytochrome c-type biogenesis protein CcmE	yes
			cytochrome c-type biogenesis protein CcmF	yes
			thiol:disulfide interchange protein	no
			cytochrome c-type biogenesis protein CcmG	yes
			cytochrome c-type biogenesis protein CcmH	no
NU357	*lpg1459*	aspartate aminotransferase	hypothetical protein	no
			single stranded DNA specific exonuclease	no
NU359	*lpg1361-63*	type II secretion protein LspF-H	type II secretion protein LspI	yes
			type II secretion protein LspJ	yes
			type II secretion protein LspK	yes
NU360	*lpg1530*	2-methylcitrate synthase	2-methylcitrate dehydratase	no
NU363	*lpc2788*	putative type I restriction enzyme	hypothetical protein	no
			hypothetical protein	no
			putative RNA helicase	no
NU366	*lpg0092*	exoribonuclease R	tRNA/rRNA methyltransferase	no
			ribose-5-phosphate isomerase A	no
			cytosolic IMP-GMP specific 5'-nucleotidase	no
NU367	*lpg1862*	trigger factor	ATP-dependent Clp protease	no

* as defined and annotated in *L. pneumophila* Philadelphia-1 or Corby genome at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi

with insertions in genes encoding for a heme export protein, an inner membrane protein, a hypothetical protein, a citrate synthase, and a putative metabolite permease, respectively, were predicted to be in the inner membrane by the same method. None of these proteins, according to PSORTb, were predicted to be secreted to the extracellular milieu. However, it can not be assumed that the predictions made by PSORTb are always correct. Therefore, some of these proteins could still potentially be secreted by type II secretion. However, other methods, such as Western blots or 2D gel analysis of strain 130b supernatants, would have to be used to determine if any of these proteins are secreted.

All the 21 mutants were replated for isolated colonies at 17°C vs. 37°C to better measure their low-temperature EOP (**Table 8**). At 37°C, only mutant strain NU361 with an insertion in the gene encoding for a citrate synthase, had a slight reduction in EOP. This strain only produced 66%±2% of strain 130b CFUs at 37°C. This mutant also had smaller colonies in 40% of experiments. At 17°C, 13 mutants (**Table 8**) had an EOP of roughly 0.1-50% of the levels of strain 130b. In addition, 8 mutants had a severe reduction in EOP, equal that seen for the previously described *lsp* mutants (**Table 8**). These mutants had insertions in the genes encoding for the type II secretion proteins, the cold shock induced ATP-dependent RNA helicase, the cold shock induced exoribonuclease R, the lipid A biosynthesis acyltransferase, the transmembrane protein and the aspartate aminotransferase. Thus, these genes are necessary for survival at low temperatures. The few bacteria that grew likely have suppressor mutations, as seen for the *lsp* mutants. This is in contrast to the mutants with an EOP of 0.1-50%, where

Table 8. 17°C growth comparison of mutants from temperature screen

Strain	Insertion in gene	EOP* at 17°C
130b	none	15% ± 10%
NU275	*lspF*	0.0004%±0.0005%
NU355	enoyl- CoA hydratase	8%±5%
NU363	putative type I restriction enzyme	4%±3%
NU358	hypothetical protein	4%±2%
NU368	unknown	3%±4%
NU367	trigger factor (*tig*)	1%±2%
NU360	2-methylcitrate dehydratase (*prpC*)	1%±1%
NU370	unknown	1%±0.5%
NU353	heme export protein (*ccmC*)	1%±0.3%
NU356	glutathione synthase (*gshB*)	0.9%±1%
NU369	unknown	0.6%±0.6%
NU364	permeases of the metabolite/drug transport	0.4%±0.1%
NU361	citrate synthase (*gltA*)	0.3%±0.5%
NU351	3-hydroacyl-CoA dehydrogenase (*fadB*)	0.1%±0.2%
NU366	exoribonuclease R (*vacB*)	0.0005%±0.0007%
NU350	lipid A biosynthesis acyltransferase (*waaM*)	0.0005%±0.0007%
NU362	*lspE*	0.0002%±0.0002%
NU359	*lspFGH*	0.0001%±0.0001%
NU365	lipid A biosynthesis acyltransferase (*waaM*)	0.0001%±0.0001%
NU354	transmembrane protein	0.00002%±0.00003%
NU352	ATP dependent helicase (*csdA*)	0%
NU357	putative aminotransferase (*yfdZ*)	0%

*EOP defined as 100 x (CFU at 17°C on day 20-21/CFU at 37°C on day 3)
Mutant strains below the thick line have EOP in the same range as the *lsp* mutants

the reduction in EOP is likely due to the existence of different subpopulations; e.g with slightly different gene expression. These differences allow only part of the population to survive and grow at low temperatures.

The mutants from the genetic screen were also plated on BCYE plates with and without the 0.25g/L ferric pyrophosphate supplement to check for improved survival and growth equal that seen for the *lsp* and *pilD* mutants on low iron plates (**data not shown**). None of the new temperature mutants had increased EOP on the BCYE plates without the iron supplement. The only exception was mutant strain NU359 and NU362, which would be expected since they have insertions in the *lsp* genes.

All the mutants were also tested in the streak assay to see if some of them would lack the ability to promote better survival and growth of the *lsp* mutants. As expected, both the *lspE* and *lspFGH* mutants behaved like the *lspF* mutant in the streak assay. Surprisingly, 3 other mutant strains, NU352, NU354, and NU366, with insertions in genes encoding for the ATP dependent helicase, an inner transmembrane protein, and the exoribonuclease R also had reduced abilities to promote survival and growth of the *lspF* mutant in the streak assay (**Figure 4-1**). The streak assay is done at RT since the ability of strain 130 to increase survival and growth of the *lsp* mutants is greatly reduced at 17°C. Since the temperature mutants have not been plated for CFUs at RT, a direct comparison between their EOP and their ability to increase survival and growth of the *lsp* mutants at RT is not possible. However, the mutants promoting less survival and growth were the ones that had the biggest reduction in EOP at 17°C. However, the two mutant strains (NU350, NU365) in the lipid A biosynthesis acyltransferase gene, that had great reductions in EOP at 17°C, did promote survival and growth of the *lspF*

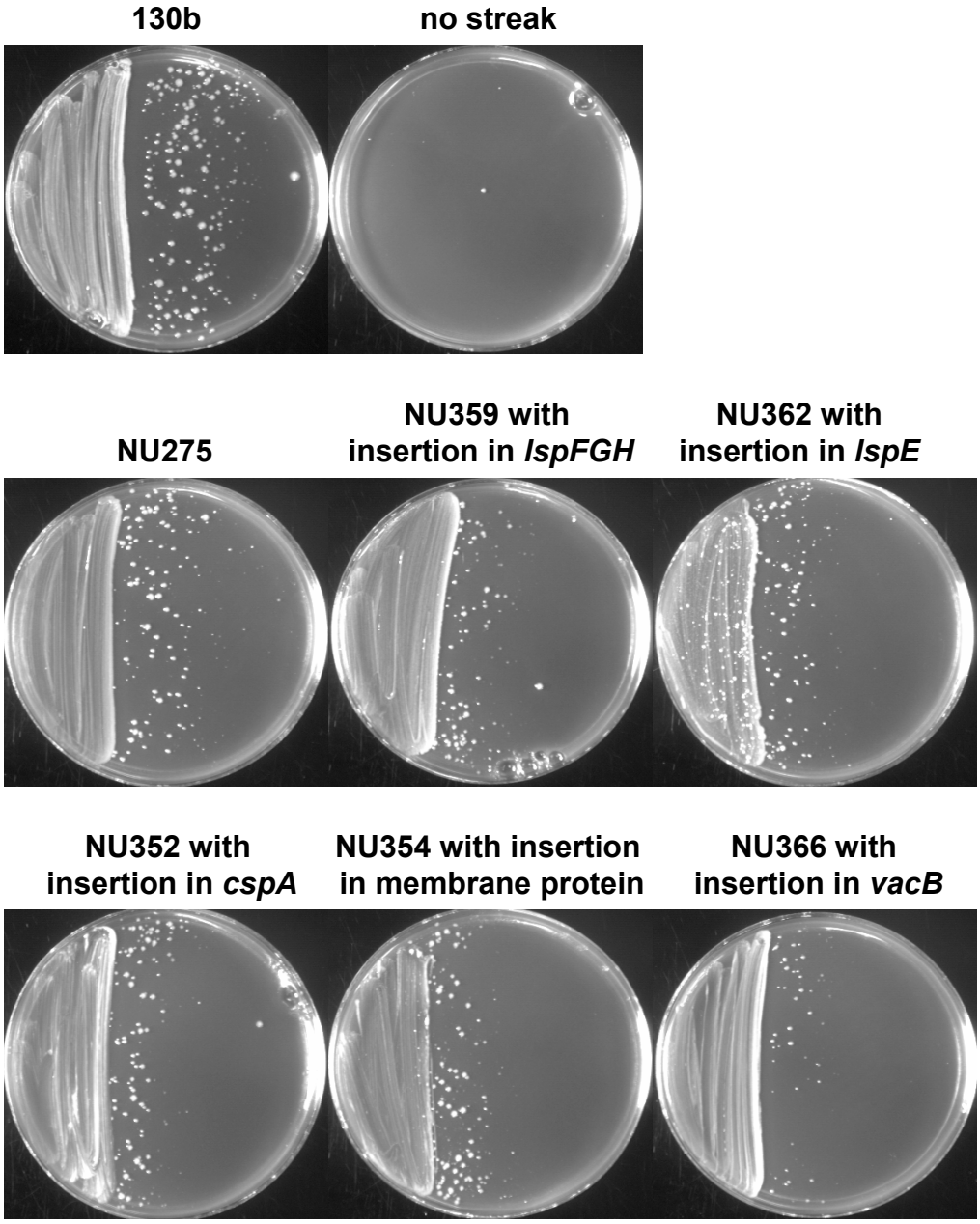

Figure 4-1. **Mutants from the genetic screen with reduced abilities to promote growth and survival of an *lspF* mutant at RT** Approximately 10^4 CFU of *lspF* mutant NU275 were plated for CFU on a series of BCYE agar plates. Some plates were then, as indicated, also inoculated in one sector with streaks of strain 130b, NU275 or mutants from the genetic screen. One set of plates (shown here) was incubated at RT, and another (data not shown) was stored at 37°C. The picture depicts the RT colonial growth of the *lspF* mutant on day 12.

mutant as well as strain 130b did (**data not shown)**. Therefore, there was not a direct correlation between poor EOP and lack of survival and growth promoting abilities.

SUMMARY

This genetic screen did give insight into what is needed for cold adaptation in *L. pneumophila* in that it did identify genes important for low temperature survival and growth. Some of these isolated genes are predicted to encode for proteins with general functions in fatty acid metabolism (3-hydroxyl-CoA dehydrogenase, lipid A biosynthesis acyltransferase, enoyl- CoA hydratase, and 2-methylcitrate synthase), amino acid metabolism (aspartate aminotransferase), general metabolism (citrate synthase) and proteins already known to be important during cold shock (ATP-dependent RNA helicase, exoribonuclease R, and trigger factor). Proteins with general functions in fatty acid, amino acid, and general metabolism have been found in other studies examining proteins important for low temperature growth and survival (147, 183, 230). Other genes might have *L. pneumophila* specific functions; e.g. strain NU358 with insertion in a gene encoding for a L. *pneumophila* specific hypothetical protein.

Unfortunately, this study did not identify any obviously secreted proteins, though it is possible that some of these proteins are secreted through pathways that are not scored for using PSORTb, like type I and type IV secretion. However, there is no evidence in the literature that any of these proteins would be secreted or in any other way be connected to type II secretion.

One reason this screen did not find secreted proteins might be because not enough colonies were screened. Therefore, additional electroporations could be done,

preferably using plasmid pMM237, since then potential mutants could be screened directly for secreted proteins by analyzing the alkaline phosphatase activity in supernatants (155). In the above study, this approach was not fully utilized, since only one gene was isolated with this *phoA* containing transposon; i.e. strain NU350 with insertion in the non-secreted gene encoding for a lipid A biosynthesis acyltransferase. However, it is also possible that if more than one type II dependent factor is needed for low temperature survival and growth, the reduction in EOP associated with the loss of one of those factors might be small. Therefore, these secreted factors might not have been found since this screen might not be sensitive enough to pick up mutants that have less than a 50% reduction in EOP.

Some of the temperature mutant strains promoted survival and growth of the *lspF* mutant less well than strain 130b; i.e., NU352, NU354, and NU366 with insertions in the gene encoding for a ATP dependent helicase, an inner transmembrane protein, and the exoribonuclease R. It is possible that even though these three proteins are not secreted, they might be needed for the synthesis of secreted factor/s responsible for promoting survival and growth at low temperatures. Perhaps, lack of the ATP dependent helicase and the exoribonuclease R that both work at the ribosome prevents these factor/s from being produced in an efficient way. Why an inner membrane transmembrane protein should be important is not clear. However, this mutant has a very weak similarity, 12% similarity, to an inner membrane protein, AsmA, in *E. coli*. This protein might be involved in the assembly of outer membrane proteins since an *asmA* mutant was isolated as an extragenic suppressor of an OmpF assembly mutant. The *asmA* mutant also had reduced levels of LPS, and it was suggested it might be involved in LPS

biogenesis (55). It is therefore possible that the assembly of/secretion through the type II secretion apparatus is decreased in the transmembrane mutant.

In summary, several proteins important for survival and growth of *L. pneumophila* at low temperatures were found, some with EOP as poor as the EOP seen for the *lsp* mutants. Probably, none of these proteins are secreted. However, at least one protein, the inner membrane protein, might be connected to type II secretion since the NU354 mutant had an EOP in the same range as the *lsp* mutants and promoted less survival and growth of the *lspF* mutant in the streak assay.

Chapter 5

IDENTIFICATION OF SECRETED PROTEINS WITH UPREGULATED EXPRESSION AT LOWER TEMPERATURES THROUGH SDS PAGE AND 2D GEL ANALYSIS OF THE 130B STRAIN SUPERNATANTS

INTRODUCTION

Previous trans-complementation experiments with 130b strain streaks and 130b strain low temperature supernatants indicated that a secreted factor/s was needed for low temperature growth. A previous temperature screen only found cell associated proteins important for growth at low temperatures. However, earlier experiments had shown lower tartrate-resistant enzyme activities in RT and 17°C vs. 37°C supernatants, indicating there are differences in secreted proteins at different temperatures. Therefore, a proteomic approach was tried. This approach involved comparing supernatants from 130b grown at 37°C, 17°C and 12°C on SDS PAGE and 2D gels. Protein bands/spots with higher intensities or only present on the gels with the low temperature supernatants were considered candidates. This approach directly targets secreted proteins, but additional work would have to be done to show if any upregulated proteins actually are important for low temperature growth.

RESULTS

To detect differences in secreted proteins at different temperatures, strain 130b and *lspF* mutant supernatants were collected from cultures grown at 37°C, RT, and 17°C. These supernatants were separated on a 10% SDS PAGE gel. When comparing the secreted protein profiles of strain 130b at these temperatures, a few differences could be seen (**Fig. 5-1**). For example, two bands had higher expression in the supernatant from 37°C cultures, while 3 bands could be detected in the supernatants from 17°C cultures but not from those grown at 37°C. Comparison with the secreted proteins from the *lspF* mutant was not possible since the protein patterns from RT and 17°C supernatants contained increased amounts of protein (**Fig. 5-1**). It is not known why this is the case but it is possible the *lspF* mutant, since it is lacking most degradative enzymes, cannot break down all the years extract components of the BYE or perhaps there is some periplasmic leakage occurring at the lower temperatures. This gel showed some promising differences between proteins secreted by the 130b strain at the different temperatures. However, since the 10% gel only resolved proteins bigger than 25kDa and the comparison was done only at one time point along the growth curve, differences in smaller proteins and possible growth phase dependent difference were not examined. Therefore, a new set of 130b strain supernatants was collected from multiple time points along the growth curve from cultures growing at 37°C and 17°C. This time the supernatants were run on a 12% SDS PAGE gel to allow for better separation of smaller proteins (**Fig. 5-2**). Again, several temperature-dependent differences could be detected in the protein secretion profile on the gel. For example,

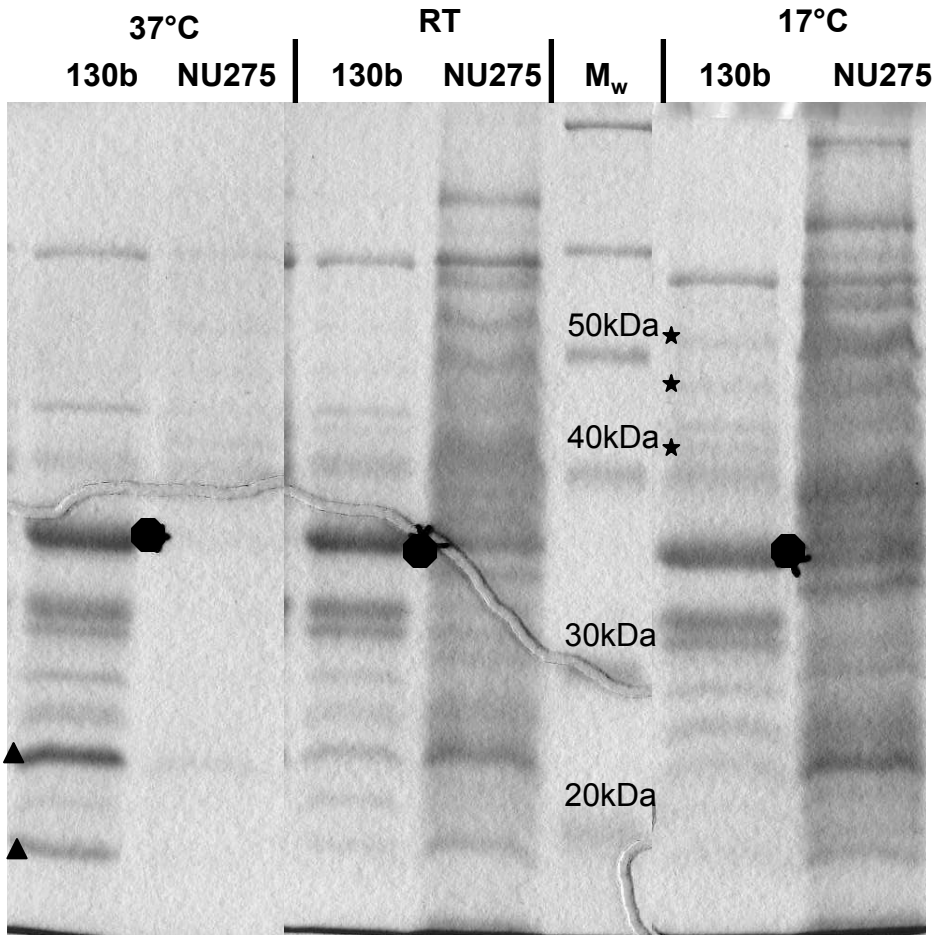

Figure 5-1. SDS PAGE gel comparison of proteins in strain 130b and *lspF* mutant supernatants. Supernatants were collected from strain 130b and *lspF* mutant NU275 grown to late log phase in BYE broth at 37°C, RT and 17°C and separated on a 10% SDS PAGE gel. Protein bands with upregulated expression at 37°C (black triangle) or 17°C (black stars) are marked on the gel. The band containing the most abundant protein the zinc metalloprotease are indicated by the big black circle.

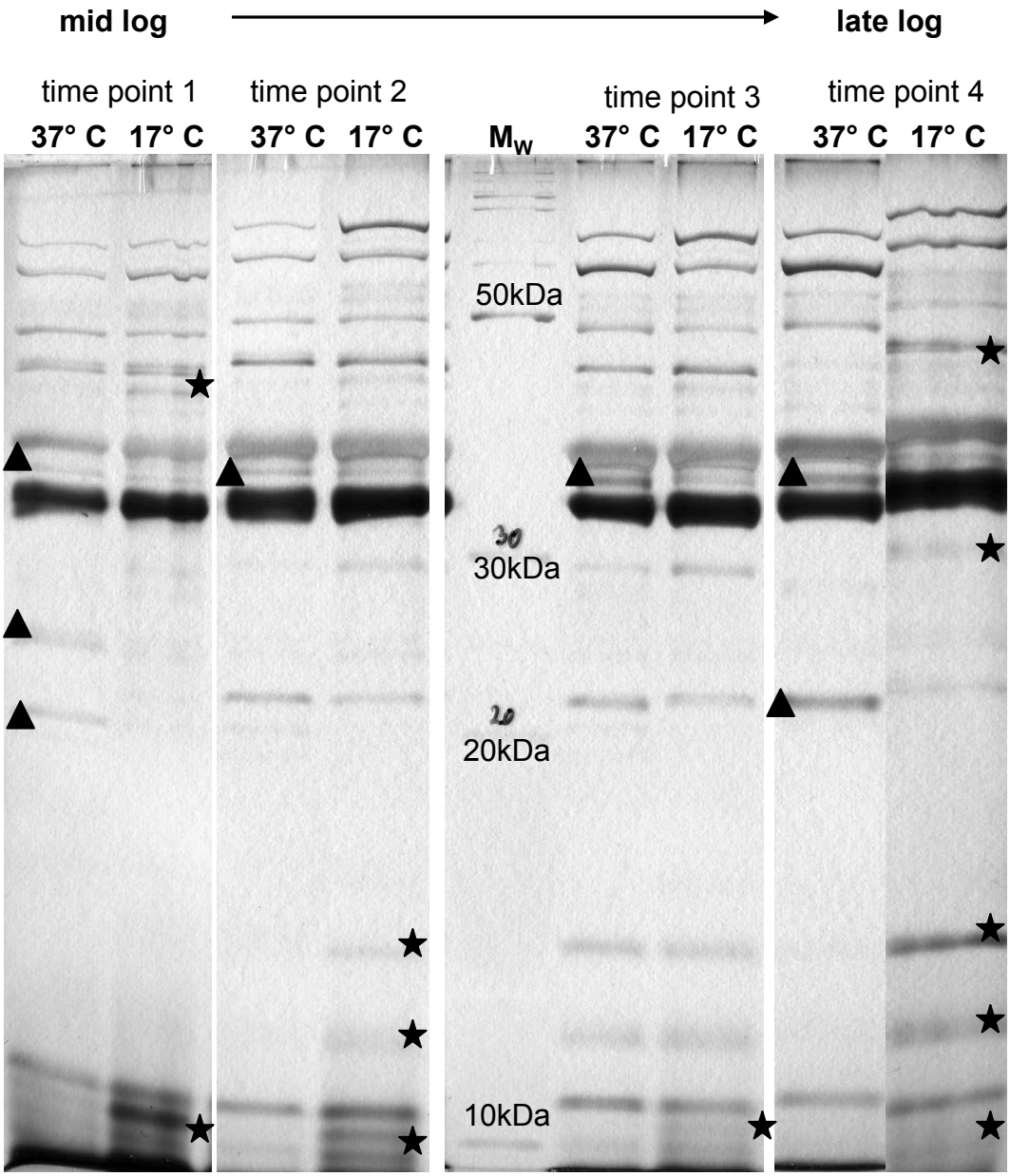

Figure 5-2. SDS PAGE gel comparison of proteins in strain 130b supernatants from 37°C and 17°C cultures. Strain 130b supernatants were collected from four time points, between mid-log to late-log phase, from both 37°C and 17°C cultures. These supernatants were separated on a 12% SDS PAGE gel. Some protein bands with upregulated expression at 37°C (black triangle) or 17°C (black stars) are marked on the gel.

several proteins between 10 and 20 kDa appeared earlier in the 17°C supernatants and with time their intensities grew stronger while at 37°C these bands disappeared.

