The novel *Legionella pneumophila* type II secretion substrate NttC contributes to infection of amoebae *Hartmannella vermiformis* and *Willaertia magna*

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The type II protein secretion (T2S) system of *Legionella pneumophila* secretes over 25 proteins, including novel proteins that have no similarity to proteins of known function. T2S is also critical for the ability of *L. pneumophila* to grow within its natural amoebal hosts, including *Acanthamoeba castellani*, *Hartmannella vermiformis* and *Naegleria lovaniensis*. Thus, T2S has an important role in the natural history of legionnaires’ disease. Our previous work demonstrated that the novel T2S substrate NttA promotes intracellular infection of *A. castellani*, whereas the secreted RNase SrnA, acyltransferase PlaC, and metalloprotease ProA all promote infection of *H. vermiformis* and *N. lovaniensis*. In this study, we determined that another novel T2S substrate that is specific to *Legionella*, designated NttC, is unique in being required for intracellular infection of *H. vermiformis* but not for infection of *N. lovaniensis* or *A. castellani*. Expanding our repertoire of amoebal hosts, we determined that *Willaertia magna* is susceptible to infection by *L. pneumophila* strains 130b, Philadelphia-1 and Paris. Furthermore, T2S and, more specifically, NttA, NttC and PlaC were required for infection of *W. magna*. Taken together, these data demonstrate that the T2S system of *L. pneumophila* is critical for infection of at least four types of aquatic amoebae and that the importance of the individual T2S substrates varies in a host cell-specific fashion. Finally, it is now clear that novel T2S-dependent proteins that are specific to the genus *Legionella* are particularly important for *L. pneumophila* infection of key, environmental hosts.

INTRODUCTION

*Legionella pneumophila* is a Gram-negative bacterium that is the agent of legionnaires’ disease, a serious form of pneumonia (Edelstein & Cianciotto, 2010). *L. pneumophila* is widespread in both natural fresh waters and man-made water systems (Alary & Joly, 1991; Fliermans et al., 1981; Mouchtouri et al., 2007). Human infection most often occurs via the inhalation of *L. pneumophila*-containing water droplets that are produced by man-made devices (Pagnier et al., 2009). The environmental persistence of *L. pneumophila* is dependent upon its ability to infect and grow in amoebae (Lau & Ashbolt, 2009; Taylor et al., 2009; Thomas et al., 2010). *Legionella*-laden amoebae or amoebal vesicles and cysts harbouring bacteria might also be part of the inoculum that precipitates lung infection (Berk et al., 1998; Brieland et al., 1997). Amoebae belonging to the genera *Acanthamoeba*, *Echinamoeba*, *Hartmannella*, *Naegleria*, *Vahlkampfia* and *Willaertia* coexist with *L. pneumophila* in water habitats, and at least 20 species representing these genera support *L. pneumophila* intracellular growth (Anand et al., 1983; Barbaree et al., 1986; Buse & Ashbolt, 2011; Cianciotto & Fields, 1992; Declerck et al., 2007; Dey et al., 2009; Fields et al., 1989; Fields, 1996; Harada et al., 2010; Harf & Monteil, 1988; Henke & Seidel, 1986; Holden et al., 1984; Hsu et al., 2011; Michel et al., 1998; Miyamoto et al., 2003; Molmeret et al., 2001; Newsome et al., 1985; Rowbotham, 1980, 1986; Tyndall & Domingue, 1982; Wadowsky et al., 1991).

Intracellular infection by *L. pneumophila* is mediated, to a large extent, by the type IV secretion system known as Dot/Icm and the type II secretion (T2S) system known as Lsp (Cianciotto, 2009; Hoffmann et al., 2014; Newton et al., 2010). Our laboratory has focused on deciphering the role of T2S in *L. pneumophila* physiology, intracellular infection and virulence (Cianciotto, 2005). T2S substrates move across the inner membrane via the Sec or Tat pathway and then a pseudopilus acts to push the proteins through an outer membrane pore (Korotkov et al., 2012). Based upon examination of the virulent strain 130b, *L. pneumophila* T2S exports ≥ 25 proteins, including 18 confirmed enzymes (Cianciotto, 2009; DeRoy et al., 2006a, b; Galka et al., 2008; Herrmann et al., 2011; Pearce & Cianciotto, 2009; Tyson et al., 2013). *L. pneumophila* mutants lacking the T2S

†These authors contributed equally to this work.