To be able to better separate the protein bands upregulated at the lower temperature it was decided to separate the proteins using 2D gels. To do this, strain 130b was grown in 300 ml broth at 37°C, 17°C or 12°C (**Fig. 5-3**). 50 ml supernatants were collected at several time points in the late log to early stationary phase. After isoelectric focusing the proteins in the supernatants were resolved on a 12% polyacrylamide SDS gel. Interesting protein spots were excised and submitted for identification to Stanford Mass Spectometry Services (Stanford University, Stanford, CA).

Most protein spots seen on the 37°C gels had previously been identified with Mass Spectrometry (51). From this analysis, only two weak spots had proteins well known for their cytoplasmic location; i.e. aconitate hydratase and DnaK. Most other proteins on the 2D gels had signal peptides and did not have a known intracellular localization. This indicated that the rest of the spots on the 37°C gel were secreted proteins and not there due to lysis of the bacteria. Overall, the 17°C gels looked a lot like the 37°C gels but most protein spots had lower intensities (**Figs. 5-4, 5-5 and 5-6**). Therefore, there was not more lysis going on at the lower temperature.

A few spots on the 37°C temperature gels had higher intensity than on the 17°C gels (**Figs. 5-4, 5-5 or 5-6**). One spot (A) had a much higher intensity on two out of three 2D gels at 37°C (**Figs. 5-5 and 5-6**). The protein in this spot was flagellin, indicating that the bacteria might be less motile or that the flagella might be shed into the medium at the lower temperature. Protein spot B was not present on the 17°C gel.

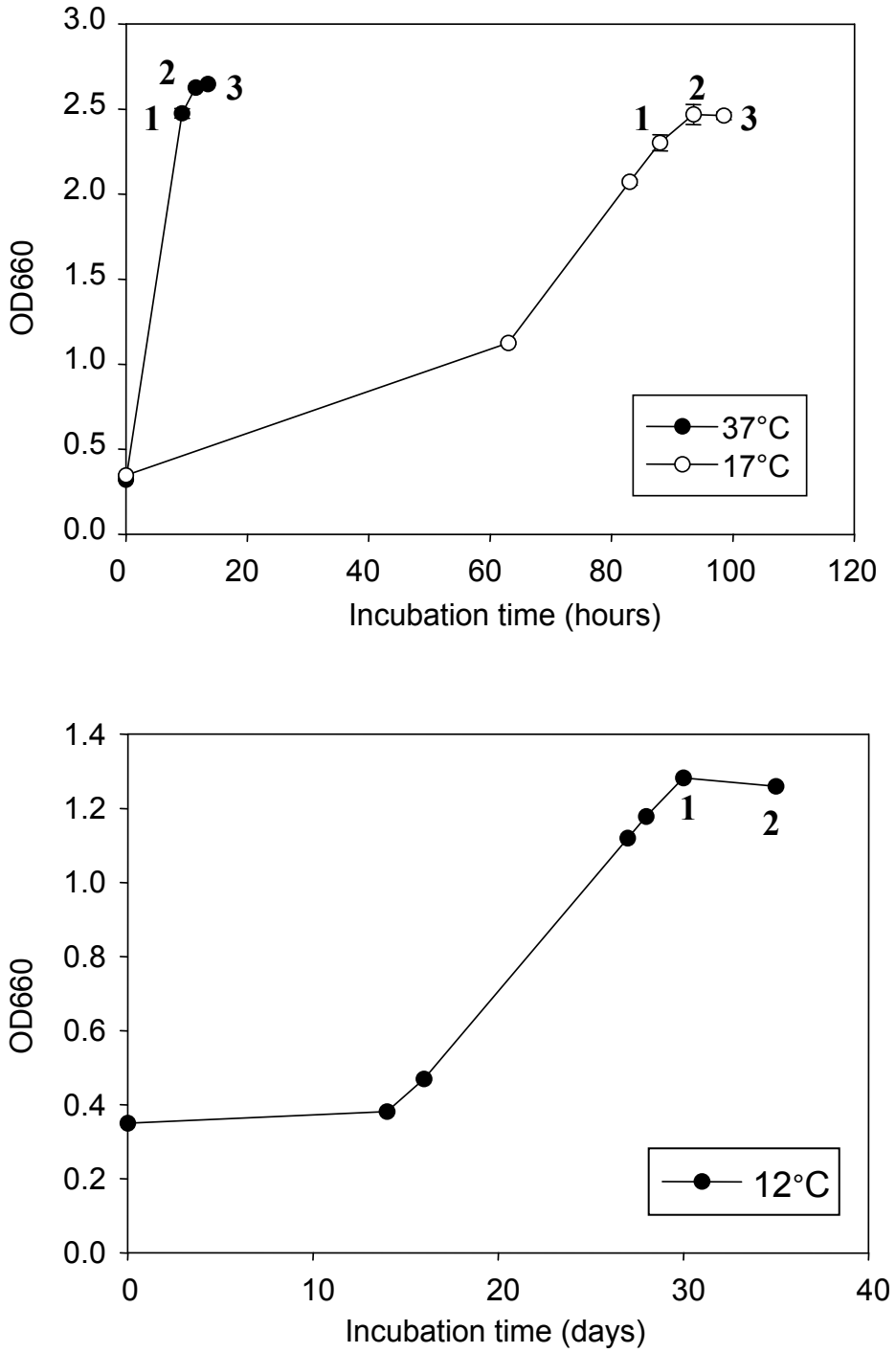

Figure 5-3. Growth curves of strain 130b grown at 37°C, 17°C and 12°C. Strain 130b was grown at 37°C, 17°C and 12°C in 300 ml of BYE and 50 ml of supernatants were collected at time point 1, 2 and 3 for 37°C and 17°C cultures and from time point 1 and 2 for 12°C cultures. The results presented are the means and standard deviations from two samples. Similar growth was seen in at least one more experiment.

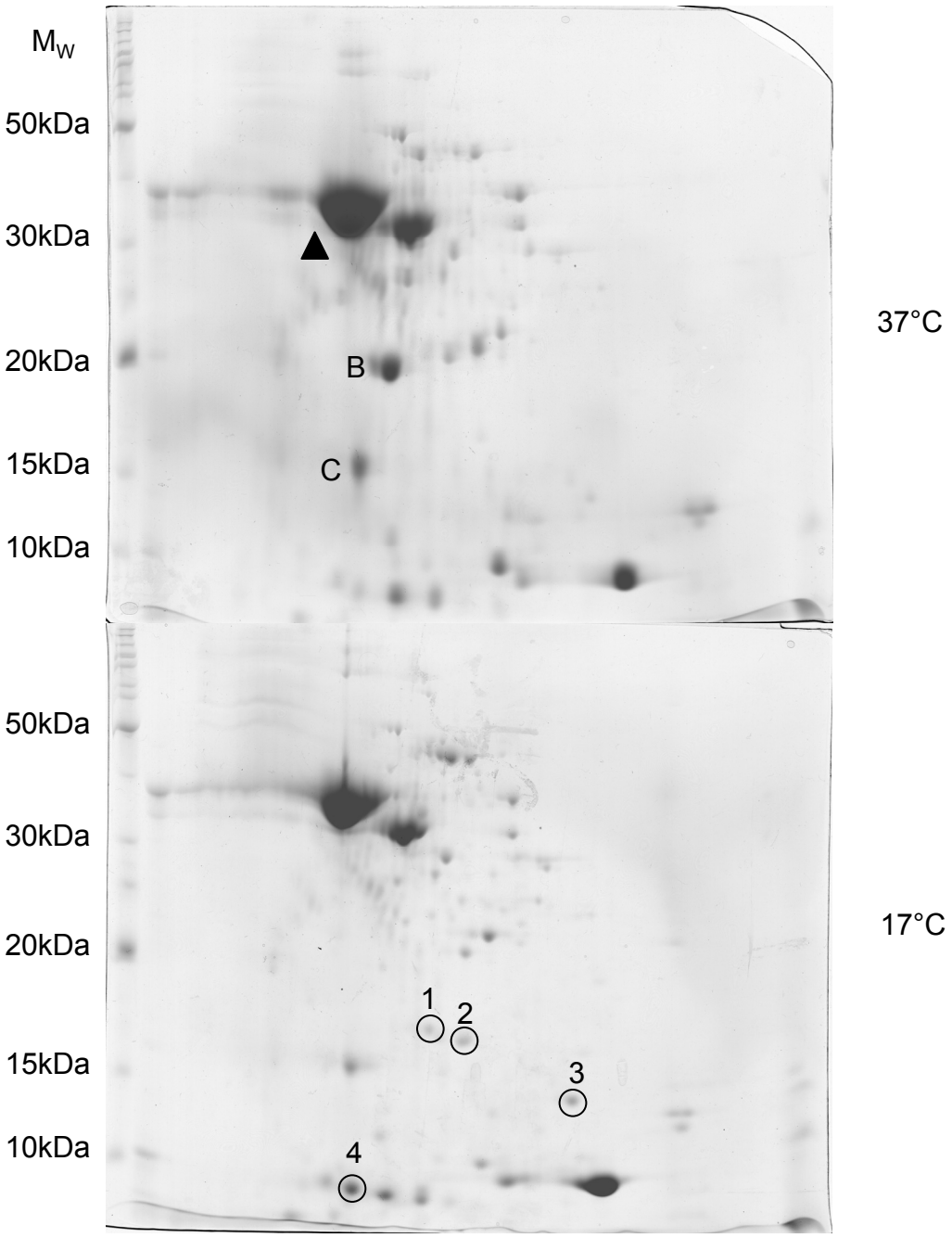

Figure 5-4. 2D gel analysis of strain 130b supernatants collected at time point 1 from cultures grown at 37°C and 17°C. Strain 130b supernatants collected at time point 1 from cultures grown at 37°C and 17°C were concentrated, focused and separated on 2D gels. Examples of proteins only present or with an upregulated expression on the 37°C gel are indicated with the letters B and C. Proteins only present or upregulated at 17°C are marked with the numbers 1, 2, 3, and 4. The spot containing the most abundant protein, the zinc metalloprotease is indicated with a black triangle. This result is representative of two independent experiments.

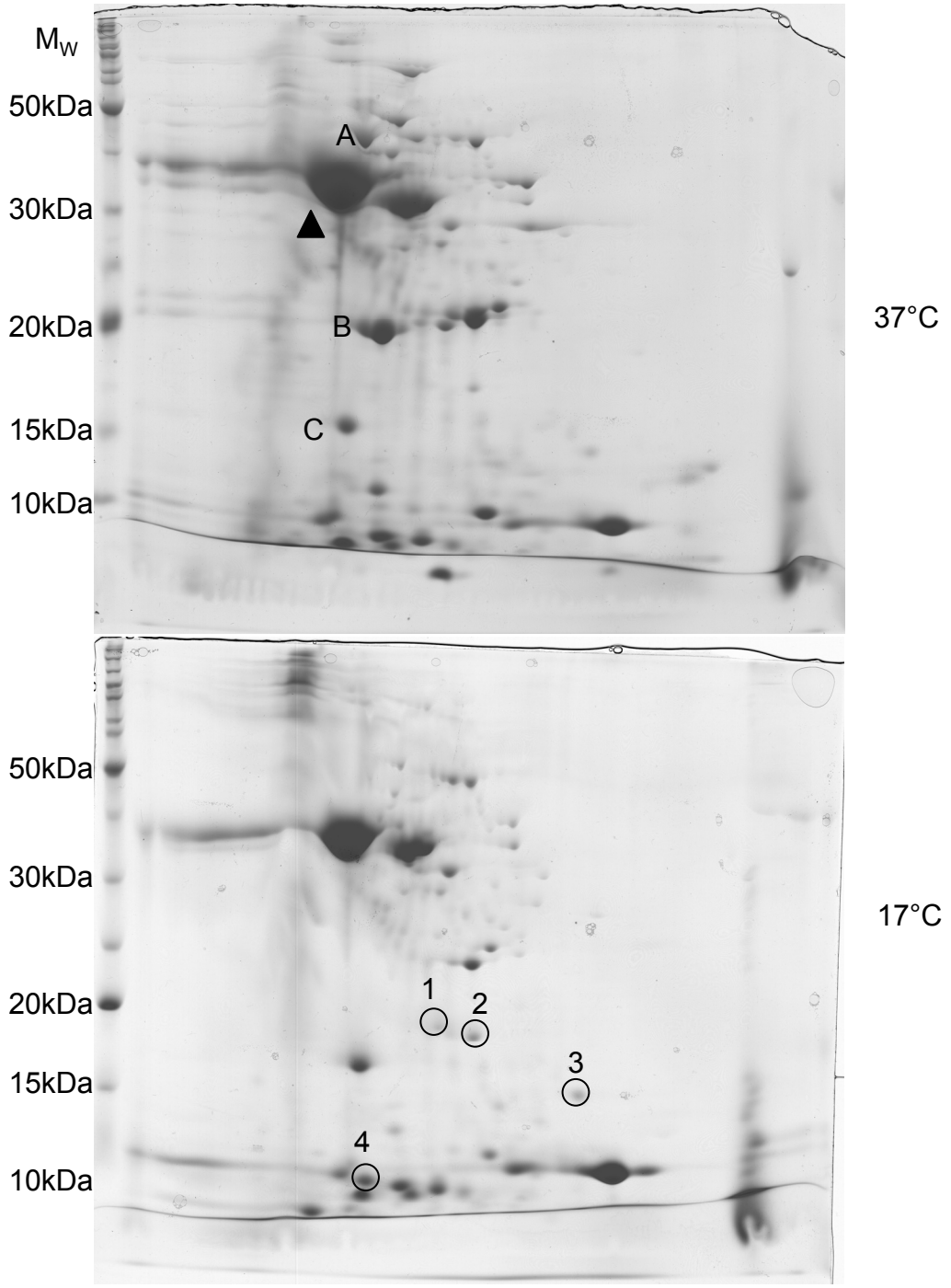

Figure 5-5. 2D gel analysis of strain 130b supernatants collected at time point 2 from cultures grown at 37°C and 17°C. Strain 130b supernatants collected at time point 2 from cultures grown at 37°C and 17°C were concentrated, focused and separated on 2D gels. Proteins only present or with an upregulated expression on the 37°C gel are indicated with the letters A, B and C. Examples of proteins only present or upregulated at 17°C are marked with the numbers 1, 2, 3, and 4. The spot containing the most abundant protein, the zinc metalloprotease is indicated with a black triangle. This result is representative of two independent experiments.

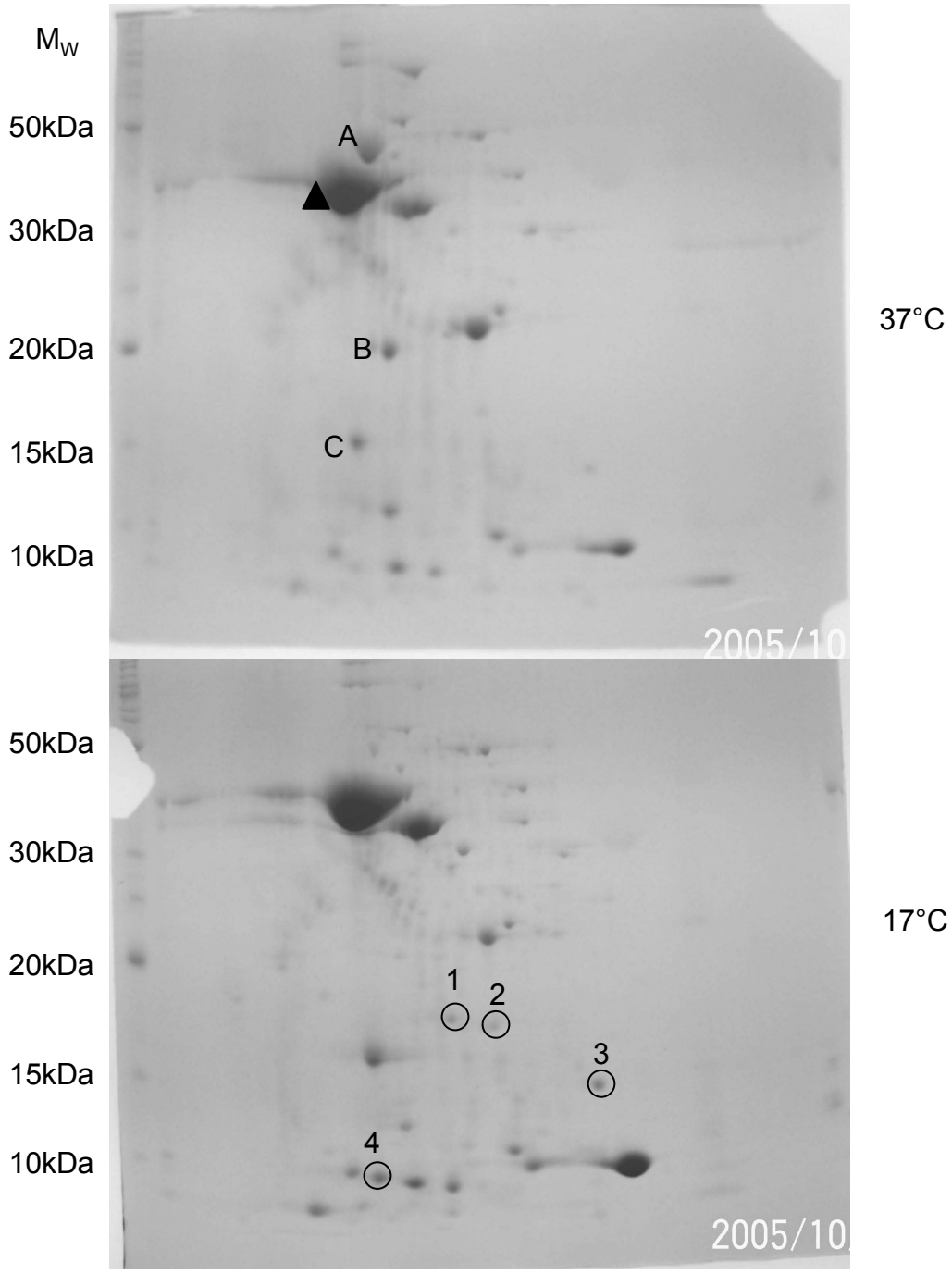

Figure 5-6. 2D gel analysis of strain 130b supernatants collected at time point 3 from cultures grown at 37°C and 17°C. Strain 130b supernatants collected at time point 3 from cultures grown at 37°C and 17°C were concentrated, focused and separated on 2D gels. Proteins only present or with an upregulated expression on the 37°C gel are indicated with the letters A, B and C. Examples of proteins only present or upregulated at 17°C are marked with the numbers 1, 2, 3, and 4. The spot containing the most abundant protein, the zinc metalloprotease is indicated with a black triangle. This result is representative of two independent experiments.

The protein in this spot was LvrE, encoded by a gene close to the *lvh* type IV secretion system operon in the *L*. *pneumophila* genome, but with unknown function (210). However, the protein in spot C was also LvrE. This is not strange since proteins often exist in multiple forms due to phosphorylation, glycosylation and/or limited proteolysis of the proteins. These data indicate that LvrE is only present in one of these forms at 17°C.

The same volume of supernatants was run for both the 37°C, 17°C and 12°C gels but since the 130b strain grew to a lower OD at 12°C than at 17°C (**Fig. 5-3**) the 12°C gel should have less intense protein spots than the 17°C gels. Comparing the gels in **Figs. 5-4, 5-5 and 5-6** and **Fig. 5-7,** this is clearly the case. However, when the spot for the zinc metalloprotease (triangle) is compared on the different gels its intensity on the 12°C gel is less than half of the intensity on the 17°C gel. Therefore, the amounts of secreted proteins are lower at 12°C than at 17°C and 37°C. This reduction in the amount of secreted proteins is probably a reflection of the slower growth.

More importantly, 4 spots had higher intensities at both 17°C and 12°C at all time points as compared to 37°C (**Figs. 5-4, 5-5, 5-6** and **Fig. 5-7**). Two of these spots (2 and 3) could be seen on the 37°C gels but had lower intensities while the other spots (1 and 4) could only be seen at 17°C and 12°C. These four spots were cut out of the 17°C gel and sent for Mass Spectometry analysis (**Table 9**). The protein in spot 3 was identified as peptidoglycan associated lipoprotein (PAL). PAL is a lipoprotein attached to the inner leaflet of the outer membrane, where it interacts with peptidoglycan. The fact this protein is found on the gel might indicate some level of periplasmic leakage, though PAL can be shed from bacteria, at least in a septic animal (142). In addition,

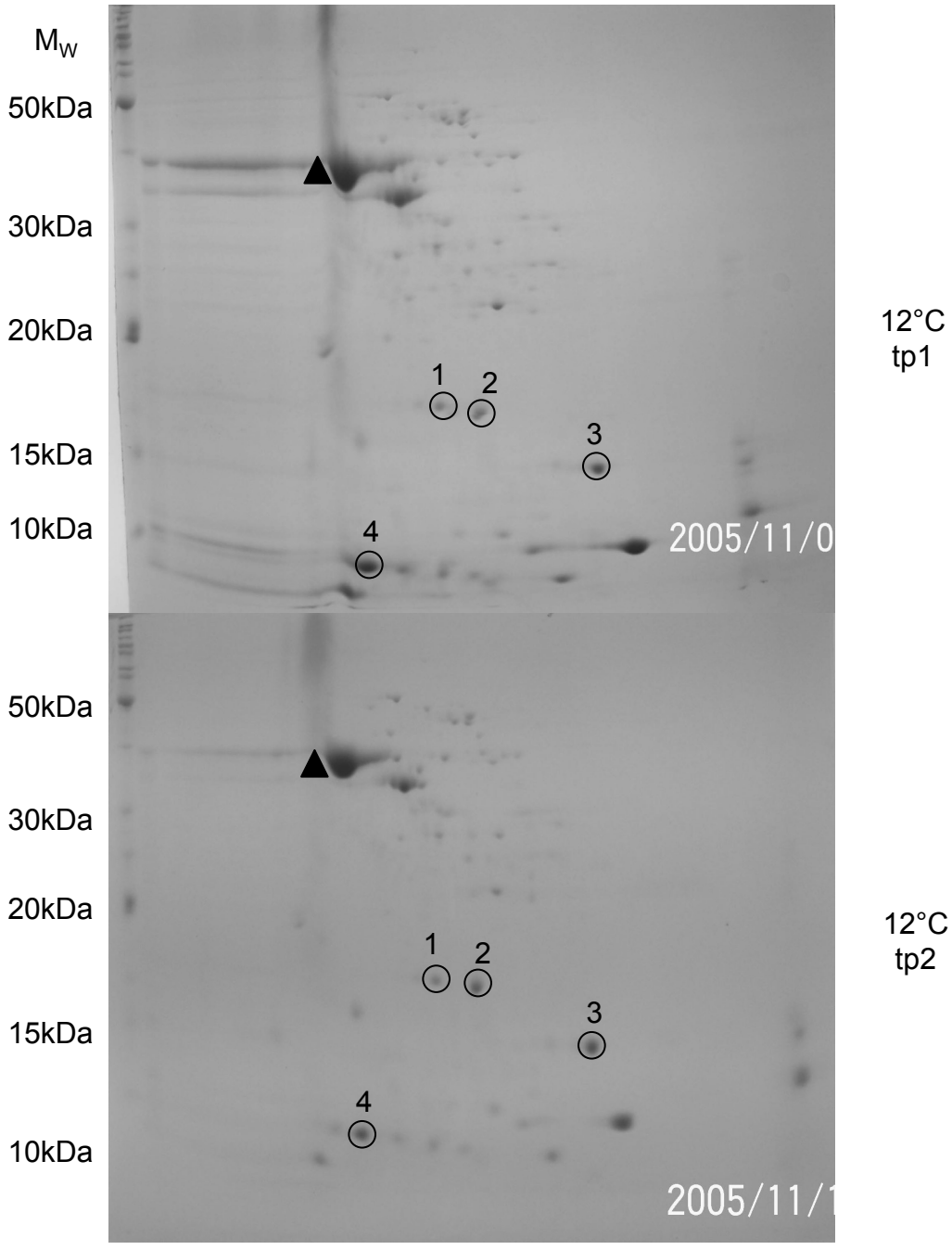

Figure 5-7. 2D gel analysis of strain 130b supernatants collected at time point 1 and 2 from cultures grown at 12°C. Strain 130b supernatants collected at time point 1 and 2 from cultures grown at 12°C were concentrated, focused and separated on 2D gels. Proteins only present or upregulated at 12-17°C are marked with the numbers 1, 2, 3, and 4. The spot containing the most abundant protein, the zinc metalloprotease is indicated with a black triangle.

Table 9. Results from mass spectrometry analysis of proteins in spots 1, 2, 3 and 4

Spot	Protein identity	Homology to gene	Score	% of protein covered	Calculated Mw	Size on Gel	Calculated PI	PI on gel	SignalP
1	PpiB	*lpg2726*	383	48	18	~19	5.3	~6	No
2	Lpg1962	*lpg1962*	163	26	20	~18	6.7	~6.5	Yes
3	peptidoglycan associated lipoprotein	*lpg2043*	295	23	19	~15	7.5	~7.5	Yes
4	hypothetical protein	*lpg0873*	119	20	16	~11	6.0	~5.5	Yes
4	hypothetical protein	*lpg1809*	95	29	14	~11	7.8	~5.5	Yes
4	hypothetical protein	*lpg0374*	78	25	15	~11	5.4	~5.5	Yes

peptidoglycan in some bacteria can be secreted and perhaps PAL since it interacts with peptidoglycan can be secreted too (42). Since the size of this protein on the 2D gel is smaller than the predicted size 15 vs 19 kDa, it is possible that this protein exists in two forms, one larger membrane attached form and one smaller secreted form.

The protein in spot 4 was actually several short proteins with signal peptides. All these proteins had a predicted size range of approximately 14-16 kDa that was slightly bigger than the 11-12 kDa seen on the gel. But since these are short proteins with only 128-141 amino acids, if the size of the cleavable signal peptide is subtracted, the size of these proteins is actually roughly 12 kDa. This is in agreement with the size seen on the gels. The recalculated pI values of these proteins with the signal peptides subtracted is in the range of 5-7, which is also in the same range as the pI of 5.5 for the spot on the gel. Therefore, it appears that all of these proteins are present in this spot. Of these proteins, the one with the highest score had homology to Lpg0873. This protein only had similarity to other short proteins in *L*. *pneumophila* strain Philadelphia, Lens and Paris (gene *lpg0035*, *lpl0035* and *lpp0034*, respectively). The other two proteins had homology to Lpg0374 and Lpg1809, respectively, but had no similarity to any other genes. None of these proteins have any predicted function, but since they are upregulated in the cold and only present in the *L*. *pneumophila* genome they might represent novel proteins with important species specific functions during cold adaptation.

The proteins in spot 1 and 2 were identified as two peptidyl prolyl cis/trans isomerases (ppiases) encoded by *lpg2726* and *lpg1962*, respectively. The ppiase with homology to Lpg1962 was a 188 amino acid long ppiase not previously described in the

Legionella literature. Since this protein had a signal peptide it could be secreted through the type II secretion system. The whole protein was predicted to only have a cyclophilin (ppiase) domain and the gene was not in an operon.

The ppiase with homology to Lpg2726 was a 164 amino acid protein that only had a one predicted domain, the cyclophilin domain. This gene was 26 base pairs downstream of a queuine tRNA-ribosyltransferase gene but probably has its own promoter based on neural network promoter predictions at http://www.fruitfly.org/seq_tools/promoter.html. If not, it would be the last gene in a possible operon with cytochrome C5, disulfide oxidoreductase, a hypothetical protein and queuine tRNA-ribosyltransferase. This gene had previously been named *lcy* for legionella cyclophilin (206). However, since this gene is annotated in the *L. pneumophila* genome as *ppiB*, it will be referred to as such. In this previous paper, PpiB was confirmed to have ppiase activity, but since activity was only tested from cell lysates of bacteria grown on plates a possible secreted role was not assessed. PpiB does not have a signal peptide predicted by psort, signalP or psortB, but the spot is repeatedly found on the low temperature gels at several time points in repeat experiments **Figs. 5-4, 5-5, 5-6** and **Fig. 5-7**). How PpiB is secreted is presently not known but it is not secreted by the *icm*/*dot* or *lvh* type IV secretion systems since the PpiB spot can be seen on 2D gels using supernatants from *dot* and *lvh* mutants (**Fig. 5-8**). However, since ppiases function by folding or regulating the activity of other proteins PpiB could very well be secreted by some other secretion pathway and still act on type II exoproteins important for low temperature growth.

One way to determine whether PpiB or Lpg1962 are really secreted by the type II secretion system would have been to make the comparison between strain 130b and

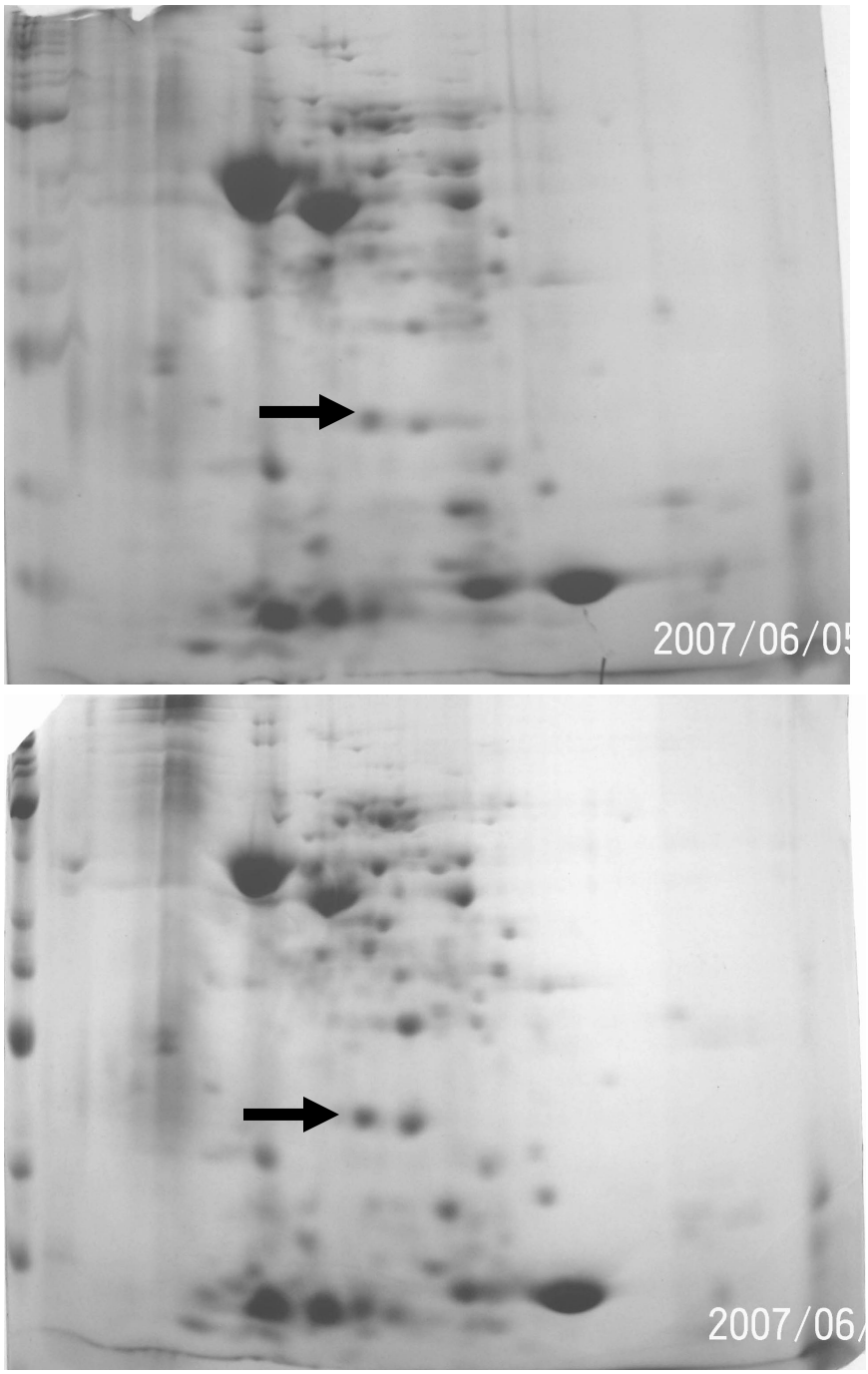

Figure 5-8. 2D gel analysis of *dotG* and *lvh* mutant supernatants from cultures grown at 17°C. The *dotG* mutant AA405 and the *lvh* mutant AA474 were grown at 17°C and supernatants collected from late log phase were concentrated, focused and separated on 2D gels. The protein spot containing PpiB is marked with an arrow.

lspF mutant supernatants grown at 37°C and 17°C. However, this kind of comparison could not be done since the *lspF* mutant has some degree of periplasmic leakage at 17°C (**Fig. 5-9**). A comparison with 12°C supernatants is not possible since the *lsp* mutants will not grow at this temperature. Spot comparison of the 130b and *lspF* mutant gels at 17°C, show roughly double the number of spots on the *lspF* gel. In comparison, an *lspF* gel with supernatants from 37°C culture is almost completely devoid of any proteins indicating most proteins are secreted in a type II dependent way (51). In addition, many of the spots on the *lspF* gel corresponded to spots on the 130b strain 17°C gel but had much lower intensities (**arrows on Fig. 5-9**). However, the number of spots was fewer than the 150 protein spots seen when *L*. *pneumophila* cell lysates were run on 2D gels (140). In conclusion, this indicates the *lspF* mutant had some degree of periplasmic leakage during growth at 17°C. This is not surprising considering the growth defect of the *lspF* mutant in BYE broth at 17°C. Similar results were seen at several different time points along the growth curve in duplicate experiments.

SUMMARY

Several differences in secreted proteins could be seen in supernatants from the 130b strain from different temperatures both on SDS PAGE and 2D gels. The 2D gel analysis identified several short hypothetical proteins and two ppiases that are secreted. Since the short hypothetical proteins are *L*. *pneumophila* specific and have a higher expression at low temperatures, they were considered to be very interesting but nothing is known about their possible functions in cold adaptation. In contrast, cell associated

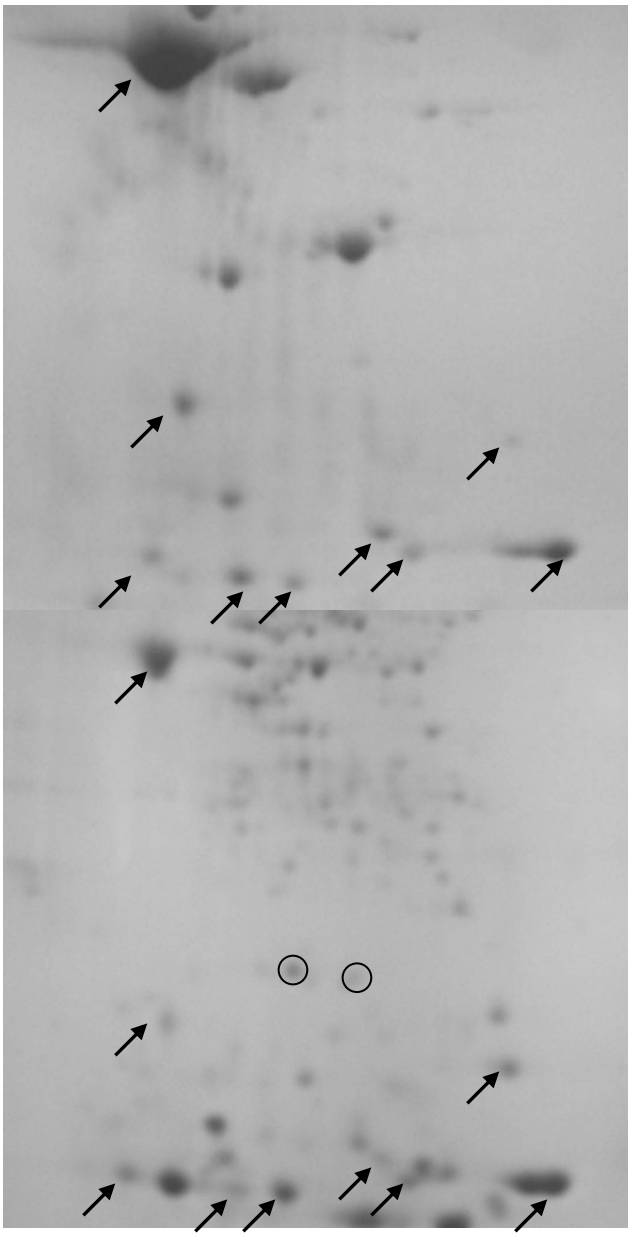

Figure 5-9. 2D gel analysis of strain 130b and *lspF* mutant supernatants from cultures grown at 17°C. Strain 130b and *lspF* mutant NU275 were grown at 17°C and supernatants collected from late log phase were concentrated, focused and separated on 2D gels. Protein spots present on both the strain 130b and the *lspF* mutant gels are indicated with arrows. The location of the two ppiases, PpiB and Lpg 1962, are circled. This result is representative of two independent experiments.

ppiases have previously been shown to be upregulated during cold exposure; e.g. RotA in *E. chrysanthemi* has a 3-fold increase in expression at 25°C vs. 37°C (184), trigger factor and PpiA are upregulated in *E. coli* after cold shock (124, 183), a FKBP ppiase is induced in *Thermococcus* after cold shock (116), PpiB has a higher level in *B. subtilis* at 15°C vs. 37°C (86), and a FKBP ppiase in the psychrotrophic bacterium *Shewanella* sp. SIB1 is increased at 4°C vs. 20°C (225). However, finding a secreted ppiase with an important function in low temperature growth would be a novel finding. Therefore, it was decided to pursue PpiB and Lpg1962 and their role in low temperature growth.

Chapter 6

SECRETED PPIASES IN *L. PNEUMOPHILA* ARE IMPORTANT FOR LOW TEMPERATURE GROWTH AND INFECTION IN THE AMOEBAE *ACANTHAMOEBA CASTELLANII*

INTRODUCTION

From the 2D gel analysis (**Chapter 5**) the spots containing the two ppiases, PPiB and Lpg1962, had higher intensities on the 12 and 17°C gels. Since finding a ppiase that is both secreted and important for low temperature growth would be a novel finding, the two ppiases were mutated and their ability to grow at lower temperatures were tested.

RESULTS

To be able to test the ppiases for a potential role in survival and growth at lower temperatures, independent *ppiB* (NU340, NU341) and *lpg1962* (NU342, NU343) mutants with both Km^R and Gm^R insertions were constructed. In addition, several independent *lpg1962*: *ppiB* (NU344, NU345), *ppiB*: *lspF* (NU346, NU347) and *lpg1962*: *lspF* (NU348, NU359) double mutants were created. To establish a role for the *lpg1962* and *ppiB* mutants in low temperature growth, we assessed their growth at 37°C and 17°C. In the initial experiment, strain 130b and *lpg1962* mutant were grown in BYE broth at 37°C and 17°C. This experiment showed that the *lpg1962* mutant did not have any growth defect at either temperature (**Fig. 6-1, Fig. 6-2 and Fig. 6-6**). This result

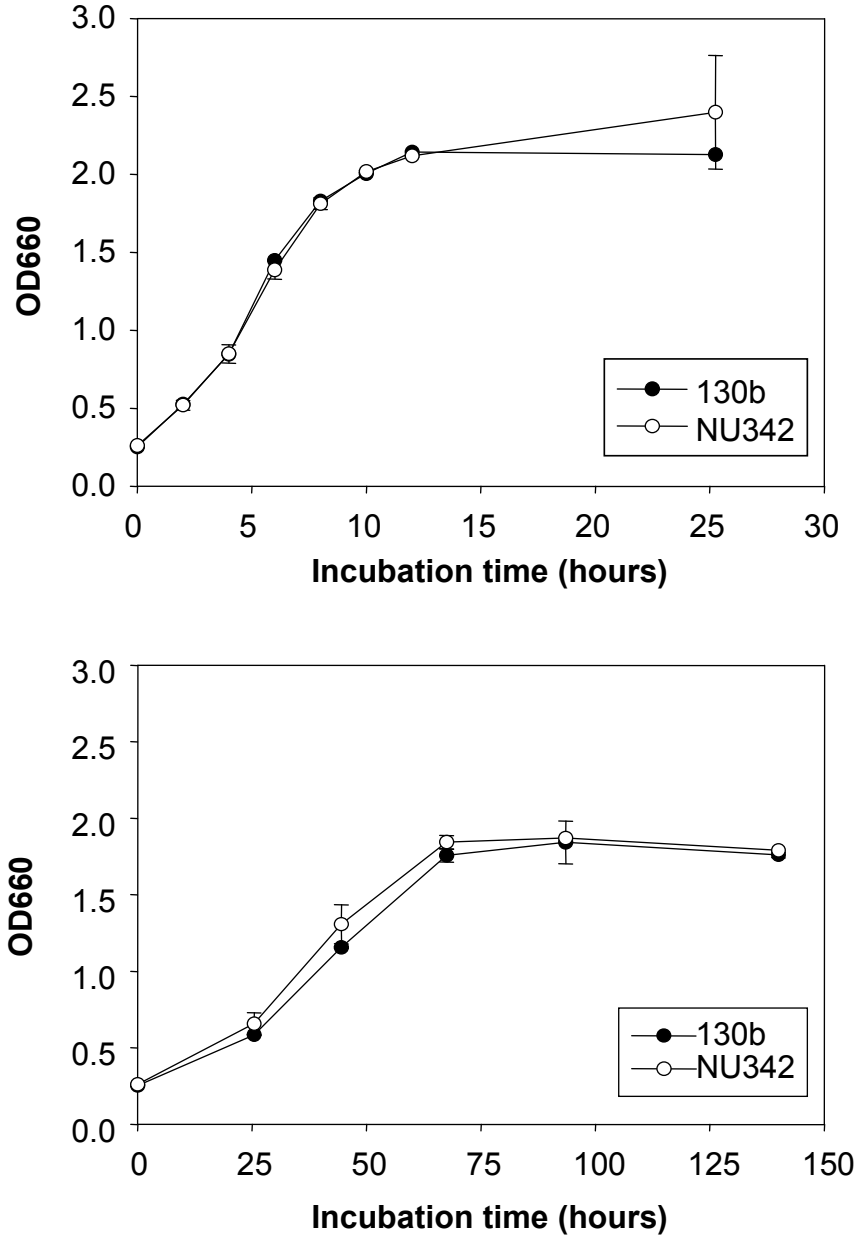

Figure 6-1. Growth of strain 130b and a *lpg1962* mutant at 37°C and 17°C in BYE broth. Log-phase strain 130b and *lpg1962* mutant NU342 bacteria were inoculated into BYE broth and then incubated at 37°C (top panel) and 17°C (bottom panel). The growth of the cultures was monitored by recording the optical density of the cultures at various times. The results presented are the means and standard deviations from duplicate cultures and are representative of at least four independent experiments.

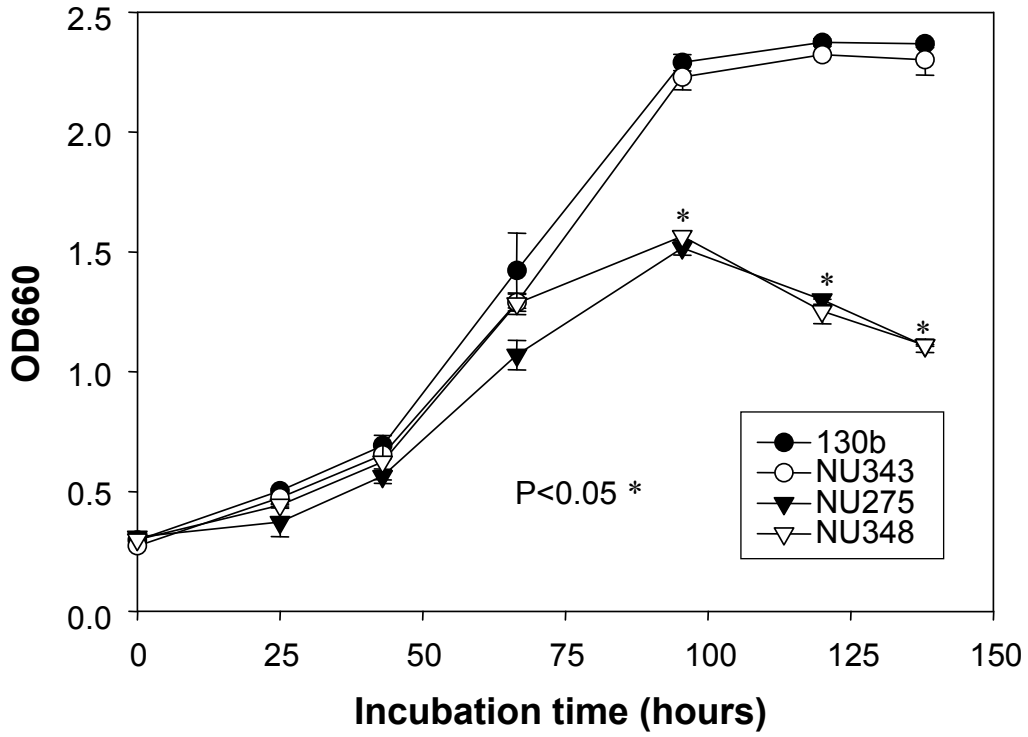

Figure 6-2. Growth of strain 130b, *lpg1962* mutant NU343, *lspF* mutant NU275 and *lpg1962*:*lspF* mutant NU348 at 17°C in BYE broth. Log-phase strain 130b and mutant bacteria were inoculated into BYE broth and then incubated at 17°C. The growth of the cultures was monitored by recording the optical density of the cultures at various times. The results presented are the means and standard deviations from duplicate cultures and are representative of at least four independent experiments.

was confirmed with two independent mutants in an additional three experiments. Next the growth of an *lpg1962*::*lspF* double mutant was compared to the growth of the *lspF* mutant at 37°C and 17°C (**data not shown**, **Fig. 6-2**). The *lpg1962*::*lspF* mutant grew like *lspF* mutant at both temperatures, which is what would be expected since the *lpg1962* single mutant was not defective for growth at 17°C.