**Abbreviations**: pNi, p-nitroanilide; qRT-PCR, quantitative reverse transcriptase PCR; T2S, type II protein secretion.

Three supplementary figures are available in the online Supplementary Material.
apparatus are greatly impaired for infection of Acanthamoeba castellanii, Hartmannella vermiformis and Naegleria lovaniensis (Hales & Shuman, 1999; Liles et al., 1999; Polesky et al., 2001; Rossier & Cianciotto, 2001, 2005; Rossier et al., 2004, 2008; Tyson et al., 2013). The mutants are not defective for entry (Söderberg et al., 2008), indicating that T2S substrates promote bacterial resistance to intracellular killing and/or facilitate replication itself. Among the many T2S substrates examined, a secreted acyltransferase (PlaC), metalloprotease (ProA) and RNase (SrnA) are required for infection of H. vermiformis and N. lovaniensis but not A. castellanii (Rossier et al., 2008, 2009; Tyson et al., 2013). In contrast, NttA, a protein that has no similarity to known proteins, promotes infection of A. castellanii but not H. vermiformis or N. lovaniensis (Tyson et al., 2013). Thus, T2S contributes to the broad host range of L. pneumophila, with the importance of individual T2S substrates being dependent on the amoeba that is being infected.

Overall, our understanding of both the interaction between L. pneumophila and its natural amoebal hosts and the bacterial factors that are involved in the infection process is derived from studies utilizing Acanthamoeba, Hartmannella and to a lesser degree Naegleria. Indeed, aside from three studies that documented the intracellular growth of L. pneumophila in Echinamoeba exudans, Vahlkampfia jugosa and Willaertia magna (Dey et al., 2009; Fields et al., 1989; Rowbotham, 1986), very little is known about how L. pneumophila grows in its other known hosts. Hence, we incorporated L. pneumophila infection of W. magna into our analysis. We report, among other things, that T2S is critical for growth within W. magna, and T2S substrates PlaC, NttA and NttC are required for optimal infection in the Willaertia host.

METHODS

Bacterial strains and bacteriological media. L. pneumophila strain 130b [American type Culture Collection (ATCC) strain number BAA-74] served as our main wild-type strain (Table 1). Mutants of strain 130b that were used in this study are listed in Table 1. Other wild-type strains that were examined are Philadelphia-1 and Paris (Table 1). Legionellae were grown at 37 °C on buffered charcoal yeast extract (BCYE) agar which, when appropriate, was supplemented with chloramphenicol at 3 μg ml⁻¹, kanamycin at 25 μg ml⁻¹ or gentamicin at 2.5 μg ml⁻¹ (Stewart et al., 2011). Escherichia coli strain DH5α (Invitrogen) was the host for recombinant plasmids. E. coli was grown in Luria–Bertani media with gentamicin (5 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹) or ampicillin (100 μg ml⁻¹).

Assessments of bacterial extracellular growth, secreted factors and motility. To monitor the extracellular growth of L. pneumophila strains, legionellae grown on BCYE agar were inoculated into buffered yeast extract (BYE) broth and then incubated at 37 °C with shaking (Chatfield et al., 2011). The optical densities of the cultures were determined at 660 nm using a DU720 spectrophotometer (Beckman Coulter). The secretion of pyomelanin was ascertained by the presence or absence of browning following bacterial growth in BYE broth to late stationary phase (Chatfield & Cianciotto, 2007). Cell-free supernatants collected from late-exponential BYE cultures were assayed for protease activity as measured by azocasein hydrolysis, for phosphatase activity as measured by the release of p-nitrophenol from p-nitrophenol phosphate, for lipase activity as measured by the release of p-nitrophenol from p-nitrophenyl caprylate, for chitinase activity as measured by the release of p-nitroaniline (pNPI) from 1-t-leucine p-NI and 1-lysine p-NI, respectively (Aragon et al., 2000, 2001; DebRoy et al., 2006b; Rossier et al., 2008). Starch-degrading activity was monitored as previously described (Herrmann et al., 2011; Tyson et al., 2013). Swimming motility was determined by wet mount, and sliding motility was assessed by examining bacteria spotted onto BCYE containing only 0.5% agar (Stewart et al., 2011).