To assess the importance of PpiB in low temperature growth, the 130b strain and the *ppiB* mutant were grown in BYE broth at 37°C and 17°C. The *ppiB* mutant grew like the parental strain at 37°C, but at 17°C the mutant reached stationary phase on average 26±4 hours after the parental strain (**Fig. 6-3, Fig. 6-4, Fig. 6-5 and Fig. 6-6**). The *ppiB* mutant sometimes grew to a slighter lower OD660 than the parental strain (**Fig. 6-6**). This growth defect was seen with two independent mutants in another four experiments. This growth defect was not due to a second site mutation, since *ppiB* mutant containing the complementing plasmid pMB3 grew like the parental strain under these same growth conditions (**Fig. 6-4**). Next, the *ppiB*:*lspF* double mutant and the *lspF* mutants were grown at 37°C and 17°C (**Fig. 6-5**). The double mutant grew like the *lsp* mutant at both temperatures in two independent experiments. The fact the double mutant was not any more defective than the *lspF* mutant is consistent with the idea that PpiB functions by regulating the activity of type II exoproteins.

Finally**,** the growth of a *ppiB*:*lpg1962* double mutant was compared to the growth of the *ppiB* mutant, the *lpg1962* mutant and strain 130b at 37°C and 17°C (**data not shown, Fig. 6-6**). The *ppiB*:*lpg1962* mutant grew like the *ppiB* mutant. This result was seen with two independent mutants in two different experiments. Therefore, Lpg1962

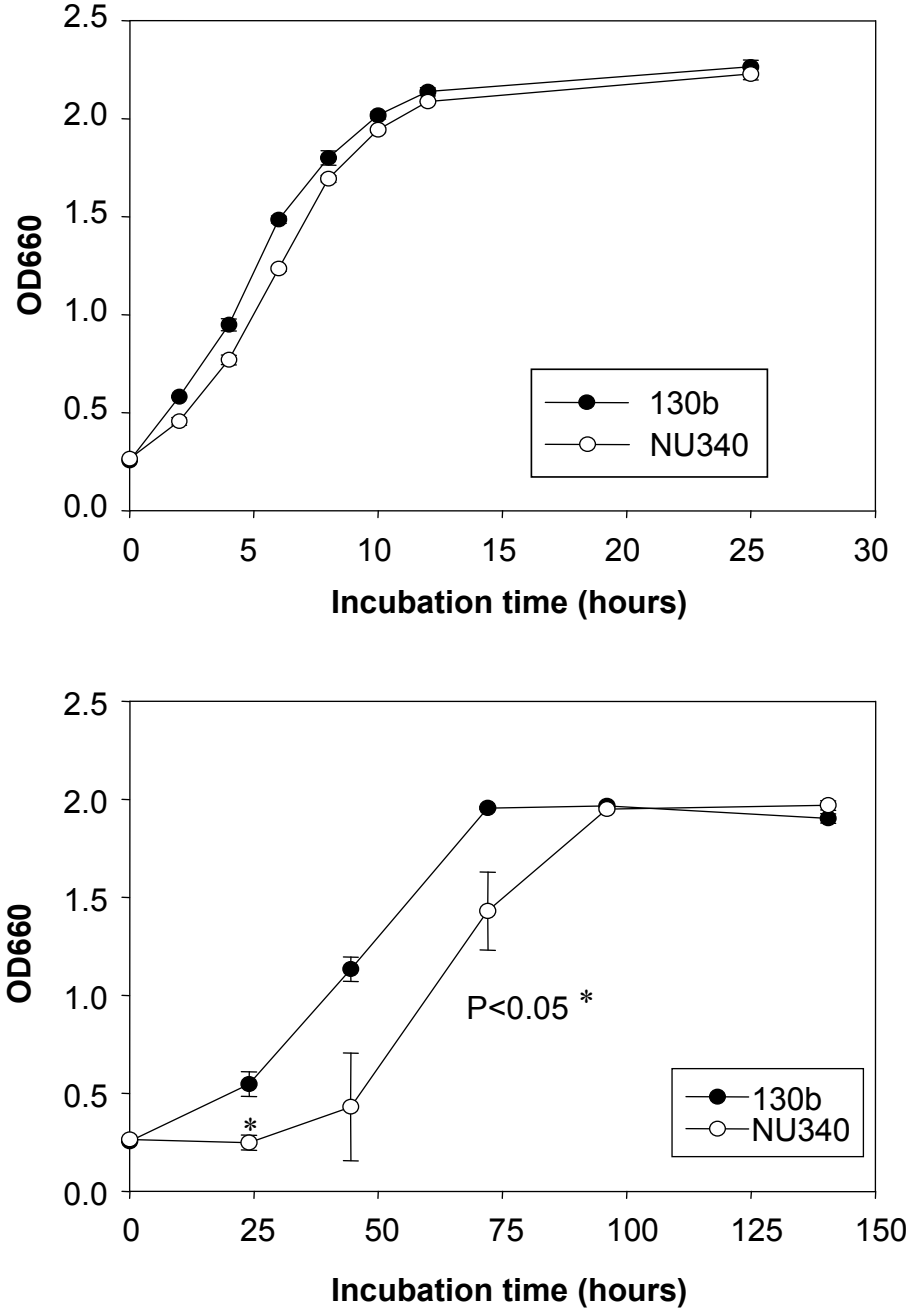

Figure 6-3. Growth of strain 130b and an *ppiB* mutant at 37°C and 17°C in BYE broth. Log-phase strain 130b and *ppiB* mutant NU340 bacteria were inoculated into BYE broth and then incubated at 37°C (top panel) and 17°C (bottom panel). The growth of the cultures was monitored by recording the optical density of the cultures at various times. The results presented are the means and standard deviations from duplicate cultures and are representative of at least five independent experiments.

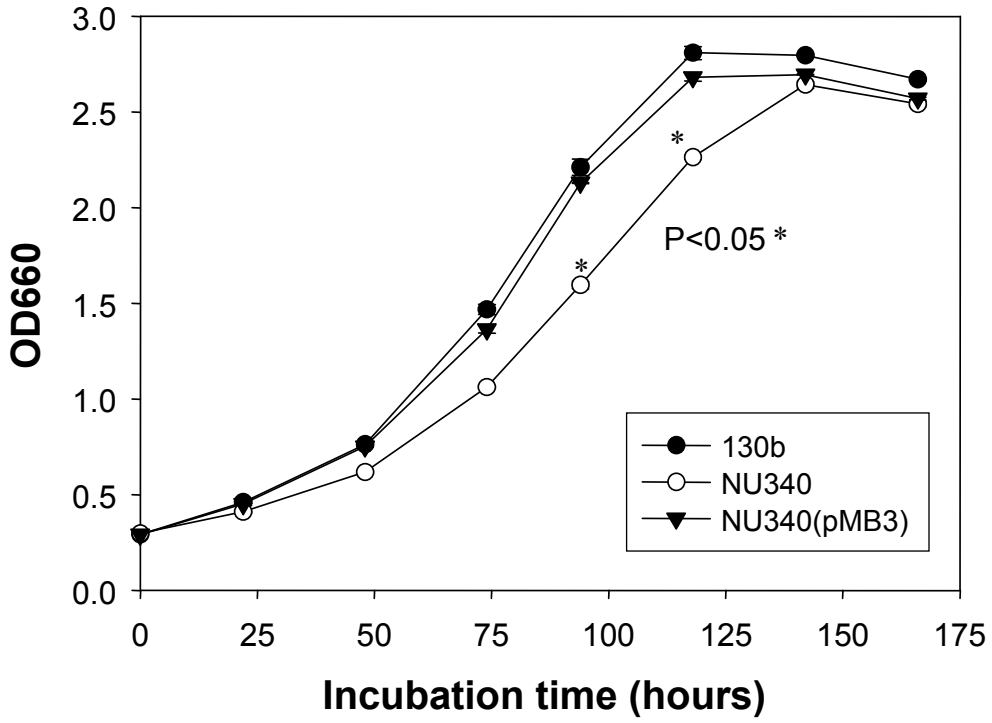

Figure 6-4. Growth of *ppiB* and a complemented *ppiB* mutant at 17°C in BYE broth. Log-phase strain 130b, *ppiB* mutant NU340 and *ppiB* mutant NU340 with the complementing plasmid pMB3 were inoculated into BYE broth and then incubated 17°C. The growth of the cultures was monitored by recording the optical density of the cultures at various times. The results presented are the means and standard deviations from duplicate cultures and are representative of two independent experiments.

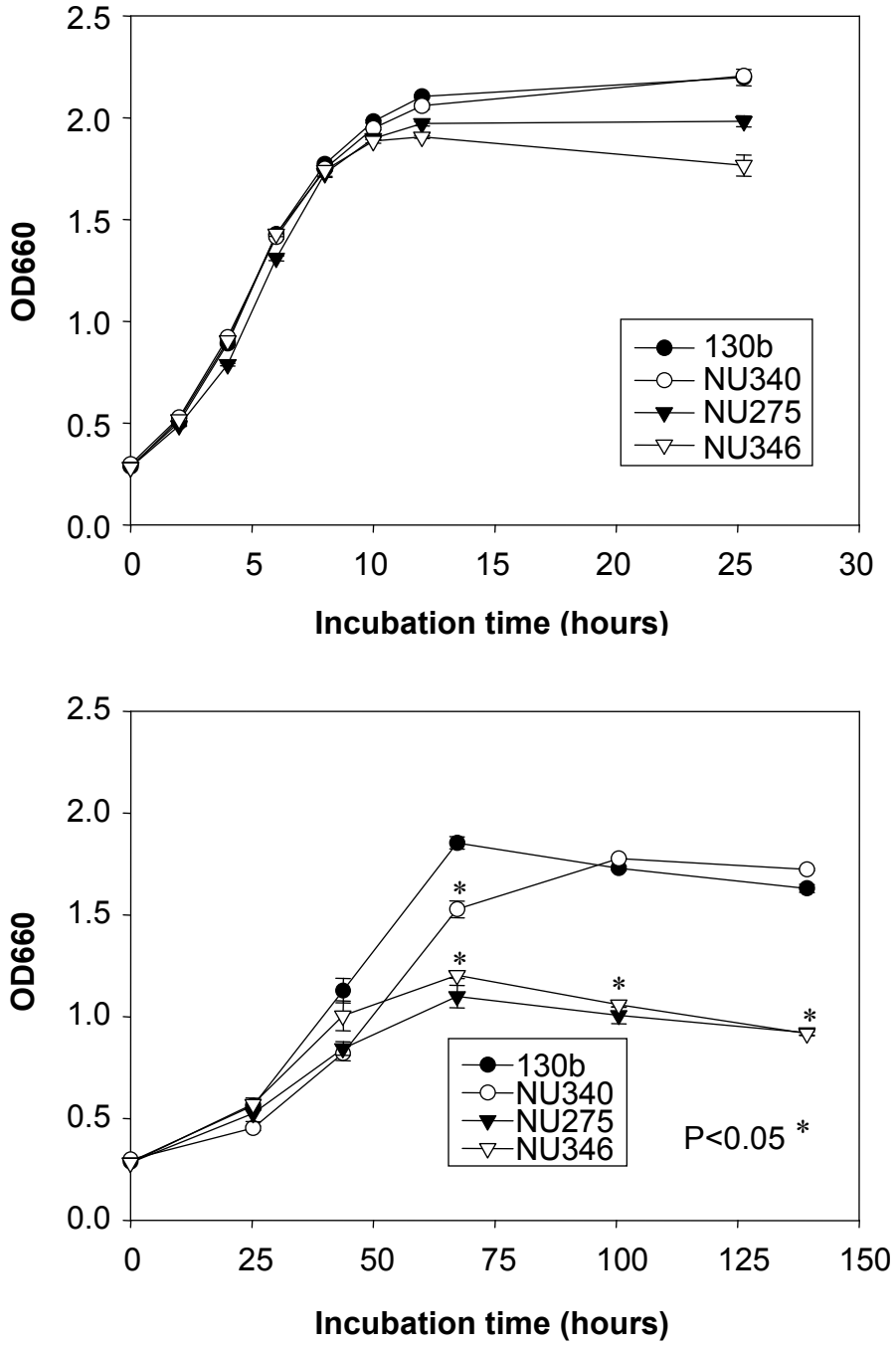

Figure 6-5. Growth of strain 130b and *ppiB* mutant NU340, *lspF* mutant NU275 and *ppiB:lspF* mutant NU346 at 37°C and 17°C in BYE broth. Log-phase strain 130b and mutant bacteria were inoculated into BYE broth and then incubated at 37°C (top panel) and 17°C (bottom panel). The growth of the cultures was monitored by recording the optical density of the cultures at various times. The results presented are the means and standard deviations from duplicate cultures and are representative of at least three independent experiments.

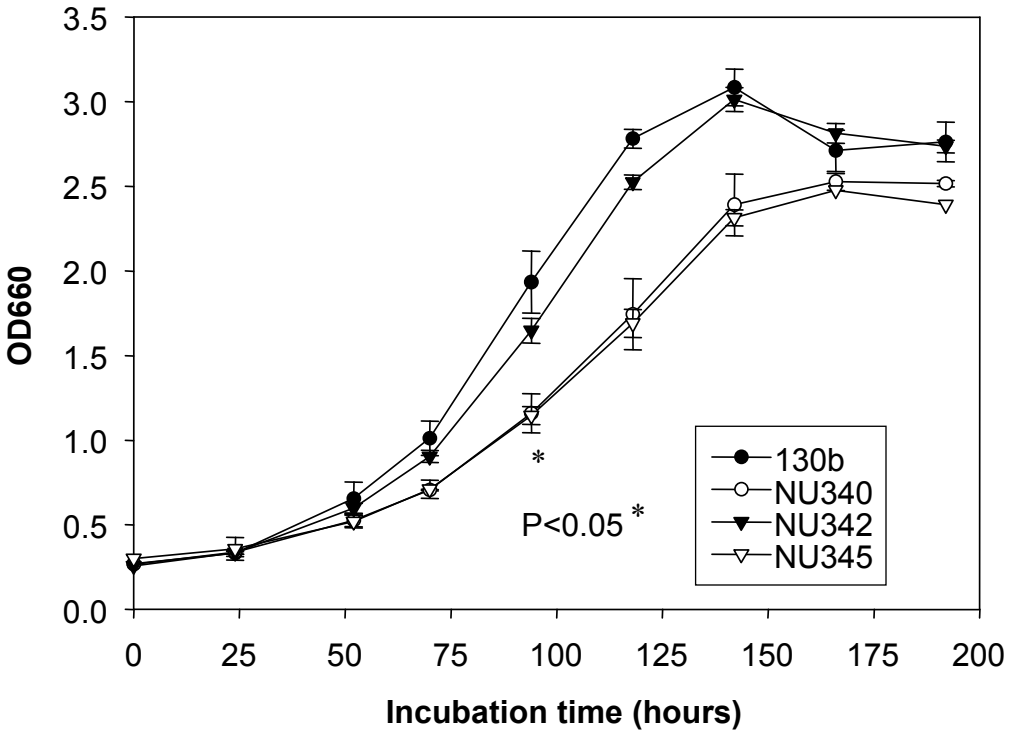

Figure 6-6. Growth of strain 130b, *ppiB*, *lpg1962*, and *ppiB:lpg1962* mutants at 17°C in BYE broth. Log-phase strain 130b, *ppiB* mutant NU340, *lpg1962* mutant NU342 and *ppiB:lpg1962* mutant NU345 were inoculated into BYE broth and then incubated 17°C. The growth of the cultures was monitored by recording the optical density of the cultures at various times. The results presented are the means and standard deviations from duplicate cultures and are representative of at least two independent experiments.

does not have any role in BYE growth at 17°C. In summary, the *ppiB* mutant has a growth defect at 17°C in BYE broth while the *lpg1962* mutant does not.

Next the ability of the ppiase mutants to survive and grow on plates at low temperatures was assessed by plating on BCYE plates at 37°C and 17°C. None of the mutants had a reduction in EOP at 37°C (**Fig. 6-7**). However, at 17°C, the *lpg1962* mutant had a small reduction in EOP that was seen five times with multiple independent mutants. This defect was sometimes as great as 1 log reduction in EOP but in a few experiments no reduction in EOP could be seen (**Fig. 6-7 and Fig. 6-8**). The fact this mutant sometimes has a reduction in EOP and sometimes not, can be explained by the prescence of different subpopulations. These subpopulations might occur due to slight changes in gene expression, changes in signaling event leading to increased/ decreased gene expression or due to a population of proteins with slightly modified structure. Sometimes a subpopulation is selected for that can survive and grow like strain 130b, while at other times a subpopulation is selected for that can not.

The *ppiB* mutant had a bigger defect than the *lpg1962* mutant usually with a 1-2 log reduction in EOP (**Fig. 6-7 and Fig. 6-8**). This result can again be explained by the presence of different subpopulations. Perhaps, in the subpopulation able to survive and grow at the low temperature, a secreted factor, due to slight changes in protein structure, might now be able to fold or regulate its own activity without the help of ppiases. This reduction in EOP of the *ppiB* mutant was not as severe as the reduction in EOP of the *lsp* mutants at lower temperatures (**Fig. 6-7)**. However, this reduction in EOP was due to the loss of *ppiB* and not a second site mutation, since the *ppiB* mutant with the complementing plasmid pMB3 had an EOP equal to the EOP of strain 130b

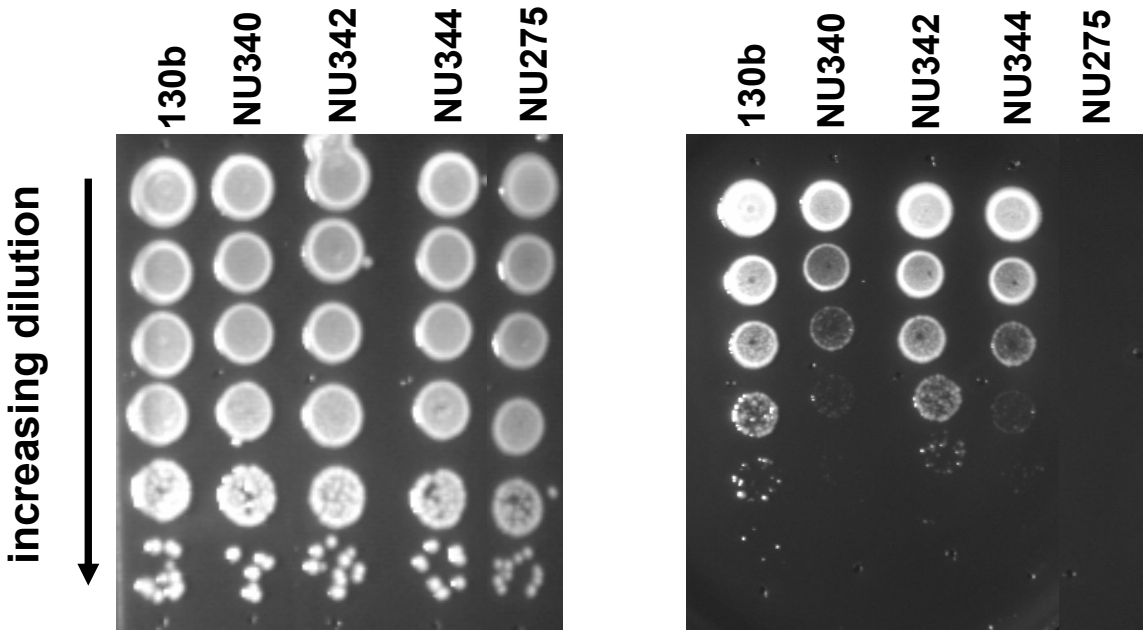

Figure 6-7. EOP of strain 130b and *ppiB*, *lpg1962*, *ppiB:lpg1962* and *lspF* mutants at 37°C and 17°C on BCYE plates. Ten-fold serial dilutions of strain 130b, and *ppiB* mutant NU340, *lpg1962* mutant NU342, *ppiB:lpg1962* mutant NU344 and *lspF* mutant NU275 containing equivalent numbers of CFU (as defined at 37°C) were spotted onto BCYE plates and then incubated at 37°C (left) and 17°C (right). Pictures were taken on day 3 for 37°C plates and on day 20 for 17°C plates. The results presented are representative of at least 3 independent experiments.

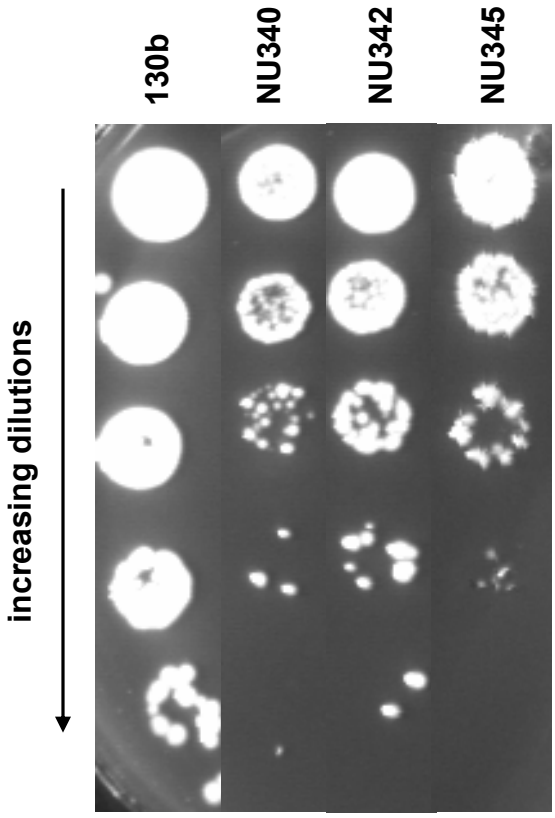

Figure 6-8. EOP of strain 130b and *ppiB*, *lpg1962* and *ppiB:lpg1962* mutants at 17°C on BCYE plates. Ten-fold serial dilutions of strain 130b, and *ppiB* mutant NU340, *lpg1962* mutant NU342 and *ppiB:lpg1962* mutantNU345 containing equivalent numbers of CFU (as defined at 37°C) were spotted onto BCYE plates and then incubated at 17°C. Pictures were taken on day 3 for 37°C plates and on day 20 for 17°C plates. The results presented are representative of at least 3 independent experiments.

with the vector pMB2002 (**Fig. 6-9**). In addition, a *ppiB*: *lpg1962* double mutant had an EOP, at 17°C, equal to the EOP of the *ppiB* mutant (**Fig. 6-7 and Fig. 6-8**). Since the *lpg1962* mutant reduction in EOP at 17°C is small, it is unclear whether PpiB and Lpg1962 work on the same subset of type II exoproteins. In summary, the *ppiB* and *lpg1962* mutants both have a reduction in EOP on plates at 17°C, but the *ppiB* mutant reduction in EOP is larger than the reduction in EOP for the *lpg1962* mutant.

The *lsp* mutants lack the secreted factor/s needed to increase survival and growth of themselves in the streak assay at RT. The two ppiase mutants were not as defective as the *lsp* mutants for growth in broth and on plates at low temperatures. However, since especially the *ppiB* mutant had a small growth defect it was still possible that these mutants would lack at least one of the secreted factors needed for promotion of growth and survival at RT and therefore both the single and double ppiase mutants were tested for promotion of growth and survival of an *lspF* mutant in the streak assay (**Fig. 6-10**). To do so, the *lsp* mutant was plated at the 10^6 dilution on BCYE plates, and then a streak of the parental strain, *lspF*, *ppiB*, *lpg1962*, or the *ppiB*:*lpg1962* mutant were added to one side of the plates. The plates were then incubated at 37°C for 3 days or at RT for 9 days. As before, no difference in survival and growth could be seen for the *lspF* mutant at 37°C with or without a streak (**data not shown**). In addition, there was no difference in the ability of the parental strain or the *ppiB*, *lpg1962* single and double mutants to promote survival and growth of the *lspF* mutant in this assay at RT. However, if the *lsp* mutant phenotype is due to the loss of several type II exoproteins, the loss of only a few of them might not be enough for a difference to be seen. In addition, the *lsp* mutants are very defective for survival and growth at RT with an EOP

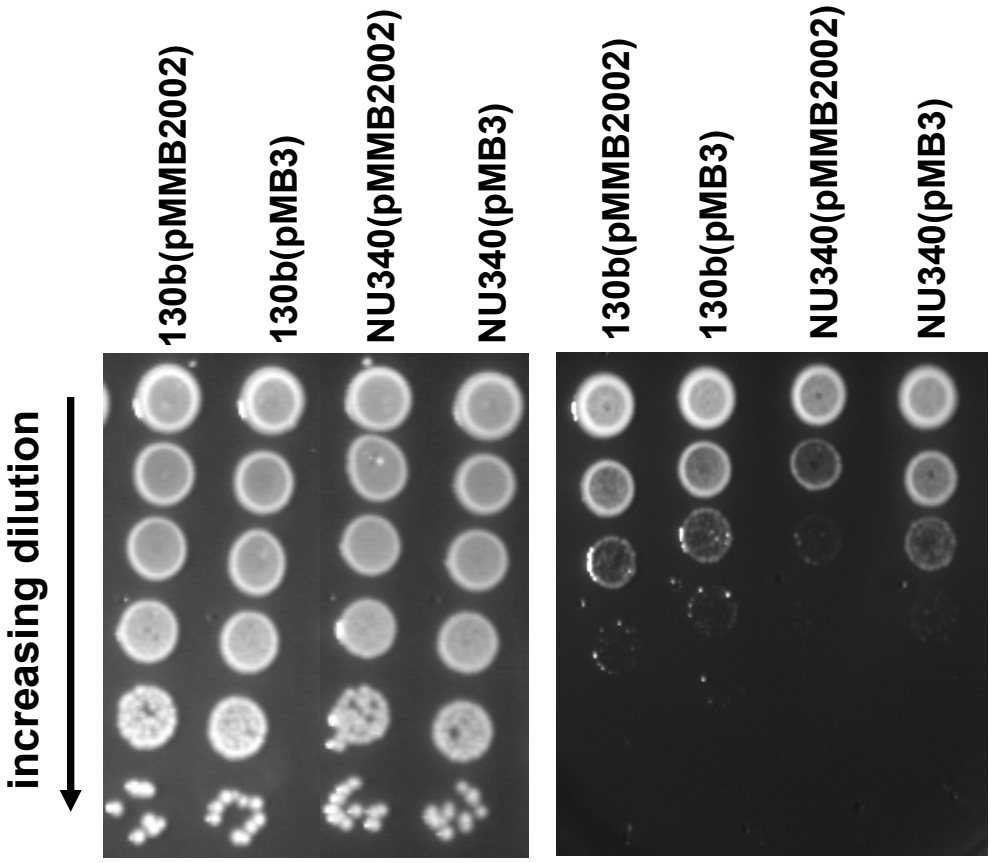

Figure 6-9. EOP of strain 130b, *ppiB* and complemented ppiB mutants at 37°C and 17°C on BCYE plates. Ten-fold serial dilutions of strain 130b and *ppiB* mutant NU340 containing either vector pMMB2002 or the complementing plasmid pMB3 were spotted onto BCYE plates and then incubated at 37°C (right) or at 17°C (left). Pictures were taken on day 3 plates and on day 20 for 17°C plates. The results presented are representative of 2 independent experiments.

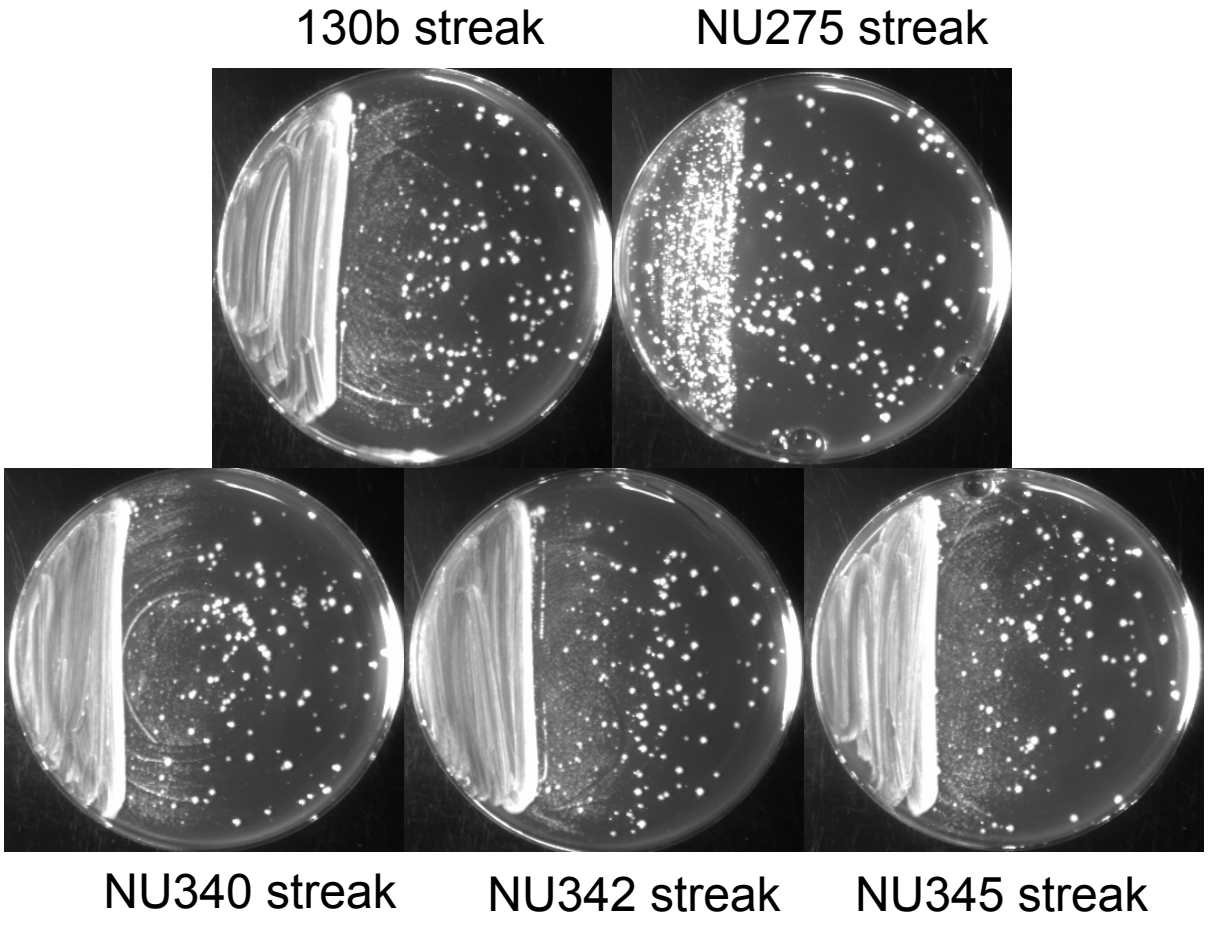

Figure 6-10. The effect of adjacent bacteria on the low-temperature survival rate of an *L. pneumophila* type II secretion mutant. Approximately 10^6 CFU of *lspF* mutant were plated for CFU on a series of BCYE agar plates. Some plates were then, as indicated, also inoculated in one sector with streaks of strain 130b or *ppiB* mutant NU340, *lpg1962* mutant NU342, *ppiB:lpg1962* mutant NU345 or *lspF* mutant NU275 bacteria. One set of plates (shown here) was incubated at RT, and another (data not shown) was stored at 37°C. The picture depicts the RT colonial growth of the *lspF* mutant on day 9. The growth promotion is the haze next to the streak. The results presented are representative of the amount of growth promoting seen on duplicate plates.

of approximately 0.01%, while the ppiase mutants do not have a reduced ability to survive and grow at RT. It is therefore possible that the *ppiB* and *lpg1962* mutants might not lack all of the cold adapted factors needed for promotion of growth and survival at this temperature.

An *lsp* mutant is very defective for growth in amoebae. It was possible that the *ppiB* and *lpg1962* mutants would have a similar growth defect and that this growth defect might be bigger if the amoeba infection was done at a lower temperature. Therefore, *H. vermiformis* and *A. castellanii* amoebae were infected at an MOI of 0.1 at the normal infection temperature of 35°C and at the lower temperature of 22°C and 17°C. Since *H. vermiformis* and *A. castellanii* had not been grown at 22°C and 17°C before in the lab, initial growth assessments were done. Both kinds of amoebae grew at 22°C, but slightly slower than at 35°C, needing 4 days instead of 3 days to form a confluent layer in the tissue culture flasks. At 17°C the amoeba usually needed about 7 days to reach confluency.

At 35°C, both the single and the double ppiase mutants grew like the parental strain in both kinds of amoeba (**Fig. 6-11 and Fig. 6-12**). In *H. vermiformis* at 22°C, the parental strain was able to infect the amoebae but needed 8 days to reach the same growth as seen at 35°C in 3 days (**Fig. 6-11**). The *ppiB*: *lpg1962* mutant grew equally well as the parental strain. At 17°C, the 130b strain could still infect the amoeba but had by day 18 only grown about 3 logs. Again, the *ppiB*: *lpg1962* mutant grew like the parental strain (**Fig. 6-11**).

During the *A. castellanii* infection at 22°C the parental strain needed 10 days to reach the same growth seen at 35°C in 3 days (**Fig. 6-12**). This time the *ppiB*: *lpg1962*

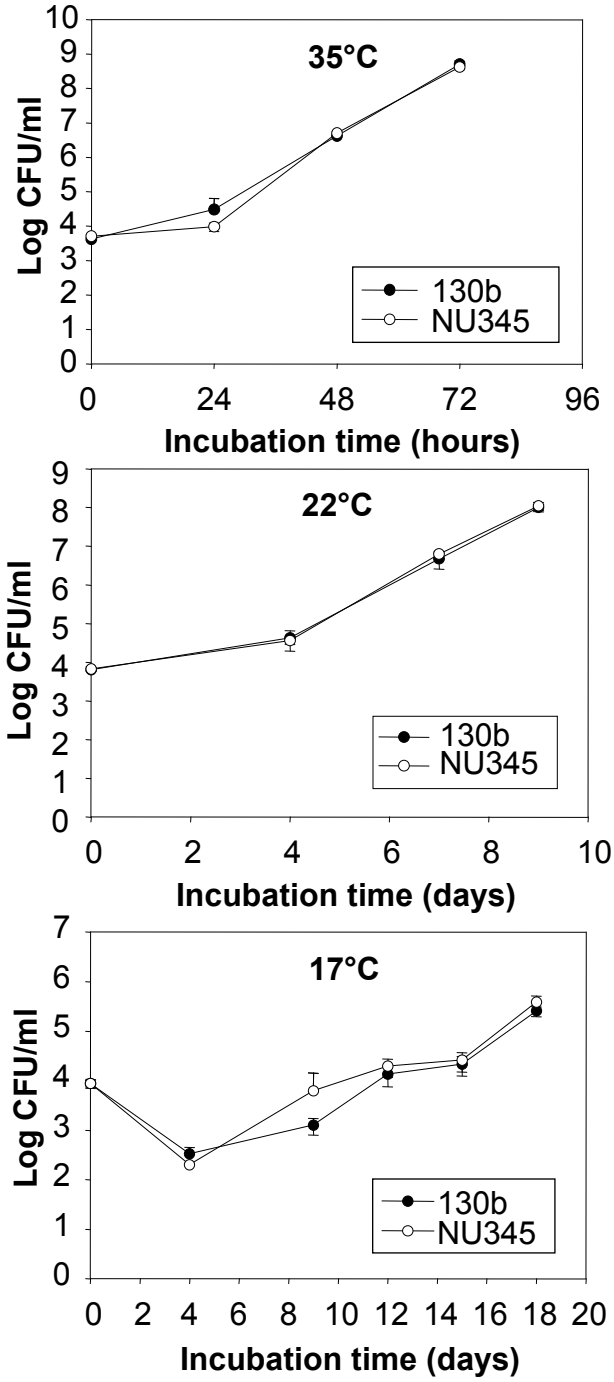

Figure 6-11. Intracellular infection of *H. vermiformis* by strain 130b and the *ppiB:lpg192* mutant NU345 at 35°C, 22°C and 17°C. Infections with *H. vermiformis* and the bacteria were carried out at 35°C, 22°C or 17°C. The numbers of bacteria per well were determined at 0, 24, 48 and 72 hours for the 35°C infection, at 0, 4, 7, 9 and 11 days for the 22°C infection and at 0, 4, 9, 12, 15 and 18 days for the 17°C infection. The values presented are the means and standard deviation obtained from four infected wells.

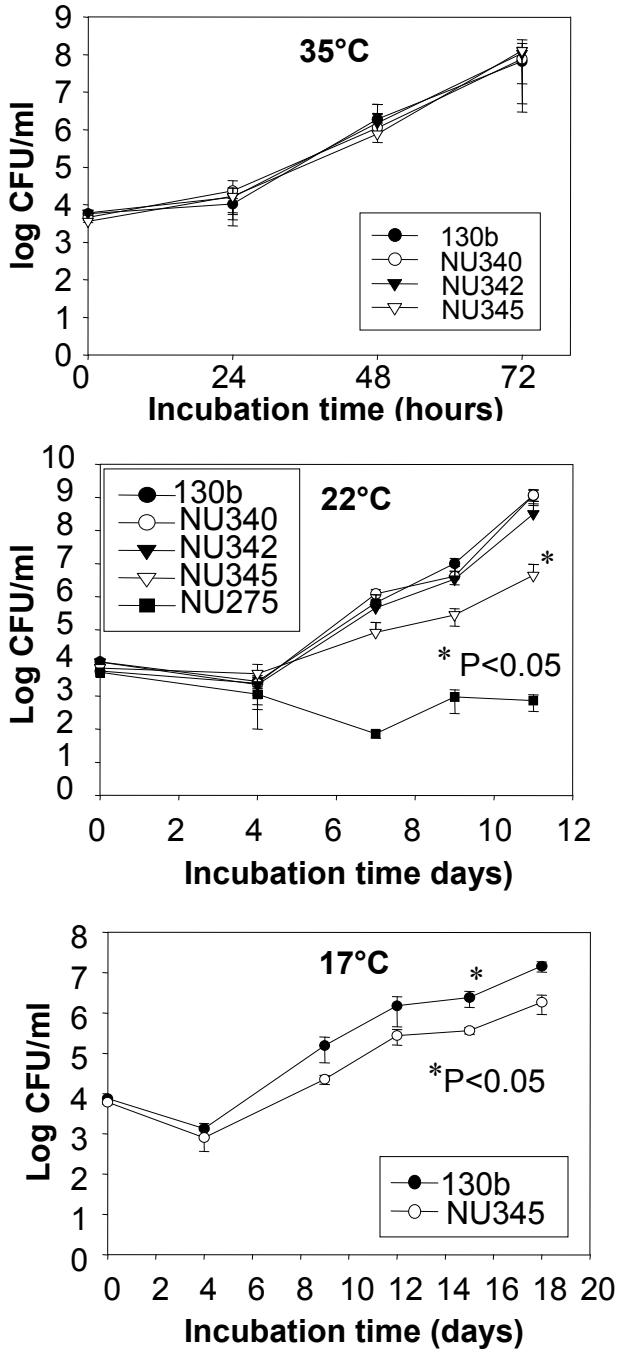

Figure 6-12. Intracellular infection of *A. castellanii* by strain 130b or *ppiB* mutant NU340, *lpg1962* mutant NU342, *ppiB:lpg1962* mutant NU345 or *lspF* mutant NU275 at 35°C, 22°C and 17°C. Infections with *A. castellanii* and the bacteria were carried out at 35°C, 22°C or 17°C. The numbers of bacteria per well were determined at 0, 24, 48 and 72 hours for the 35°C infection, at 0, 4, 7, 9 and 11 days for the 22°C infection and at 0, 4, 9, 12, 15 and 18 days for the 17°C infection. The values presented are the means and standard deviation obtained from four infected wells.

double mutant had a 2-3 log reduction in growth after 11 days of growth. This growth defect was not seen for the single mutants. In a repeat experiment, the double ppiase mutant was again defective for growth, this time lagging almost 3 logs behind the 130b strain (**data not shown**). At 17°C the 130b strain still grew but had only grown about 4 logs by day 18. At this temperature, the *ppiB*: *lpg1962* double mutant had about 1 log defect compared to the 130b strain. The fact the defect at 17°C is smaller than the defect at 22°C might reflect that a different subset of proteins that need ppiases for their activites are secreted at the two temperatures. This difference in growth was not due to an extracellular growth defect of the bacteria since 130b and the double mutant did not grow during the infections in media alone. It is also highly unlikely that the bacterial growth seen is extracellular growth due to amoebal cell content spilling out into the media, because amoebae when stressed do not lyse but become encysted (152). In addition, at the time intracellular growth at the lower temperatures started the amoebae still look healthy. These *A*. *castellanii* low temperature infections need to be repeated with a complemented *ppiB:lpg1962* mutant, containing *ppiB* or *lpg1962* in *trans*, to rule out a possible second site mutation. However, since a defect is only seen at 17°C-22°C, and not at 35°C, this difference is probably due to a reduced ability of the double mutant to grow within the amoebae. In conclusion, neither PpiB nor Lpg1962 is important for growth in *H*. *vermiformis* at any temperature. In *A*. *castellanii*, PpiB and Lpg1962 have overlapping substrate pools and a defect is only seen when both these proteins are absent.

The *ppiB*: *lpg1962* double mutant was also tested in a competition assay with the 130b strain in the A/J mouse model (**Fig. 6-13**) (26, 191, 198). More specifically, mice

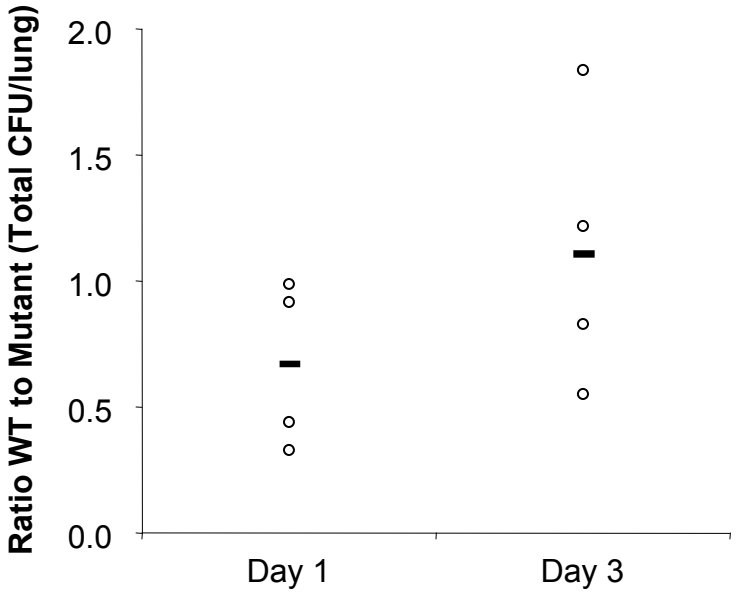

Figure 6-13. Pulmonary infections of A/J mice with *L. pneumophila*. Strain 130b and *ppiB:lpg1962* mutant NU345 were introduced into the lungs of A/J mice by intratracheal inoculation in a 1:1 ratio. At days, 1 and 3 post-inoculation, the ratio of 130b to *lcy*:*ppiA* in infected lungs was determined. Data are representative of actual values obtained per mouse (n=4), where a solid bar indicates the median value. This experiment was done by Jenny Dao.

were inoculated with 10^6 CFU of a 1:1 ratio of the 130b strain and the mutant. Since *L*. *pneumophila* were recovered from the lungs of these mice at a ratio close to 1 on both day 1 and 3, the *ppiB and lpg1962* are not required for *L. pneumophila* growth in the lungs of A/J mice.

To test if the *ppiB* and *lpg1962* mutants, like the *lsp* mutants, have a reduced survivability in tap water, the ppiase mutants and the 130b strain from late log BYE cultures were centrifuged, rinsed twice with sterile tap water and then inoculated into 50 ml of sterile tap water. Then, these flasks were incubated at 37°C or 17°C while shaking at 225 rpm. At 0, 38 and 104 days, a sample was taken out and the CFUs were determined (**Fig. 6-14**). The *lpg1962* mutant survived as well as the 130b strain at all time points. In contrast, the *ppiB* mutant had lower survivability than the 130b strain and by day 104 this difference had grown to more than 2 logs. Therefore, the survivability of the *ppiB* mutant might equal that of the *lsp* mutants in water cultures.