DNA and protein sequence analysis. DNA was isolated from L. pneumophila as described by Stewart et al. (2011). Primers used for sequencing or PCR were obtained from Integrated DNA Technologies. DNA sequences were analysed using Lasergene (DNASTAR), and protein alignments were done using the CLUSTAL method. Phyre (www.sbg.bio.ic.ac.uk/~phyre/) and BLAST homology searches were done using GenBank at the NCBI and the other L. pneumophila databases at http://genolist.pasteur.fr/LegioList/.

Reverse transcriptase PCR (RT-PCR). To compare levels of lwp18401 (nttC) RNA in different L. pneumophila cultures, quantitative RT-PCR (qRT-PCR) was done essentially as before (Gunderson & Cianciotto, 2013). The primer pair JS135 (5’-GGCTACATAGCAG-GCAGCAT) and JS136 (5’-GACGGCATTGCAGCATTTAG) was used for nttC. Three biological replicates were obtained, with each one being tested in triplicate. L. pneumophila lwpw00031 (gyrB) and lwp16991 were used as reference genes to normalize gene expression, as previously done (Gunderson & Cianciotto, 2013). The level of gene expression was assessed by determining the cycle at which the amplification curve crossed the detection threshold, and the relative change in gene expression was calculated using the 2^ΔΔCt method (Livak & Schmittgen, 2001). To determine whether nttC was co-transcribed with its neighbouring ORF, standard RT-PCR was done as before (Gunderson & Cianciotto, 2013). To begin, cDNA was made using 130b RNA as template and primer JS89 (5’-TTGTTGTCCGGATTAATAACG), nttC-specific primers were JS93 (5’-GGCTACATAAGGACGAGCAT) and JS94 (5’-TTAGGCTTTCAGTCTTATGCTTA), and the lwp18391-specific primer pair was JS95 (5’-GAGAGGCGGGTCAGTACACA) and JS96 (5’-TCAGCATGACTTACCGTGT). DNA products amplified by PCR were separated by agarose-gel electrophoresis and detected with ethidium bromide staining.

Mutant construction. New mutants were made by the allelic exchange method (Rossier et al., 2008; Stewart et al., 2011; Tyson et al., 2013). To obtain a mutant lacking nttC, an 810 bp fragment containing the 3’ end of the gene was amplified using primers JS15 (5’-ACAGGCCGTTAGCTGTGG) and JS16 (5’-AAAAAAGGG-CCTGGCTGATGTGAGAACACAC, with Stul site underlined), and an 823 bp fragment containing the 3’ end of the gene was amplified using primers JS17 (5’-AAAAAGGCGGTCTGTGCATATG), DNA products amplified by PCR with each primer were separately ligated into pGEM-T Easy (Promega), yielding plg18401a and plg18401b, respectively. Next, the gentamicin-resistance gene from pX1918-GE (Tyson et al., 2013) and the 823 bp fragment from plg18401b were ligated into SacI/Stul-digested plg18401a, yielding plg18401::Gt. Finally, plg18401::Gt was introduced into strain 130b by transformation (Stewart et al., 2011), and mutant colonies were obtained on BCYE agar containing gentamicin. Two nttC mutants, NU425 and NU426, were independently obtained by this method. Verification of the nttC mutants’ genotype was done by PCR, using primers JS15 and JS18.

Genetic complementation. Complementation analysis of the nttC mutants was done by reintroducing an intact copy of nttC on a
Table 1. L. pneumophila strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<td>Stewart et al. (2009)</td>
</tr>
<tr>
<td>Philadelphia-1</td>
<td>Clinical isolate, serogroup 1</td>
<td>Bremer et al. (1979)</td>
</tr>
<tr>
<td>Paris</td>
<td>Clinical isolate, serogroup 1</td>
<td>Cazalet et al. (2004)</td>
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<td>proA mutant of 130b</td>
<td>Moffatt et al. (1994)</td>
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<td>AA200 (pMproA)</td>
<td>Complemented AA200 with plasmid-encoded proA</td>
<td>Rossier et al. (2008)</td>
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<td>map mutant of 130b</td>
<td>Aragon et al. (2001)</td>
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<td>Flieger et al. (2002)</td>
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<td>chaA mutant of 130b</td>
<td>DebRoy et al. (2006b)</td>
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<td>Rossier et al. (2009)</td>
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<td>Complemented NU328 with plasmid-encoded srnA</td>
<td>This study</td>
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<td>celA mutant of 130b</td>
<td>Pearce &amp; Cianciotto (2009)</td>
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<td>McCoy-Simandle et al. (2011)</td>
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<td>nttB mutant of 130b</td>
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<td>legP mutant of 130b</td>
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<td>NU419</td>
<td>gamA mutant of 130b</td>
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<td>NU425, NU426</td>
<td>nttC mutants of 130b</td>
<td>This study</td>
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<tr>
<td>NU425 (pM18401)</td>
<td>Complemented NU425 with plasmid-encoded nttC</td>
<td>This study</td>
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</table>