SUMMARY

Since Lpg1962 has a signal peptide, it is most likely secreted through the type II secretion machinery and the secreted proteins it acts on when grown on BCYE plates, but not in BYE, are important for survival and growth at 17°C. In addition, Lpg1962 has a role in intracellular growth in *A*. *castellanii*, but was dispensable for survival in tap water at low temperatures. In contrast, PpiB is important for survival and growth on BCYE plates, in BYE broth and for growth in *A*. *castellanii* at low temperatures. The *ppiB* mutant data from the 35°C infection in *A*. *castellanii* were surprising since the *ppiB* mutant previously made in *L*. *pneumophila* strain Corby had shown a small growth

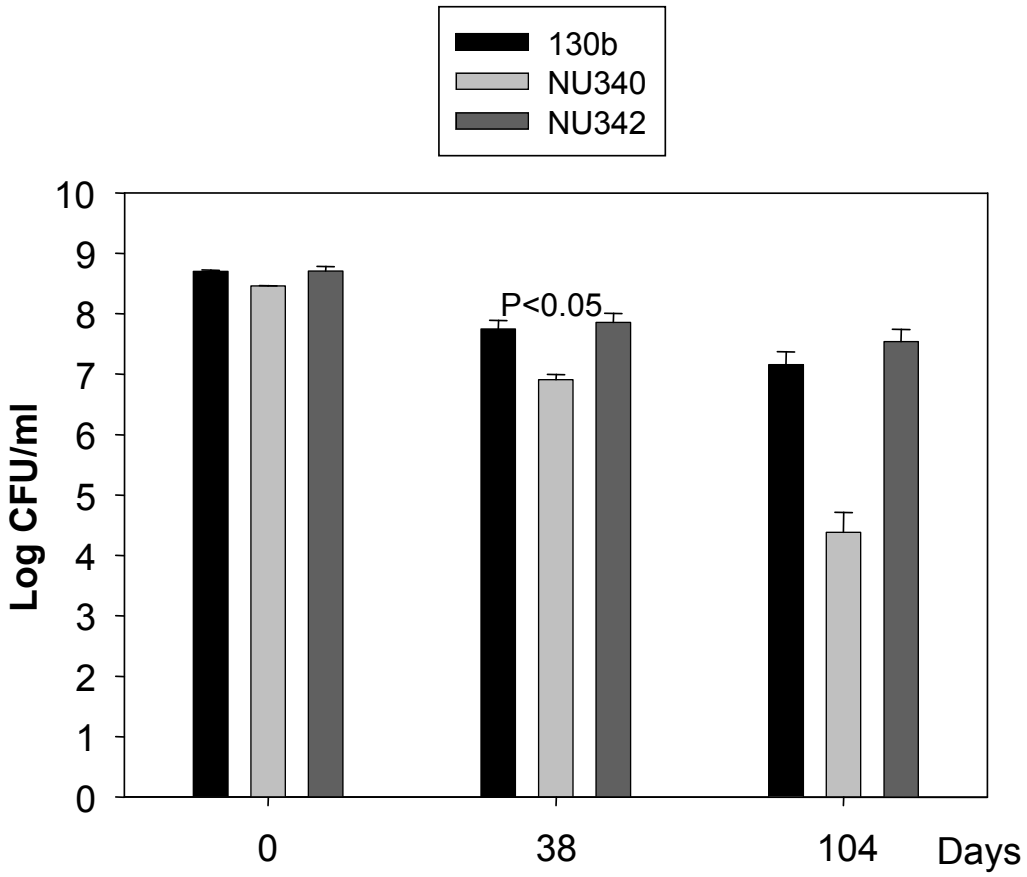

Figure 6-14. Survival of strain 130b, *ppiB* mutant NU340, and *lpg1962* mutant NU342 in sterile tap water 12°C. Log-phase bacteria from BYE cultures were spun down, rinsed twice with sterile tap water and then inoculated into 50 ml of sterile tap water before incubation at 12°C. At indicated days a sample was taken out and the CFU count was determined. The results presented are the mean and standard deviation of triplicate samples.

defect during a 35°C infection (206). However, since the *ppiB* mutant had fewer CFUs entering the amoebae, these data might be more a reflection of an entry defect than an actual growth defect (206). In addition, the differences in data could possibly be explained by strain differences between our strain, 130b and the Corby strain.

Interestingly, PpiB have an important role for survival in tap water at low temperatures. However, *ppiB* and *lspF* mutants would have to be examined in the same experiment to see if the loss in survivability seen for *ppiB* mutant can explain the whole defect seen for *lsp* mutants at the lower temperature or not. In addition, complementation would have to be done to exclude potential second site mutations in the *ppiB* mutant.

Since PpiB does not have a signal peptide, it is probably not secreted through the type II secretion system. However, since ppiases work on folding/regulating other proteins, PpiB can very well be secreted in some other way and still fold/regulate the activity of type II secreted substrates. However, the *ppiB* mutant reduction in EOP at low temperatures is not as severe as the reduction seen for the type II secretion mutants. Therefore, other presently unknown secreted factors are also required for survival and growth at low temperatures.

DISCUSSION

L. *pneumophila* is an environmental pathogen with the ability to survive in a wide variety of aquatic habitats (72, 73, 95, 172). Its success for survival in these environments is at least in part due to its ability to survive over a wide range of temperatures; i.e. 5-63°C (72). But since the bacteria grow best in the laboratory at 32-37°C, there are few data on what is important for low temperature growth/survival (133, 136, 249). However, understanding the ability of *Legionella* to grow and survive in low temperature aquatic environments is important since it can increase our knowledge about the natural history of Legionellosis.

Even though studies identify warm water sources in cases of Legionnaires' disease (68, 166, 194, 200, 221), many other studies show that *L*. *pneumophila* has the capability to grow and survive in these water sources at temperatures in the range of 20-30°C (141, 193, 243). In fact, *L*. *pneumophila* can probably grow and survive in these water systems at even lower temperatures since this study has shown for the first time that *L*. *pneumophila* can grow and survive at temperatures below 20°C; i.e. at least down to 12°C. In addition, at least some studies do not find a difference in the concentration of *Legionella* in the hot vs. cold water supplies (190). Outside a lab, the ability of Legionella to replicate is largely dependent on amoebae, which have been found in up to 88% of cold tap water samples, indicating the ability of man-made cold water systems to support growth of *L*. *pneumophila* (97). In addition, my data have shown for the first time that *L*. *pneumophila* has the ability to infect and to grow within both *H*. *vermiformis* and *A. castellani* amoeba at temperatures as low as 17-22°C. On

this note, this study has also found that RT grown bacteria are as infectious as 37°C grown bacteria, a finding that correlates with *L*. *pneumophila* from both an ice machine and cold water supplies being able to cause infections in humans (85, 102, 103, 120). In summary, *L*. *pneumophila* has the capacity to both grow and be infectious at lower temperatures and therefore the ability of bacteria from man-made cold water supply to infect humans should not be ignored in the natural history of Legionellosis.

There are very few studies in *Legionella* regarding what is important for growth or survival at temperatures below 37°C. However, the *lvh* type IV secretion system is important for infection of macrophages at lower temperatures since a *lvh* mutant grown at 30°C but not 37°C had a 100-fold reduction in entry and intracellular infection as compared to a 130b strain (189). In addition, the flagella gene *flaA*, has a higher expression at 30°C as compared to 37°C (100). The *pilBCD* operon also has a higher expression at 30 vs. 37°C, which correlates with the bacteria being more piliated at the lower temperatures (145). This study has now shown an even higher expression of the prepilin peptidase gene *pilD*, at temperatures below 25°C, an increase which correlates with the type II secretion being important for growth/survival at temperatures between 12-25°C both in bacteriological media and in tap water cultures. However, it is currently unknown if the type II secretion subunits and exoproteins also have higher expression at lower temperatures. Since *flaA* and *pilD* are regulated by temperature and growth phase, it is possible that a common cold activation signal exist regulating flagellation, piliation, and perhaps the components and exoproteins of the type II secretion system itself. However, the nature of this signal is presently unknown, but is likely to involve changes in DNA supercoiling, changes in RNA stability or changes in protein

conformation (113). There is no homologue in *L*. *pneumophila* of the *B*. *subtilus* and cyanobacterial low temperature activated two component regulator DesK (151). But since *L*. *pneumophila* does not have a desaturase gene another regulator or other signal might exist.

Type II secreted factors in *L. pneumophila* are probably needed for efficient growth at lower temperatures since both streaks and supernatants from the 130b strain can rescue the poor growth of the *lsp* mutants. Surprisingly, some species in the genus *Legionella* grow as poorly at lower temperatures as the *L. pneumophila lsp* mutants. This growth defect, at least in the few species examined, correlated with the absence of type II exoproteins. Interestingly, a *P*. *aeruginosa pilD* mutant and an *E*. *chrysanthemi hofQ* mutant do not show the same growth defect at lower temperatures even though at least HofQ had an increased expression at 18°C vs. 28°C (84). Therefore, the exoproteins and the route of export of the proteins needed for low temperature growth might differ in different species of bacteria. However, since many other bacteria possess type II secretion systems, including pathogens such as *Aeromonas hydrophila*, *Burkholderia* spp., and *Vibrio* spp. it is possible that at least some of them need the same type II exoproteins as *L. pneumophila* for efficient growth at lower temperatures. For *L. pneumophila*, this becomes especially important in that type II secretion is not only important for growth on bacteriological media but have a role in long-term water survival. This importance might stem from simple inability of the *lsp* mutant to acquire nutrients from the environment, which might be more critical at low temperatures. But, it is also possible that small amounts of iron in the water produce radicals that type II mutants cannot protect themselves against. This would not be surprising since the

growth defect of the *lsp* mutants at low temperatures is bigger on bacteriological media when iron is present. It is also possible that this greater sensitivity might stem from a type II exoprotein having a function in protection against biocides added to the water. Further studies are clearly needed to distinguish between these possibilities.

In addition to the very important role for type II secretion in low temperature growth/survival, this study identified several other genes important for low temperature growth. Some of these identified genes have general functions in fatty acid, amino acid, general metabolism and already known cold shock genes (147, 183, 230). More importantly, several genes encoding hypothetical proteins specific for *L. pneumophila* were found e.g. both strain NU358 from the temperature screen and the secreted small hypothetical protein from the 2D gel analysis indicate *L. pneumophila* employs novel strategies for low temperature growth and survival. Since nothing is known about these proteins and their function at low temperatures, future studies are needed. In addition to these proteins, two secreted ppiases were found in the 2D gel analysis. The ppiase Lpg1962 is found in supernatants from both 37 and 17°C grown strain 130b cultures but has higher expression at the lower temperature. Since it has a signal peptide, it is most likely secreted through type II secretion, though it is impossible to conclude this for certain since an *lspF* mutant has some degree of periplasmic leakage. Lpg1962 does not have an important function for growth in bacteriological media or survival in water, but it is possible other ppiases have overlapping functions and the importance of this gene is masked. This was the case in low temperature infections with *A. castellanii*, where only the double ppiase mutant is defective for growth.

On the other hand, the second ppiase, PpiB, is important for growth on bacteriological media at lower temperatures. Since this protein lacks a signal peptide, its secretion pathway is unclear. However, the PpiB protein spot is repeatedly found on the 17°C and 12°C 2D gels at multiple time points in repeat experiments. In contrast, this spot is never seen on the 37°C gel even though another study did detect its activity in cell extracts at this temperature (206). Why is this? It is possible that PpiB is secreted at a very low concentration at 37°C but our method is not sensitive enough to detect it. It is also possible that PpiB might be differentially secreted at some temperatures but not at other. However, the fact that PpiB is important for growth and is found secreted at low temperatures but not at 37°C indicates that a secreted PpiB has a role in cold adaptation. Since this protein does not have a signal peptide predicted by psort or signalP, it does not cross the inner membrane using the Sec system. In addition, it likely does not cross the inner membrane using the Tat pathway since it does not have the signature twin arginine motif in the N-terminal end (175). In addition, the Mass Spectrometry data shows peptide hits from the N-terminal end of the protein indicating that the whole protein is secreted. Unless other mechanisms exist for transport through the inner membrane, PpiB is therefore highly unlikely to be a substrate for the type II secretion system. However, if PpiB could be transported through the inner membrane by an unknown mechanism, it could be transported through T2S or perhaps the T4P apparatus since cyclophilin ppiases have high beta sheet content, a feature that is thought to be important for type II exoprotein secretion (69, 203). PpiB is probably not an auto transporter since the protein is too small to contain both a pore and a transported protein. In addition, PpiB is not likely transported through the T1S since it is

not in an operon with ABC transport genes, neither is it glycine rich or has a pI of 4 (53, 104). It is likely not secreted through the *icm*/*dot* or *lvh* type IV secretion systems since PpiB can be found in supernatants from both *dotG* and *lvh* mutants. However, a *dot*:*lvh* double mutant has not been tested. There is also the newly discovered type VI secretion system (186), but this secretion system is probably not present in *L. pneumophila* since it at least is lacking two genes involved in T6S; i.e., the *vasA* and the *vgrG-2* genes. In addition, secretion through vesicle mediated transport exists, but has never been shown to occur in *L. pneumophila* (134). Therefore, the way PpiB is secreted can only be speculated upon at this time point. *Legionella* does not have a type III secretion system but it does have a flagella apparatus and in other organisms secretion through this apparatus is possible and maybe this is the route of choice for PpiB (122). The fact, the 2D gel data show more flagellin on the 37°C gel, indicating that the bacteria might be less motile in the cold, does not mean that the flagella apparatus itself has to be down regulated at lower temperatures. This hypothesis could easily be tested by 2D analysis of supernatants from a *L. pneumophila* strain with a mutation in the flagella apparatus. However, the way PpiB is secreted is not important since ppiases work by folding and regulating the activity of other proteins. Then what substrates does PpiB work on? It is possible that if PpiB functions by regulating the activity of type II exoproteins analyzing the sequences of known type II exoproteins to see if any of those have a “proline switch” motif, consisting of a hydrophobic stretch of 3-4 amino acids followed by glutamate, any amino acid and a proline, found in three proteins with known structural changes due to proline *cis*/*trans* isomerization could give

a clue (7). If this approach is unsuccessful, crosslinking and immunoprecipitation of PpiB with its interacting proteins might be possible from low temperature supernatants.

However, since a *ppiB* mutant is not as defective for growth at low temperatures as the type II secretion mutants, other secreted factors important for low temperature growth must exist. One approach to finding those factors that has not been tested is the biochemical approach where the growth promoting substance in the 130b strain low temperature supernatants could be isolated. To do this, the assay first needs to be optimized perhaps by using concentrated supernatants, experimenting with supernatants from different growth temperatures and different growth stages or perhaps by fractionation of supernatants to get rid of a possible inhibitory factor.

Another clue to why the type II secretion is important for low temperature comes from some recent preliminary data that shows about 1 log less growth for a double mutant in *lipA* and *lipB* genes at 17°C vs. 37°C (**data not shown**). This defect was missed before since this mutant was never plated at a temperature below RT. In addition, a more recently constructed chitinase mutant also has about 1 log less growth at 17°C vs. 37°C (**data not shown**). Why are lipolytic activities important for low temperature growth? The temperature screen did find several genes involved in fatty acid metabolism and it is possible that *L. pneumophila* uses fatty acids as an energy source at lower temperatures or perhaps it needs lipid components for membrane synthesis. It is hard to say why a chitinase would be important for growth on bacteriological media at lower temperatures. However, this chitinase is also important for growth inside the A/J mouse and it was hypothesized that it might act on *O*-GlcNAcylated proteins in eukaryotic cells (51).

In conclusion, this study has found a previously unknown connection of type II secretion with low temperature growth. Since this secretion system is important not only for growth on bacteriological media but also for survival in water at low temperatures this has implications in understanding what is importance for survival of *L. pneumophila* in low temperature environmental niches. In addition, an important role for secreted ppiases for efficient low temperature growth has also been found. Clearly, this study has increased our understanding of how L*. pneumophila* is able to persist in low temperature aquatic environments.

REFERENCES

1. 1997. Summary of notifiable diseases, United States, 1996. MMWR Morb Mortal Wkly Rep **45:**1-87.

2. **Abu Kwaik, Y.** 1996. The phagosome containing Legionella pneumophila within the protozoan Hartmannella vermiformis is surrounded by the rough endoplasmic reticulum. Appl Environ Microbiol **62:**2022-8.

3. **Agnihotri, G., and H. W. Liu.** 2003. Enoyl-CoA hydratase. reaction, mechanism, and inhibition. Bioorg Med Chem **11:**9-20.

4. **Alary, M., and J. R. Joly.** 1991. Risk factors for contamination of domestic hot water systems by legionellae. Appl Environ Microbiol **57:**2360-7.

5. **Allard, K. A., V. K. Viswanathan, and N. P. Cianciotto.** 2006. lbtA and lbtB are required for production of the Legionella pneumophila siderophore legiobactin. J Bacteriol **188:**1351-63.

6. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J Mol Biol **215:**403-10.

7. **Andreotti, A. H.** 2003. Native state proline isomerization: an intrinsic molecular switch. Biochemistry **42:**9515-24.

8. **Andreotti, A. H.** 2006. Opening the pore hinges on proline. Nat Chem Biol **2:**13-4.
9. **Angelidis, A. S., L. T. Smith, L. M. Hoffman, and G. M. Smith.** 2002. Identification of opuC as a chill-activated and osmotically activated carnitine transporter in Listeria monocytogenes. Appl Environ Microbiol **68:**2644-50.

10. **Aragon, V., S. Kurtz, and N. P. Cianciotto.** 2001. Legionella pneumophila major acid phosphatase and its role in intracellular infection. Infect Immun **69:**177-85.

11. **Aragon, V., S. Kurtz, A. Flieger, B. Neumeister, and N. P. Cianciotto.** 2000. Secreted enzymatic activities of wild-type and pilD-deficient Legionella pneumophila. Infect Immun **68:**1855-63.

12. **Aragon, V., O. Rossier, and N. P. Cianciotto.** 2002. Legionella pneumophila genes that encode lipase and phospholipase C activities. Microbiology **148:**2223-31.

13. **Ausubel, F. M., Brent, R.B., Kingston, R.E., Moore, D.D.,, and J. G. Seidman, Smith, J.A., k. Struhl** 1989. Current Protocols in Molecular Biology. New York: John Wiley & Sons.

14. **Backert, S., and T. F. Meyer.** 2006. Type IV secretion systems and their effectors in bacterial pathogenesis. Curr Opin Microbiol **9:**207-17.

15. **Baine, W. B.** 1985. Cytolytic and phospholipase C activity in Legionella species. J Gen Microbiol **131:**1383-91.

16. **Baine, W. B.** 1988. A phospholipase C from the Dallas 1E strain of Legionella pneumophila serogroup 5: purification and characterization of conditions for optimal activity with an artificial substrate. J Gen Microbiol **134:**489-98.

17. **Bandyopadhyay, P., S. Liu, C. B. Gabbai, Z. Venitelli, and H. M. Steinman.** 2007. Environmental mimics and the Lvh type IVA secretion system contribute to virulence-related phenotypes of Legionella pneumophila. Infect Immun **75:**723-35.

18. **Barbaree, J. M., B. S. Fields, J. C. Feeley, G. W. Gorman, and W. T. Martin.** 1986. Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of Legionella pneumophila. Appl Environ Microbiol **51:**422-4.

19. **Basak, C., S. K. Pathak, A. Bhattacharyya, S. Pathak, J. Basu, and M. Kundu.** 2005. The secreted peptidyl prolyl cis,trans-isomerase HP0175 of Helicobacter pylori induces apoptosis of gastric epithelial cells in a TLR4- and apoptosis signal-regulating kinase 1-dependent manner. J Immunol **174:**5672-80.

20. **Begic, S., and E. A. Worobec.** 2006. Regulation of Serratia marcescens ompF and ompC porin genes in response to osmotic stress, salicylate, temperature and pH. Microbiology **152:**485-91.

21. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol **340:**783-95.

22. **Berg, J. D., J. C. Hoff, P. V. Roberts, and A. Matin.** 1985. Growth of Legionella pneumophila in continuous culture. Appl Environ Microbiol **49:**1534-7.

23. **Berks, B. C., T. Palmer, and F. Sargent.** 2005. Protein targeting by the bacterial twin-arginine translocation (Tat) pathway. Curr Opin Microbiol **8:**174-81.

24. **Borezee, E., E. Pellegrini, and P. Berche.** 2000. OppA of Listeria monocytogenes, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. Infect Immun **68:**7069-77.

25. **Bozue, J. A., and W. Johnson.** 1996. Interaction of Legionella pneumophila with Acanthamoeba castellanii: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. Infect Immun **64:**668-73.

26. **Brieland, J., P. Freeman, R. Kunkel, C. Chrisp, M. Hurley, J. Fantone, and C. Engleberg.** 1994. Replicative Legionella pneumophila lung infection in intratracheally

inoculated A/J mice. A murine model of human Legionnaires' disease. Am J Pathol **145:**1537-46.

27. **Budde, I., L. Steil, C. Scharf, U. Volker, and E. Bremer.** 2006. Adaptation of Bacillus subtilis to growth at low temperature: a combined transcriptomic and proteomic appraisal. Microbiology **152:**831-53.

28. **Byrne, B., and M. S. Swanson.** 1998. Expression of Legionella pneumophila virulence traits in response to growth conditions. Infect Immun **66:**3029-34.

29. **Cairrao, F., A. Cruz, H. Mori, and C. M. Arraiano.** 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. Mol Microbiol **50:**1349-60.

30. **Carmel-Harel, O., and G. Storz.** 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the Escherichia coli and saccharomyces cerevisiae responses to oxidative stress. Annu Rev Microbiol **54:**439-61.

31. **Carratala, J., F. Gudiol, R. Pallares, J. Dorca, R. Verdaguer, J. Ariza, and F. Manresa.** 1994. Risk factors for nosocomial Legionella pneumophila pneumonia. Am J Respir Crit Care Med **149:**625-9.

32. **Cazalet, C., C. Rusniok, H. Bruggemann, N. Zidane, A. Magnier, L. Ma, M. Tichit, S. Jarraud, C. Bouchier, F. Vandenesch, F. Kunst, J. Etienne, P. Glaser, and C. Buchrieser.** 2004. Evidence in the Legionella pneumophila genome for exploitation of host cell functions and high genome plasticity. Nat Genet **36:**1165-73.

33. **Chapon-Herve, V., M. Akrim, A. Latifi, P. Williams, A. Lazdunski, and M. Bally.** 1997. Regulation of the xcp secretion pathway by multiple quorum-sensing modulons in Pseudomonas aeruginosa. Mol Microbiol **24:**1169-78.

34. **Chien, M., I. Morozova, S. Shi, H. Sheng, J. Chen, S. M. Gomez, G. Asamani, K. Hill, J. Nuara, M. Feder, J. Rineer, J. J. Greenberg, V. Steshenko, S. H. Park, B. Zhao, E. Teplitskaya, J. R. Edwards, S. Pampou, A. Georghiou, I. C. Chou, W. Iannuccilli, M. E. Ulz, D. H. Kim, A. Geringer-Sameth, C. Goldsberry, P. Morozov, S. G. Fischer, G. Segal, X. Qu, A. Rzhetsky, P. Zhang, E. Cayanis, P. J. De Jong, J. Ju, S. Kalachikov, H. A. Shuman, and J. J. Russo.** 2004. The genomic sequence of the accidental pathogen Legionella pneumophila. Science **305:**1966-8.

35. **Chintalapati, S., M. D. Kiran, and S. Shivaji.** 2004. Role of membrane lipid fatty acids in cold adaptation. Cell Mol Biol (Noisy-le-grand) **50:**631-42.

36. **Chrisope, G. L., C. W. Fox, and R. T. Marshall.** 1976. Lecithin agar for detection of microbial phospholipases. Appl Environ Microbiol **31:**784-6.

37. **Christie, P. J., and E. Cascales.** 2005. Structural and dynamic properties of bacterial type IV secretion systems (review). Mol Membr Biol **22:**51-61.

38. **Cianciotto, N. P.** 2005. Type II secretion: a protein secretion system for all seasons. Trends Microbiol **13:**581-8.