plasmid, analogously to what we have done before for other 130b mutants (Rossier et al., 2008). To that end, a 554 bp fragment containing nttC as its only intact reading frame was amplified from 130b DNA using primers JS122 (5'-AAAAAAGTGCAGTGAATCAATAATCAAATCAAGA; SacI site underlined) and JS123 (5'-AAAAAAGGATCCATCTCCTTCCCAATTGA; SalI site underlined) and then digested with SacI and SalI. The resulting fragment was ligated into SalI/SalI-digested pMMB2002, a plasmid that encodes chloramphenicol resistance and is stably maintained in L. pneumophila (Rossier et al., 2008). The resulting plasmid, pM18401, was electroporated into the nttC mutant NU425, and transformants were obtained by plating on BCYE agar containing chloramphenicol. For complementation of a plaC mutant, a 1512 bp fragment with plaC as the only intact ORF was amplified from 130b DNA using primers JS25 (5'-AAAAAAGTGCAGTGAATCAATAATCAAATCAAGA; SacI site underlined) and JS26 (5'-AAAAAAGGATCCATCTCCTTCCCAATTGA; SalI site underlined) and then digested with KpnI and XhoI, whose restriction sites were built into JS25 and JS26, respectively. The resulting fragment was ligated into KpnI/XhoI-digested pMMB2002, yielding pMplaC. Finally, pMplaC was electroporated into plaC mutant NU367 (Table 1) and transformants were obtained by plating on BCYE agar containing chloramphenicol. For complementation of an srnA mutant, a 1235 bp fragment with srnA as the only intact ORF was amplified using JS76 (5'-AAAAAAGAGTTCACTCTCCCTCCCTCAGTAA) and JS77 (5'-AAAAAAGAGTTCACTCTCCCTCCCTCAGTAA) and digested with KpnI and KpnI, whose restriction sites were built into JS76 and JS77, respectively. The resulting fragment was ligated into KpnI/KpnI-digested pMMB2002, yielding pMsrnA. pMsrnA was then electroporated into srnA mutant NU283 (Table 1) and transformants were selected on BCYE agar containing chloramphenicol.

Amoebal strains and their growth media. The aquatic strain T5[S]4 of W. magna was obtained from the ATCC (ATCC 50036). Other amoebae that we obtained previously from the ATCC were A. castellanii (ATCC 30234), H. vermiformis (ATCC 50237) and N. lovaniensis (ATCC 30569). As recommended by the ATCC, willaertiai, hartmannellae and naegleriae were axenically grown and maintained in ATCC medium 1034 at 35 °C. Acanthamoebae were grown and maintained in 712 PYG medium at 35 °C, as recommended by the ATCC.

Intracellular infection assays. L. pneumophila infection of A. castellanii, H. vermiformis and N. lovaniensis was done as previously described (Pearce & Cianciotto, 2009; Rossier et al., 2008; Stewart et al., 2011; Tyson et al., 2013). Infection of W. magna was done in the same way as described for H. vermiformis and N. lovaniensis (Tyson et al., 2013). Briefly, bacteria were added, at a variety of bacteria to host cell ratios, to tissue culture wells containing 1 × 10⁶ amoebae in ATCC medium 1034 that lacked its serum component, and then at various times post-inoculation the legionellae were enumerated by dilution-plating aliquots from the well on BCYE agar. L. pneumophila does not grow in the medium, even after the medium is conditioned following bacterial infection of the amoebae, and thus all increases in bacterial numbers result from intracellular infection and growth. Student’s t-test was used to determine the statistical significance of differences in bacterial numbers obtained when comparing infection with various mutant strains with that of infection by the parental wild-type strain.

RESULTS

Definition of NttC, a novel protein that is secreted by L. pneumophila T2S

We began this study with the further examination of a T2S-dependent, 14 kDa protein that we had previously found to be present in the culture supernatants of wild-type L. pneumophila.
**Legionella type II secretion and Willaertia pneumophila** strain 130b but absent from supernatants of a 130b *lspF* mutant that lacks T2S (DebRoy et al., 2006b). According to the now-completed genome database of strain 130b, the secreted protein is encoded by ORF lpw18401 (Fig. 1) (Schroeder et al., 2010). RT-PCR analysis determined that lpw18401 is monocistronic in 130b (data not shown). This is consistent with a transcriptional start site detected just upstream of the homologous ORF in the Lens strain of *L. pneumophila* (Sahr et al., 2012). Importantly, current searches did not yield any similarities to the Lpw18401 protein outside of the *Legionella* database. Thus, lpw18401 was designated *nttC*, for novel type two secreted protein C, following the nomenclature used to designate *nttA* and *nttB* (Tyson et al., 2013). The *nttC* gene was found in all other sequenced strains of *L. pneumophila*, with the corresponding NttC proteins sharing 97–100% amino acid identity with the 130b protein (Table 2). A BLAST-analysis identified an *nttC*-like gene in the genomes of 11 other sequenced *Legionella* species (Table 2). These included both environmental and clinical isolates, and the NttC proteins shared 50–72% identity and 70–84% similarity with NttC of *L. pneumophila* 130b (Table 2). In contrast, *Legionella geestiana* (Dennis et al., 1993), another environmental *Legionella* species that has been recently sequenced, did not contain *nttC* although it did carry *lspF* and *proA* (encoding a different T2S substrate). In summary, NttC appears to be conserved within *L. pneumophila*, present in some but not all other *Legionella* species and absent outside of the genus *Legionella*. In agreement with our past proteomic assessment of T2S (DebRoy et al., 2006b), qRT-PCR analysis determined that *nttC* is expressed when strain 130b is cultured in BYE broth at 37 °C. Although the gene was expressed in all stages of growth, it was most highly expressed in the exponential phase (Fig. 1). The level of *nttC* expression in the exponential phase was statistically greater than those during early and late stationary phase (*P*<0.05, Student’s *t*-test).

**L. pneumophila** NttC mutants

As the first step toward determining the role of NttC in *L. pneumophila* growth and infection, we made and characterized a mutant of strain 130b specifically lacking *nttC*. The mutant, designated NU425, grew in BYE broth like the wild-type (Fig. 1c), indicating that the strain does not have a generalized growth defect and that *nttC* is not needed for extracellular growth. Supernatants obtained from the

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**Fig. 1.** The *nttC* locus and expression of *nttC* during extracellular growth. (a) Depiction of the region of the 130b chromosome containing *nttC*. The horizontal arrows denote the locations, orientations and relative sizes of the *L. pneumophila* genes. The ‘lpw’ numbers placed within the arrows are ORF designations used in the database. The gene names and annotations appear under the arrows. The sizes of the genes (bp) are given above the arrows, and sizes of the intergenic regions are given between the arrows. (b) Expression of *nttC* during *L. pneumophila* growth in broth culture. After 130b was grown in BYE broth at 37 °C, qRT-PCR was used to assess the fold change in *nttC* transcript levels during early stationary and late stationary phases as compared with exponential (E) phase. The data are shown as mean ± SD from triplicate cultures (*n* = 3) or RNA samples. Results are representative of two independent experiments. (c) Wild-type (WT) strain 130b and its *nttC* mutant derivative, NU425, were inoculated into BYE broth, and then the cultures were incubated at 37 °C with shaking. At various times post-inoculation, the extent of bacterial growth was monitored spectrophotometrically. The data points represent the mean and SD of triplicate cultures, and the results presented are representative of three independent experiments.
mutant cultures had normal levels of acid phosphatase, aminopeptidase, chitinase, lipase, protease and starch-degrading activity (data not shown), indicating that loss of nttC does not result in a generalized defect in T2S. The mutant was also similar to wild-type strain 130b in terms of colony morphology, cell shape, swimming and sliding motility, and pyomelanin levels.