39. **Cianciotto, N. P., P. Cornelis, and C. Baysse.** 2005. Impact of the bacterial type I cytochrome c maturation system on different biological processes. Mol Microbiol **56:**1408-15.

40. **Cianciotto, N. P., and B. S. Fields.** 1992. Legionella pneumophila mip gene potentiates intracellular infection of protozoa and human macrophages. Proc Natl Acad Sci U S A **89:**5188-91.

41. **Cirillo, J. D., S. Falkow, and L. S. Tompkins.** 1994. Growth of Legionella pneumophila in Acanthamoeba castellanii enhances invasion. Infect Immun **62:**3254-61.

42. **Cloud-Hansen, K. A., S. B. Peterson, E. V. Stabb, W. E. Goldman, M. J. McFall-Ngai, and J. Handelsman.** 2006. Breaching the great wall: peptidoglycan and microbial interactions. Nat Rev Microbiol **4:**710-6.

43. **Colgan, J., M. Asmal, M. Neagu, B. Yu, J. Schneidkraut, Y. Lee, E. Sokolskaja, A. Andreotti, and J. Luban.** 2004. Cyclophilin A regulates TCR signal strength in CD4+ T cells via a proline-directed conformational switch in Itk. Immunity **21:**189-201.

44. **Cornelis, G. R.** 2006. The type III secretion injectisome. Nat Rev Microbiol **4:**811-25.

45. **Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier.** 1998. The virulence plasmid of Yersinia, an antihost genome. Microbiol Mol Biol Rev **62:**1315-52.

46. **Cronan, J. E.** 2006. A bacterium that has three pathways to regulate membrane lipid fluidity. Mol Microbiol **60:**256-9.

47. **Datry, T., F. Malard, and J. Gibert.** 2004. Dynamics of solutes and dissolved oxygen in shallow urban groundwater below a stormwater infiltration basin. Sci Total Environ **329:**215-29.

48. **De Buck, E., L. Maes, E. Meyen, L. Van Mellaert, N. Geukens, J. Anne, and E. Lammertyn.** 2005. Legionella pneumophila Philadelphia-1 tatB and tatC affect intracellular replication and biofilm formation. Biochem Biophys Res Commun **331:**1413-20.

49. **de Keyzer, J., C. van der Does, and A. J. Driessen.** 2003. The bacterial translocase: a dynamic protein channel complex. Cell Mol Life Sci **60:**2034-52.

50. **Debroy, S., V. Aragon, S. Kurtz, and N. P. Cianciotto.** 2006. Legionella pneumophila Mip, a surface-exposed peptidylproline cis-trans-isomerase, promotes the presence of phospholipase C-like activity in culture supernatants. Infect Immun **74:**5152-60.

51. **DebRoy, S., J. Dao, M. Soderberg, O. Rossier, and N. P. Cianciotto.** 2006. Legionella pneumophila type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. Proc Natl Acad Sci U S A **103:**19146-51.

52. **Declerck, P., J. Behets, V. van Hoef, and F. Ollevier.** 2007. Replication of Legionella pneumophila in Floating Biofilms. Curr Microbiol **55:**435-40.

53. **Delepelaire, P.** 2004. Type I secretion in gram-negative bacteria. Biochim Biophys Acta **1694:**149-61.

54. **Den Boer, J. W., P. F. Yzerman, and M. Conyn-Van Spaendonck.** 2002. A large outbreak of Legionnaire's disease at a flower show, the Netherlands, 1999. Emergin Infectious Diseases **8:**37-44.

55. **Deng, M., and R. Misra.** 1996. Examination of AsmA and its effect on the assembly of Escherichia coli outer membrane proteins. Mol Microbiol **21:**605-12.

56. **Dornan, J., P. Taylor, and M. D. Walkinshaw.** 2003. Structures of immunophilins and their ligand complexes. Curr Top Med Chem **3:**1392-409.

57. **Eckert, B., A. Martin, J. Balbach, and F. X. Schmid.** 2005. Prolyl isomerization as a molecular timer in phage infection. Nat Struct Mol Biol **12:**619-23.

58. **Edelstein, P. H.** 1981. Improved semiselective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. J Clin Microbiol **14:**298-303.

59. **Edelstein, P. H., K. B. Beer, and E. D. DeBoynton.** 1987. Influence of growth temperature on virulence of Legionella pneumophila. Infect Immun **55:**2701-5.

60. **Edelstein, P. H., and R. D. Meyer.** 1984. Legionnaires' disease. A review. Chest **85:**114-20.

61. **Edwards, R. A., L. H. Keller, and D. M. Schifferli.** 1998. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene **207:**149-57.

62. **Elend, C., C. Schmeisser, H. Hoebenreich, H. L. Steele, and W. R. Streit.** 2007. Isolation and characterization of a metagenome-derived and cold-active lipase with high stereospecificity for (R)-ibuprofen esters. J Biotechnol **130:**370-7.

63. **Engleberg, N. C., D. J. Drutz, and B. I. Eisenstein.** 1984. Cloning and expression of Legionella pneumophila antigens in Escherichia coli. Infect Immun **44:**222-7.

64. **Feeley, J. C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Rasheed, D. C. Mackel, and W. B. Baine.** 1979. Charcoal-yeast extract agar: primary isolation medium for Legionella pneumophila. J Clin Microbiol **10:**437-41.

65. **Feeley, J. C., G. W. Gorman, R. E. Weaver, D. C. Mackel, and H. W. Smith.** 1978. Primary isolation media for Legionnaires disease bacterium. J Clin Microbiol **8:**320-5.

66. **Feller, G., and C. Gerday.** 2003. Psychrophilic enzymes: hot topics in cold adaptation. Nat Rev Microbiol **1:**200-8.

67. **Feller, G., E. Narinx, J. L. Arpigny, Z. Zekhnini, J. Swings, and C. Gerday.** 1994. Temperature dependence of growth, enzyme secretion and activity of psychrophilic Antarctic bacteria. Applied Microbiology and Biotechnology **41:**477-479.

68. **Fields, B. S., R. F. Benson, and R. E. Besser.** 2002. Legionella and Legionnaires' disease: 25 years of investigation. Clin Microbiol Rev **15:**506-26.

69. **Filloux, A.** 2004. The underlying mechanisms of type II protein secretion. Biochim Biophys Acta **1694:**163-79.

70. **Flieger, A., S. Gong, M. Faigle, S. Stevanovic, N. P. Cianciotto, and B. Neumeister.** 2001. Novel lysophospholipase A secreted by Legionella pneumophila. J Bacteriol **183:**2121-4.

71. **Flieger, A., B. Neumeister, and N. P. Cianciotto.** 2002. Characterization of the gene encoding the major secreted lysophospholipase A of Legionella pneumophila and its role in detoxification of lysophosphatidylcholine. Infect Immun **70:**6094-106.

72. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, S. J. Smith, D. L. Tison, and D. H. Pope.** 1981. Ecological distribution of Legionella pneumophila. Appl Environ Microbiol **41:**9-16.

73. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, and L. Thacker.** 1979. Isolation of Legionella pneumophila from nonepidemic-related aquatic habitats. Appl Environ Microbiol **37:**1239-42.

74. **Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, and P. S. Brachman.** 1977. Legionnaires' disease: description of an epidemic of pneumonia. N Engl J Med **297:**1189-97.

75. **Galan, J. E., and H. Wolf-Watz.** 2006. Protein delivery into eukaryotic cells by type III secretion machines. Nature **444:**567-73.

76. **Gao, L. Y., O. S. Harb, and Y. Abu Kwaik.** 1997. Utilization of similar mechanisms by Legionella pneumophila to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. Infect Immun **65:**4738-46.

77. **Gardy, J. L., M. R. Laird, F. Chen, S. Rey, C. J. Walsh, M. Ester, and F. S. Brinkman.** 2005. PSORTb v.2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. Bioinformatics **21:**617-23.

78. **Garsoux, G., J. Lamotte, C. Gerday, and G. Feller.** 2004. Kinetic and structural optimization to catalysis at low temperatures in a psychrophilic cellulase from the Antarctic bacterium Pseudoalteromonas haloplanktis. Biochem J **384:**247-53.

79. **Ghelardi, E., F. Celandroni, S. Salvetti, D. J. Beecher, M. Gominet, D. Lereclus, A. C. Wong, and S. Senesi.** 2002. Requirement of flhA for swarming differentiation, flagellin export, and secretion of virulence-associated proteins in Bacillus thuringiensis. J Bacteriol **184:**6424-33.

80. **Gibson, F. C., 3rd, A. O. Tzianabos, and F. G. Rodgers.** 1994. Adherence of Legionella pneumophila to U-937 cells, guinea-pig alveolar macrophages, and MRC-5 cells by a novel, complement-independent binding mechanism. Can J Microbiol **40:**865-72.

81. **Glick, T. H., M. B. Gregg, B. Berman, G. Mallison, W. W. Rhodes, Jr., and I. Kassanoff.** 1978. Pontiac fever. An epidemic of unknown etiology in a health department: I. Clinical and epidemiologic aspects. Am J Epidemiol **107:**149-60.

82. **Golbik, R., G. Fischer, and A. R. Fersht.** 1999. Folding of barstar C40A/C82A/P27A and catalysis of the peptidyl-prolyl cis/trans isomerization by human cytosolic cyclophilin (Cyp18). Protein Sci **8:**1505-14.

83. **Gothel, S. F., and M. A. Marahiel.** 1999. Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. Cell Mol Life Sci **55:**423-36.

84. **Goyer, C., and M. S. Ullrich.** 2006. Identification of low-temperature-regulated genes in the fire blight pathogen Erwinia amylovora. Can J Microbiol **52:**468-75.

85. **Graman, P. S., G. A. Quinlan, and J. A. Rank.** 1997. Nosocomial legionellosis traced to a contaminated ice machine. Infect Control Hosp Epidemiol **18:**637-40.

86. **Graumann, P., K. Schroder, R. Schmid, and M. A. Marahiel.** 1996. Cold shock stress-induced proteins in Bacillus subtilis. J Bacteriol **178:**4611-9.

87. **Grindley, N. D., and C. M. Joyce.** 1980. Genetic and DNA sequence analysis of the kanamycin resistance transposon Tn903. Proc Natl Acad Sci U S A **77:**7176-80.

88. **Gualerzi, C. O., A. M. Giuliodori, and C. L. Pon.** 2003. Transcriptional and post-transcriptional control of cold-shock genes. J Mol Biol **331:**527-39.

89. **Hager, A. J., D. L. Bolton, M. R. Pelletier, M. J. Brittnacher, L. A. Gallagher, R. Kaul, S. J. Skerrett, S. I. Miller, and T. Guina.** 2006. Type IV pili-mediated secretion modulates Francisella virulence. Mol Microbiol **62:**227-37.

90. **Hales, L. M., and H. A. Shuman.** 1999. Legionella pneumophila contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. Infect Immun **67:**3662-6.

91. **Hammer, B. K., and M. S. Swanson.** 1999. Co-ordination of legionella pneumophila virulence with entry into stationary phase by ppGpp. Mol Microbiol **33:**721-31.

92. **Hammer, B. K., E. S. Tateda, and M. S. Swanson.** 2002. A two-component regulator induces the transmission phenotype of stationary-phase Legionella pneumophila. Mol Microbiol **44:**107-18.

93. **Hayano, T., N. Takahashi, S. Kato, N. Maki, and M. Suzuki.** 1991. Two distinct forms of peptidylprolyl-cis-trans-isomerase are expressed separately in periplasmic and cytoplasmic compartments of Escherichia coli cells. Biochemistry **30:**3041-8.

94. **Hayashi, H., H. Mizuguchi, I. Miyahara, Y. Nakajima, K. Hirotsu, and H. Kagamiyama.** 2003. Conformational change in aspartate aminotransferase on substrate binding induces strain in the catalytic group and enhances catalysis. J Biol Chem **278:**9481-8.

95. **Heller, R., C. Holler, R. Sussmuth, and K. O. Gundermann.** 1998. Effect of salt concentration and temperature on survival of Legionella pneumophila. Lett Appl Microbiol **26:**64-8.

96. **Henderson, I. R., F. Navarro-Garcia, M. Desvaux, R. C. Fernandez, and D. Ala'Aldeen.** 2004. Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev **68:**692-744.

97. **Henke, M., and K. M. Seidel.** 1986. Association between Legionella pneumophila and amoebae in water. Isr J Med Sci **22:**690-5.

98. **Hermans, P. W., P. V. Adrian, C. Albert, S. Estevao, T. Hoogenboezem, I. H. Luijendijk, T. Kamphausen, and S. Hammerschmidt.** 2006. The streptococcal lipoprotein rotamase A (SlrA) is a functional peptidyl-prolyl isomerase involved in pneumococcal colonization. J Biol Chem **281:**968-76.

99. **Heuner, K., L. Bender-Beck, B. C. Brand, P. C. Luck, K. H. Mann, R. Marre, M. Ott, and J. Hacker.** 1995. Cloning and genetic characterization of the flagellum subunit gene (flaA) of Legionella pneumophila serogroup 1. Infect Immun **63:**2499-507.

100. **Heuner, K., B. C. Brand, and J. Hacker.** 1999. The expression of the flagellum of Legionella pneumophila is modulated by different environmental factors. FEMS Microbiol Lett **175:**69-77.

101. **Hickey, E. K., and N. P. Cianciotto.** 1997. An iron- and fur-repressed Legionella pneumophila gene that promotes intracellular infection and encodes a protein with similarity to the Escherichia coli aerobactin synthetases. Infect Immun **65:**133-43.

102. **Hoebe, C. J., J. J. Cluitmans, and J. H. Wagenvoort.** 1998. Two fatal cases of nosocomial Legionella pneumophila pneumonia associated with a contaminated cold water supply. Eur J Clin Microbiol Infect Dis **17:**740.

103. **Hoebe, C. J., J. J. Cluitmans, J. H. Wagenvoort, W. J. van Leeuwen, and M. A. Bilkert-Mooiman.** 1999. [Cold tap water as a source of fatal nosocomial pneumonia due to Legionella pneumophila in a rehabilitation center]. Ned Tijdschr Geneeskd **143:**1041-5.

104. **Holland, I. B., L. Schmitt, and J. Young.** 2005. Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review). Mol Membr Biol **22:**29-39.

105. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (Legionella pneumophila) in human monocytes. J Exp Med **158:**1319-31.

106. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (Legionella pneumophila) inhibits phagosome-lysosome fusion in human monocytes. J Exp Med **158:**2108-26.

107. **Horwitz, M. A.** 1984. Phagocytosis of the Legionnaires' disease bacterium (Legionella pneumophila) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell **36:**27-33.

108. **Horwitz, M. A., and F. R. Maxfield.** 1984. Legionella pneumophila inhibits acidification of its phagosome in human monocytes. J Cell Biol **99:**1936-43.

109. **Hossain, M. M., and H. Nakamoto.** 2002. HtpG plays a role in cold acclimation in cyanobacteria. Curr Microbiol **44:**291-6.

110. **Hou, Y., T. Wang, H. Long, and H. Zhu.** 2007. Cloning, sequencing and expression analysis of the first cellulase gene encoding cellobiohydrolase 1 from a cold-adaptive Penicillium chrysogenum FS010. Acta Biochim Biophys Sin (Shanghai) **39:**101-7.

111. **Hou, Y. H., T. H. Wang, H. Long, and H. Y. Zhu.** 2006. Novel cold-adaptive Penicillium strain FS010 secreting thermo-labile xylanase isolated from Yellow Sea. Acta Biochim Biophys Sin (Shanghai) **38:**142-9.

112. **Howard, S. P., J. Critch, and A. Bedi.** 1993. Isolation and analysis of eight exe genes and their involvement in extracellular protein secretion and outer membrane assembly in Aeromonas hydrophila. J Bacteriol **175:**6695-703.

113. **Hurme, R., and M. Rhen.** 1998. Temperature sensing in bacterial gene regulation--what it all boils down to. Mol Microbiol **30:**1-6.

114. **Hussong, D., R. R. Colwell, and W. D. Burge.** 1987. Viable Legionella pneumophila not detectable by culture on agar media. Nature Biotechnology **5:**947-950.

115. **Huston, A. L., B. Methe, and J. W. Deming.** 2004. Purification, characterization, and sequencing of an extracellular cold-active aminopeptidase produced by marine psychrophile Colwellia psychrerythraea strain 34H. Appl Environ Microbiol **70:**3321-8.

116. **Ideno, A., T. Yoshida, T. Iida, M. Furutani, and T. Maruyama.** 2001. FK506-binding protein of the hyperthermophilic archaeum, Thermococcus sp. KS-1, a cold-shock-inducible peptidyl-prolyl cis-trans isomerase with activities to trap and refold denatured proteins. Biochem J **357:**465-71.

117. **Jacob-Dubuisson, F., R. Fernandez, and L. Coutte.** 2004. Protein secretion through autotransporter and two-partner pathways. Biochim Biophys Acta **1694:**235-57.

118. **Jacobi, S., and K. Heuner.** 2003. Description of a putative type I secretion system in Legionella pneumophila. Int J Med Microbiol **293:**349-58.

119. **Jernigan, D. B., J. Hofmann, M. S. Cetron, C. A. Genese, J. P. Nuorti, B. S. Fields, R. F. Benson, R. J. Carter, P. H. Edelstein, I. C. Guerrero, S. M. Paul, H. B. Lipman, and R. Breiman.** 1996. Outbreak of Legionnaires' disease among cruise ship passengers exposed to a contaminated whirlpool spa. Lancet **347:**494-9.

120. **Johansson, P. J., K. Andersson, T. Wiebe, C. Schalen, and S. Bernander.** 2006. Nosocomial transmission of Legionella pneumophila to a child from a hospital's cold-water supply. Scand J Infect Dis **38:**1023-7.

121. **Johnson, T. L., J. Abendroth, W. G. Hol, and M. Sandkvist.** 2006. Type II secretion: from structure to function. FEMS Microbiol Lett **255:**175-86.

122. **Journet, L., K. T. Hughes, and G. R. Cornelis.** 2005. Type III secretion: a secretory pathway serving both motility and virulence (review). Mol Membr Biol **22:**41-50.

123. **Kandror, O., A. DeLeon, and A. L. Goldberg.** 2002. Trehalose synthesis is induced upon exposure of Escherichia coli to cold and is essential for viability at low temperatures. Proc Natl Acad Sci U S A **99:**9727-32.

124. **Kandror, O., and A. L. Goldberg.** 1997. Trigger factor is induced upon cold shock and enhances viability of Escherichia coli at low temperatures. Proc Natl Acad Sci U S A **94:**4978-81.

125. **Kaneda, T.** 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev **55:**288-302.

126. **Kannan, K., K. L. Janiyani, S. Shivaji, and M. K. Ray.** 1998. Histidine utilisation operon (hut) is upregulated at low temperature in the antarctic psychrotrophic bacterium Pseudomonas syringae. FEMS Microbiol Lett **161:**7-14.

127. **Kawahara, H., N. Koda, M. Oshio, and H. Obata.** 2000. A cold acclimation protein with refolding activity on frozen denatured enzymes. Biosci Biotechnol Biochem **64:**2668-74.

128. **Kennan, R. M., O. P. Dhungyel, R. J. Whittington, J. R. Egerton, and J. I. Rood.** 2001. The type IV fimbrial subunit gene (fimA) of Dichelobacter nodosus is essential for virulence, protease secretion, and natural competence. J Bacteriol **183:**4451-8.

129. **Kim, N., D. L. Weeks, J. M. Shin, D. R. Scott, M. K. Young, and G. Sachs.** 2002. Proteins released by Helicobacter pylori in vitro. J Bacteriol **184:**6155-62.

130. **Kirn, T. J., N. Bose, and R. K. Taylor.** 2003. Secretion of a soluble colonization factor by the TCP type 4 pilus biogenesis pathway in Vibrio cholerae. Mol Microbiol **49:**81-92.

131. **Kohler, R., J. Fanghanel, B. Konig, E. Luneberg, M. Frosch, J. U. Rahfeld, R. Hilgenfeld, G. Fischer, J. Hacker, and M. Steinert.** 2003. Biochemical and functional analyses of the Mip protein: influence of the N-terminal half and of peptidylprolyl isomerase activity on the virulence of Legionella pneumophila. Infect Immun **71:**4389-97.

132. **Konkel, M. E., J. D. Klena, V. Rivera-Amill, M. R. Monteville, D. Biswas, B. Raphael, and J. Mickelson.** 2004. Secretion of virulence proteins from Campylobacter jejuni is dependent on a functional flagellar export apparatus. J Bacteriol **186:**3296-303.

133. **Kramer, M. H., and T. E. Ford.** 1994. Legionellosis: ecological factors of an environmentally 'new' disease. Zentralbl Hyg Umweltmed **195:**470-82.

134. **Kuehn, M. J., and N. C. Kesty.** 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev **19:**2645-55.

135. **Kuroki, H., H. Miyamoto, K. Fukuda, H. Iihara, Y. Kawamura, M. Ogawa, Y. Wang, T. Ezaki, and H. Taniguchi.** 2007. Legionella impletisoli sp. nov. and Legionella yabuuchiae sp. nov., isolated from soils contaminated with industrial wastes in Japan. Syst Appl Microbiol **30:**273-9.

136. **Kusnetsov, J. M., E. Ottoila, and P. J. Martikainen.** 1996. Growth, respiration and survival of Legionella pneumophila at high temperatures. J Appl Bacteriol **81:**341-7.

137. **La Scola, B., R. J. Birtles, G. Greub, T. J. Harrison, R. M. Ratcliff, and D. Raoult.** 2004. Legionella drancourtii sp. nov., a strictly intracellular amoebal pathogen. Int J Syst Evol Microbiol **54:**699-703.

138. **Lambert, M. A., and C. W. Moss.** 1989. Cellular fatty acid compositions and isoprenoid quinone contents of 23 Legionella species. J Clin Microbiol **27:**465-73.

139. **LaPointe, C. F., and R. K. Taylor.** 2000. The type 4 prepilin peptidases comprise a novel family of aspartic acid proteases. J Biol Chem **275:**1502-10.

140. **Lebeau, I., E. Lammertyn, E. De Buck, L. Maes, N. Geukens, L. Van Mellaert, L. Arckens, J. Anne, and S. Clerens.** 2005. First proteomic analysis of Legionella pneumophila based on its developing genome sequence. Res Microbiol **156:**119-29.

141. **Lee, J. V., and A. A. West.** 1991. Survival and growth of Legionella species in the environment. Soc Appl Bacteriol Symp Ser **20:**121S-129S.

142. **Liang, M. D., A. Bagchi, H. S. Warren, M. M. Tehan, J. A. Trigilio, L. K. Beasley-Topliffe, B. L. Tesini, J. C. Lazzaroni, M. J. Fenton, and J. Hellman.** 2005. Bacterial peptidoglycan-associated lipoprotein: a naturally occurring toll-like receptor 2 agonist that is shed into serum and has synergy with lipopolysaccharide. J Infect Dis **191:**939-48.

143. **Liles, M. R.** 1998. Type II secretion of virulence factors and iron acquisition mechanisms of Legionella pneumophila. Dissertation at Northwestern University.

144. **Liles, M. R., P. H. Edelstein, and N. P. Cianciotto.** 1999. The prepilin peptidase is required for protein secretion by and the virulence of the intracellular pathogen Legionella pneumophila. Mol Microbiol **31:**959-70.

145. **Liles, M. R., V. K. Viswanathan, and N. P. Cianciotto.** 1998. Identification and temperature regulation of Legionella pneumophila genes involved in type IV pilus biogenesis and type II protein secretion. Infect Immun **66:**1776-82.

146. **Lindeberg, M., and A. Collmer.** 1992. Analysis of eight out genes in a cluster required for pectic enzyme secretion by Erwinia chrysanthemi: sequence comparison with secretion genes from other gram-negative bacteria. J Bacteriol **174:**7385-97.

147. **Liu, S., J. E. Graham, L. Bigelow, P. D. Morse, 2nd, and B. J. Wilkinson.** 2002. Identification of Listeria monocytogenes genes expressed in response to growth at low temperature. Appl Environ Microbiol **68:**1697-705.

148. **Lu, Y. J., Y. M. Zhang, and C. O. Rock.** 2004. Product diversity and regulation of type II fatty acid synthases. Biochem Cell Biol **82:**145-55.

149. **Lummis, S. C., D. L. Beene, L. W. Lee, H. A. Lester, R. W. Broadhurst, and D. A. Dougherty.** 2005. Cis-trans isomerization at a proline opens the pore of a neurotransmitter-gated ion channel. Nature **438:**248-52.

150. **Mahoney, F. J., C. W. Hoge, T. A. Farley, J. M. Barbaree, R. F. Breiman, R. F. Benson, and L. M. McFarland.** 1992. Communitywide outbreak of Legionnaires' disease associated with a grocery store mist machine. J Infect Dis **165:**736-9.

151. **Mansilla, M. C., and D. de Mendoza.** 2005. The Bacillus subtilis desaturase: a model to understand phospholipid modification and temperature sensing. Arch Microbiol **183:**229-35.

152. **Marciano-Cabral, F., and G. Cabral.** 2003. Acanthamoeba spp. as agents of disease in humans. Clin Microbiol Rev **16:**273-307.

153. **Marston, B. J., H. B. Lipman, and R. F. Breiman.** 1994. Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. Arch Intern Med **154:**2417-22.

154. **Mauchline, W. S., B. W. James, R. B. Fitzgeorge, P. J. Dennis, and C. W. Keevil.** 1994. Growth temperature reversibly modulates the virulence of Legionella pneumophila. Infect Immun **62:**2995-7.

155. **McClain, M. S., and N. C. Engleberg.** 1996. Construction of an alkaline phosphatase fusion-generating transposon, mTn10phoA. Gene **170:**147-8.

156. **McDade, J. E., D. J. Brenner, and F. M. Bozeman.** 1979. Legionnaires' disease bacterium isolated in 1947. Ann Intern Med **90:**659-61.

157. **McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, and W. R. Dowdle.** 1977. Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. N Engl J Med **297:**1197-203.

158. **Moffat, J. F., P. H. Edelstein, D. P. Regula, Jr., J. D. Cirillo, and L. S. Tompkins.** 1994. Effects of an isogenic Zn-metalloprotease-deficient mutant of Legionella pneumophila in a guinea-pig pneumonia model. Mol Microbiol **12:**693-705.

159. **Moffat, J. F., and L. S. Tompkins.** 1992. A quantitative model of intracellular growth of Legionella pneumophila in Acanthamoeba castellanii. Infect Immun **60:**296-301.

160. **Molofsky, A. B., and M. S. Swanson.** 2004. Differentiate to thrive: lessons from the Legionella pneumophila life cycle. Mol Microbiol **53:**29-40.

161. **Molofsky, A. B., and M. S. Swanson.** 2003. Legionella pneumophila CsrA is a pivotal repressor of transmission traits and activator of replication. Mol Microbiol **50:**445-61.

162. **Morgan-Kiss, R. M., J. C. Priscu, T. Pocock, L. Gudynaite-Savitch, and N. P. Huner.** 2006. Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. Microbiol Mol Biol Rev **70:**222-52.

163. **Muder, R. R., V. L. Yu, and A. H. Woo.** 1986. Mode of transmission of Legionella pneumophila. A critical review. Arch Intern Med **146:**1607-12.

164. **Munoz-Elias, E. J., and J. D. McKinney.** 2006. Carbon metabolism of intracellular bacteria. Cell Microbiol **8:**10-22.

165. **Murga, R., T. S. Forster, E. Brown, J. M. Pruckler, B. S. Fields, and R. M. Donlan.** 2001. Role of biofilms in the survival of Legionella pneumophila in a model potable-water system. Microbiology **147:**3121-6.

166. **Neill, M. A., G. W. Gorman, C. Gibert, A. Roussel, A. W. Hightower, R. M. McKinney, and C. V. Broome.** 1985. Nosocomial legionellosis, Paris, France. Evidence for transmission by potable water. Am J Med **78:**581-8.

167. **Neumann, B., A. Pospiech, and H. U. Schairer.** 1992. Rapid isolation of genomic DNA from gram-negative bacteria. Trends Genet **8:**332-3.

168. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng **10:**1-6.

169. **Ninio, S., and C. R. Roy.** 2007. Effector proteins translocated by Legionella pneumophila: strength in numbers. Trends Microbiol **15:**372-80.

170. **Nudleman, E., and D. Kaiser.** 2004. Pulling together with type IV pili. J Mol Microbiol Biotechnol **7:**52-62.

171. **O'Connell, W. A., J. M. Bangsborg, and N. P. Cianciotto.** 1995. Characterization of a Legionella micdadei mip mutant. Infect Immun **63:**2840-5.

172. **Ortiz-Roque, C. M., and T. C. Hazen.** 1987. Abundance and distribution of Legionellaceae in Puerto Rican waters. Appl Environ Microbiol **53:**2231-6.

173. **Osterholm, M. T., T. D. Chin, D. O. Osborne, H. B. Dull, A. G. Dean, D. W. Fraser, P. S. Hayes, and W. N. Hall.** 1983. A 1957 outbreak of Legionnaire's disease associated with a meat packing plant. American Journal of Epidemiology **117:**60-67.

174. **Ott, M., P. Messner, J. Heesemann, R. Marre, and J. Hacker.** 1991. Temperature-dependent expression of flagella in Legionella. J Gen Microbiol **137:**1955-61.

175. **Palmer, T., F. Sargent, and B. C. Berks.** 2005. Export of complex cofactor-containing proteins by the bacterial Tat pathway. Trends Microbiol **13:**175-80.

176. **Park, M. Y., K. S. Ko, H. K. Lee, M. S. Park, and Y. H. Kook.** 2003. Legionella busanensis sp. nov., isolated from cooling tower water in Korea. Int J Syst Evol Microbiol **53:**77-80.

177. **Payne, N. R., and M. A. Horwitz.** 1987. Phagocytosis of Legionella pneumophila is mediated by human monocyte complement receptors. J Exp Med **166:**1377-89.

178. **Peabody, C. R., Y. J. Chung, M. R. Yen, D. Vidal-Ingigliardi, A. P. Pugsley, and M. H. Saier, Jr.** 2003. Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. Microbiology **149:**3051-72.

179. **Pedersen, L. L., M. Radulic, M. Doric, and Y. Abu Kwaik.** 2001. HtrA homologue of Legionella pneumophila: an indispensable element for intracellular infection of mammalian but not protozoan cells. Infect Immun **69:**2569-79.

180. **Pepe, J. C., and S. Lory.** 1998. Amino acid substitutions in PilD, a bifunctional enzyme of Pseudomonas aeruginosa. Effect on leader peptidase and N-methyltransferase activities in vitro and in vivo. J Biol Chem **273:**19120-9.

181. **Pessi, G., C. Blumer, and D. Haas.** 2001. lacZ fusions report gene expression, don't they? Microbiology **147:**1993-5.

182. **Phadtare, S., J. Alsina, and M. Inouye.** 1999. Cold-shock response and cold-shock proteins. Curr Opin Microbiol **2:**175-80.

183. **Phadtare, S., and M. Inouye.** 2004. Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-csp-deletion strains of Escherichia coli. J Bacteriol **186:**7007-14.

184. **Pissavin, C., and N. Hugouvieux-Cotte-Pattat.** 1997. Characterization of a periplasmic peptidyl-prolyl cis-trans isomerase in Erwinia chrysanthemi. FEMS Microbiol Lett **157:**59-65.

185. **Porankiewicz, J., and A. K. Clarke.** 1997. Induction of the heat shock protein ClpB affects cold acclimation in the cyanobacterium Synechococcus sp. strain PCC 7942. J Bacteriol **179:**5111-7.

186. **Pukatzki, S., A. T. Ma, A. T. Revel, D. Sturtevant, and J. J. Mekalanos.** 2007. Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. Proc Natl Acad Sci U S A **104:**15508-13.

187. **Pukatzki, S., A. T. Ma, D. Sturtevant, B. Krastins, D. Sarracino, W. C. Nelson, J. F. Heidelberg, and J. J. Mekalanos.** 2006. Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system. Proc Natl Acad Sci U S A **103:**1528-33.

188. **Rey, S., M. Acab, J. L. Gardy, M. R. Laird, K. deFays, C. Lambert, and F. S. Brinkman.** 2005. PSORTdb: a protein subcellular localization database for bacteria. Nucleic Acids Res **33:**D164-8.

189. **Ridenour, D. A., S. L. Cirillo, S. Feng, M. M. Samrakandi, and J. D. Cirillo.** 2003. Identification of a gene that affects the efficiency of host cell infection by Legionella pneumophila in a temperature-dependent fashion. Infect Immun **71:**6256-63.

190. **Rivera, J. M., L. Aguilar, J. J. Granizo, A. Vos-Arenilla, M. J. Gimenez, J. M. Aguiar, and J. Prieto.** 2007. Isolation of Legionella species/serogroups from water cooling systems compared with potable water systems in Spanish healthcare facilities. J Hosp Infect.

191. **Robey, M., and N. P. Cianciotto.** 2002. Legionella pneumophila feoAB promotes ferrous iron uptake and intracellular infection. Infect Immun **70:**5659-69.

192. **Rodgers, F. G., P. W. Greaves, A. D. Macrae, and M. J. Lewis.** 1980. Electron microscopic evidence of flagella and pili on Legionella pneumophila. J Clin Pathol **33:**1184-8.

193. **Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil.** 1994. Influence of temperature and plumbing material selection on biofilm formation and growth of Legionella pneumophila in a model potable water system containing complex microbial flora. Appl Environ Microbiol **60:**1585-92.

194. **Roig, J., C. Domingo, and J. Morera.** 1994. Legionnaires' disease. Chest **105:**1817-25.

195. **Rossier, O., and N. P. Cianciotto.** 2005. The Legionella pneumophila tatB gene facilitates secretion of phospholipase C, growth under iron-limiting conditions, and intracellular infection. Infect Immun **73:**2020-32.

196. **Rossier, O., and N. P. Cianciotto.** 2001. Type II protein secretion is a subset of the PilD-dependent processes that facilitate intracellular infection by Legionella pneumophila. Infect Immun **69:**2092-8.

197. **Rossier, O., J. Dao, and N. P. Cianciotto.** 2007. The Type II Secretion System of Legionella pneumophila Elaborates Two Aminopeptidases as well as a Metalloprotease

That Contributes to Differential Infection Among Protozoan Hosts. Epub. doi:10.1128/AEM.02512-07.

198. **Rossier, O., S. R. Starkenburg, and N. P. Cianciotto.** 2004. Legionella pneumophila type II protein secretion promotes virulence in the A/J mouse model of Legionnaires' disease pneumonia. Infect Immun **72:**310-21.

199. **Russell, N. J.** 2000. Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles **4:**83-90.

200. **Sabria, M., and V. L. Yu.** 2002. Hospital-acquired legionellosis: solutions for a preventable infection. Lancet Infect Dis **2:**368-73.

201. **Sakamoto, T., and N. Murata.** 2002. Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress. Curr Opin Microbiol **5:**208-10.

202. **Sandkvist, M.** 2001. Biology of type II secretion. Mol Microbiol **40:**271-83.

203. **Sandkvist, M.** 2001. Type II secretion and pathogenesis. Infect Immun **69:**3523-35.

204. **Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey, V. J. DiRita, and M. Bagdasarian.** 1997. General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in Vibrio cholerae. J Bacteriol **179:**6994-7003.

205. **Saunders, N. F., C. Ng, M. Raftery, M. Guilhaus, A. Goodchild, and R. Cavicchioli.** 2006. Proteomic and computational analysis of secreted proteins with type I signal peptides from the Antarctic archaeon Methanococcoides burtonii. J Proteome Res **5:**2457-64.

206. **Schmidt, B., T. Tradler, J. U. Rahfeld, B. Ludwig, B. Jain, K. Mann, K. P. Rucknagel, B. Janowski, A. Schierhorn, G. Kullertz, J. Hacker, and G. Fischer.** 1996. A cyclophilin-like peptidyl-prolyl cis/trans isomerase from Legionella pneumophila--characterization, molecular cloning and overexpression. Mol Microbiol **21:**1147-60.

207. **Schulze-Robbecke, R., M. Rodder, and M. Exner.** 1987. [Multiplication and killing temperatures of naturally occurring legionellas]. Zentralbl Bakteriol Mikrobiol Hyg [B] **184:**495-500.

208. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and xylE fusion analysis in Pseudomonas aeruginosa. Gene **158:**15-22.

209. **Segal, G., M. Feldman, and T. Zusman.** 2005. The Icm/Dot type-IV secretion systems of Legionella pneumophila and Coxiella burnetii. FEMS Microbiol Rev **29:**65-81.

210. **Segal, G., J. J. Russo, and H. A. Shuman.** 1999. Relationships between a new type IV secretion system and the icm/dot virulence system of Legionella pneumophila. Mol Microbiol **34:**799-809.

211. **Sheehan, K. B., J. M. Henson, and M. J. Ferris.** 2005. Legionella species diversity in an acidic biofilm community in Yellowstone National Park. Appl Environ Microbiol **71:**507-11.

212. **Shi, C., V. Forsbach-Birk, R. Marre, and T. L. McNealy.** 2006. The Legionella pneumophila global regulatory protein LetA affects DotA and Mip. Int J Med Microbiol **296:**15-24.

213. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy lac-based cloning vectors for protein and operon fusions. Gene **53:**85-96.

214. **Skaliy, P., and H. V. McEachern.** 1979. Survival of the Legionnaires' disease bacterium in water. Ann Intern Med **90:**662-3.

215. **Soderberg, M. A., O. Rossier, and N. P. Cianciotto.** 2004. The type II protein secretion system of Legionella pneumophila promotes growth at low temperatures. J Bacteriol **186:**3712-20.

216. **Song, Y. C., S. Jin, H. Louie, D. Ng, R. Lau, Y. Zhang, R. Weerasekera, S. Al Rashid, L. A. Ward, S. D. Der, and V. L. Chan.** 2004. FlaC, a protein of Campylobacter jejuni TGH9011 (ATCC43431) secreted through the flagellar apparatus, binds epithelial cells and influences cell invasion. Mol Microbiol **53:**541-53.

217. **Starkenburg, S. R., J. M. Casey, and N. P. Cianciotto.** 2004. Siderophore activity among members of the Legionella genus. Curr Microbiol **49:**203-7.

218. **Steele, T. W., J. Lanser, and N. Sangster.** 1990. Isolation of Legionella longbeachae serogroup 1 from potting mixes. Appl Environ Microbiol **56:**49-53.

219. **Steinert, M., L. Emody, R. Amann, and J. Hacker.** 1997. Resuscitation of viable but nonculturable Legionella pneumophila Philadelphia JR32 by Acanthamoeba castellanii. Appl Environ Microbiol **63:**2047-53.

220. **Stone, B. J., and Y. Abu Kwaik.** 1998. Expression of multiple pili by Legionella pneumophila: identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. Infect Immun **66:**1768-75.

221. **Stout, J. E., and V. L. Yu.** 1997. Legionellosis. N Engl J Med **337:**682-7.

222. **Strom, M. S., P. Bergman, and S. Lory.** 1993. Identification of active-site cysteines in the conserved domain of PilD, the bifunctional type IV pilin leader peptidase/N-methyltransferase of Pseudomonas aeruginosa. J Biol Chem **268:**15788-94.

223. **Strom, M. S., and S. Lory.** 1993. Structure-function and biogenesis of the type IV pili. Annu Rev Microbiol **47:**565-96.

224. **Sturgill-Koszycki, S., and M. S. Swanson.** 2000. Legionella pneumophila replication vacuoles mature into acidic, endocytic organelles. J Exp Med **192:**1261-72.

225. **Suzuki, Y., M. Haruki, K. Takano, M. Morikawa, and S. Kanaya.** 2004. Possible involvement of an FKBP family member protein from a psychrotrophic bacterium Shewanella sp. SIB1 in cold-adaptation. Eur J Biochem **271:**1372-81.

226. **Swanson, M. S., and B. K. Hammer.** 2000. Legionella pneumophila pathogesesis: a fateful journey from amoebae to macrophages. Annu Rev Microbiol **54:**567-613.

227. **Terranova, W., M. L. Cohen, and D. W. Fraser.** 1978. 1974 outbreak of Legionnaires' Disease diagnosed in 1977. Clinical and epidemiological features. Lancet **2:**122-4.

228. **Thacker, S. B., J. V. Bennett, T. F. Tsai, D. W. Fraser, J. E. McDade, C. C. Shepard, K. H. Williams, Jr., W. H. Stuart, H. B. Dull, and T. C. Eickhoff.** 1978. An outbreak in 1965 of severe respiratory illness caused by the Legionnaires' disease bacterium. J Infect Dis **138:**512-9.

229. **Thanassi, D. G., C. Stathopoulos, A. Karkal, and H. Li.** 2005. Protein secretion in the absence of ATP: the autotransporter, two-partner secretion and chaperone/usher pathways of gram-negative bacteria (review). Mol Membr Biol **22:**63-72.

230. **Thieringer, H. A., P. G. Jones, and M. Inouye.** 1998. Cold shock and adaptation. Bioessays **20:**49-57.

231. **Tiaden, A., T. Spirig, S. S. Weber, H. Bruggemann, R. Bosshard, C. Buchrieser, and H. Hilbi.** 2007. The Legionella pneumophila response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. Cell Microbiol.

232. **Tilney, L. G., O. S. Harb, P. S. Connelly, C. G. Robinson, and C. R. Roy.** 2001. How the parasitic bacterium Legionella pneumophila modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J Cell Sci **114:**4637-50.

233. **Tison, D. L., D. H. Pope, W. B. Cherry, and C. B. Fliermans.** 1980. Growth of Legionella pneumophila in association with blue-green algae (cyanobacteria). Appl Environ Microbiol **39:**456-9.

234. **Touati, D.** 2000. Iron and oxidative stress in bacteria. Arch Biochem Biophys **373:**1-6.

235. **Tsai, T. F., D. R. Finn, B. D. Plikaytis, W. McCauley, S. M. Martin, and D. W. Fraser.** 1979. Legionnaires' disease: clinical features of the epidemic in Philadelphia. Ann Intern Med **90:**509-17.

236. **Turkiewicz, M., M. Pazgier, H. Kalinowska, and S. Bielecki.** 2001. Properties of cold-adapted subtilisine-like proteinase of endemic yeast Leucosporidium antarcticum. Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet **66:**329-32.

237. **Ullrich, M., and C. L. Bender.** 1994. The biosynthetic gene cluster for coronamic acid, an ethylcyclopropyl amino acid, contains genes homologous to amino acid-activating enzymes and thioesterases. J Bacteriol **176:**7574-86.

238. **Viswanathan, V. K., P. H. Edelstein, C. D. Pope, and N. P. Cianciotto.** 2000. The Legionella pneumophila iraAB locus is required for iron assimilation, intracellular infection, and virulence. Infect Immun **68:**1069-79.

239. **von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem **133:**17-21.

240. **Vorachek-Warren, M. K., S. M. Carty, S. Lin, R. J. Cotter, and C. R. Raetz.** 2002. An Escherichia coli mutant lacking the cold shock-induced palmitoleoyltransferase of lipid A biosynthesis: absence of unsaturated acyl chains and antibiotic hypersensitivity at 12 degrees C. J Biol Chem **277:**14186-93.

241. **Voulhoux, R., G. Ball, B. Ize, M. L. Vasil, A. Lazdunski, L. F. Wu, and A. Filloux.** 2001. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. Embo J **20:**6735-41.

242. **Wadowsky, R. M., L. J. Butler, M. K. Cook, S. M. Verma, M. A. Paul, B. S. Fields, G. Keleti, J. L. Sykora, and R. B. Yee.** 1988. Growth-supporting activity for Legionella pneumophila in tap water cultures and implication of hartmannellid amoebae as growth factors. Appl Environ Microbiol **54:**2677-82.

243. **Wadowsky, R. M., R. Wolford, A. M. McNamara, and R. B. Yee.** 1985. Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring Legionella pneumophila in potable water. Appl Environ Microbiol **49:**1197-205.

244. **Wadowsky, R. M., and R. B. Yee.** 1983. Satellite growth of Legionella pneumophila with an environmental isolate of Flavobacterium breve. Appl Environ Microbiol **46:**1447-9.

245. **Wagner, C., A. S. Khan, T. Kamphausen, B. Schmausser, C. Unal, U. Lorenz, G. Fischer, J. Hacker, and M. Steinert.** 2007. Collagen binding protein Mip enables Legionella pneumophila to transmigrate through a barrier of NCI-H292 lung epithelial cells and extracellular matrix. Cell Microbiol **9:**450-62.

246. **Weber, M. H., and M. A. Marahiel.** 2003. Bacterial cold shock responses. Sci Prog **86:**9-75.

247. **Woo, A. H., A. Goetz, and V. L. Yu.** 1992. Transmission of Legionella by respiratory equipment and aerosol generating devices. Chest **102:**1586-90.

248. **Yaffe, M. B., M. Schutkowski, M. Shen, X. Z. Zhou, P. T. Stukenberg, J. U. Rahfeld, J. Xu, J. Kuang, M. W. Kirschner, G. Fischer, L. C. Cantley, and K. P. Lu.** 1997. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science **278:**1957-60.

249. **Yee, R. B., and R. M. Wadowsky.** 1982. Multiplication of Legionella pneumophila in unsterilized tap water. Appl Environ Microbiol **43:**1330-4.

250. **Young, G. M., D. H. Schmiel, and V. L. Miller.** 1999. A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. Proc Natl Acad Sci U S A **96:**6456-61.

251. **Zink, S. D., L. Pedersen, N. P. Cianciotto, and Y. Abu-Kwaik.** 2002. The Dot/Icm type IV secretion system of Legionella pneumophila is essential for the induction of apoptosis in human macrophages. Infect Immun **70:**1657-63.

252. **Zinkevich, V., L. Popova, V. Kryukov, A. Abadjieva, I. Bogdarina, P. Janscak, and K. Firman.** 1997. The HsdR subunit of R.EcoR124II: cloning and over-expression of the gene and unexpected properties of the subunit. Nucleic Acids Res **25:**503-11.