**NttC is required for L. pneumophila infection of H. vermiformis**

To determine the importance of NttC for *L. pneumophila* infection of protozoan hosts, we assessed the relative ability of nttC mutant NU425 to infect *N. lovaniensis*, *H. vermiformis* and *A. castellanii*. The mutant grew similarly to its parent in *N. lovaniensis* (Fig. 2a), indicating that NttC is not required for optimal infection of the *Naegleria* host. However, NU425 was defective for infection of *H. vermiformis*. At 48 and 72 h post-inoculation, the mutant-infected *H. vermiformis* cultures contained approximately 20-fold fewer bacteria (Fig. 2b; *P*<0.05 at 24 and 48 h, and *P*<0.01 at 72 h). The mutant did not exhibit reduced survivability when incubated in the 1034 assay medium, the spent medium taken from infected amoebal cultures or the spent medium taken from uninfected amoebal cultures (data not shown), indicating that the strain's reduced recovery is a result of impaired intracellular growth. Because a second, independently derived nttC mutant (i.e. NU426) had the same infection defect as NU425 (Fig. 2b; *P*<0.05 at 24 and 48 h, and *P*<0.01 at 72 h), we concluded that NttC is required for optimal infection in *H. vermiformis*. This was confirmed when the mutant defect was complemented by reintroduction of nttC (Fig. 2c); recovery of the nttC mutant was significantly less than that of both the wild-type and the complemented mutant (*P*<0.01 at 48 h, and *P*<0.05 at 72 h). The nttC mutants also had reduced recovery upon infection of *A. castellanii* (Fig. 2d, and data not shown). However, this phenotype could not be complemented, even though nttC is monocistronic; therefore, the importance of NttC in *A. castellanii* infection remains unclear. Taken together, these data indicated that the novel T2S substrate NttC is required for optimal infection of some but not all amoebal hosts. That NttC is required for infection of *H. vermiformis* but not for infection of *N. lovaniensis* represents a unique finding, as all T2S substrates (i.e. PlaC, ProA, SmA) that had been found to be required for infection of *Hartmannella* amoebae were also necessary for optimal

<table>
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<th>Species/strain</th>
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<td>llb0603</td>
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<td>NSW150</td>
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<td>Czalet et al. (2010)</td>
<td>lio104</td>
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<td>Campbell et al. (1984)</td>
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<td>Thacker et al. (1992)</td>
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<td>loa01056</td>
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<td>lor56c12680</td>
<td>52, 70</td>
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<td><em>L. fairfieldensis</em></td>
<td>Environmental</td>
<td>Thacker et al. (1991)</td>
<td>WP_028387797</td>
<td>54, 70</td>
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</table>

* Percentage amino acid identity and percentage amino acid similarity compared with the sequence of NttC from strain 130b, as determined by BLAST analysis.
† Also representative of strains Thunder Bay, LPE509, HL06041035 and Lorraine.
‡ Also representative of strains 121004, Leg01/53, Leg01/11 and Leg01/20.
infection of the Naegleria host (Table 3; Fig. S1, available in the online Supplementary Material).

**T2S promotes L. pneumophila infection of W. magna**

As the analysis of NttC revealed a new means by which T2S influences the interaction between L. pneumophila and amoebae, we studied a fourth amoebal model, W. magna. Initially, we examined the ability of wild-type 130b to grow within W. magna T5 [S]44, utilizing the bacteria to host cell ratio of 0.1 that has been routinely used for infection of A. castellanii, H. vermiformis and N. lovaniensis (Tyson et al., 2013). However, this level of inoculation did not result in an increase in bacterial numbers as measured by c.f.u. within the co-culture. When the bacteria to host cell ratio was increased to 1.0, there was still no growth of 130b, indicating that W. magna is a less permissive host as compared with A. castellanii, H. vermiformis and N. lovaniensis. Yet, when we increased the bacteria to host cell ratio to 5.0, there was evidence of bacterial growth in W. magna with the increases in c.f.u. ranging from ~1.5 to 2.5 log units (Fig. 4, below). Even at the bacteria to host cell ratio of 5.0, the rate and level of 130b growth in W. magna tended to be less than what was obtained when using the other amoebal hosts (Fig. 3b–d).

To determine if the results obtained with 130b were typical, we performed infections using strains Paris and Philadelphia-1, two other clinical isolates that are widely studied in the Legionella field. Both strains grew within W. magna and to a degree that was comparable to strain 130b (Fig. 4a), suggesting that the capacity to grow within W. magna is a common trait within L. pneumophila. An lspF mutant of strain 130b was greatly impaired for infection of W. magna T5 [S]44 (Fig. 4b). Recovery of the lspF mutant was significantly lower than that of the wild-type at 24, 72

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**Fig. 2.** Infection of N. lovaniensis, H. vermiformis and A. castellanii by wild-type strain 130b, nttC mutants and a complemented nttC mutant. N. lovaniensis (a), H. vermiformis (b, c) and A. castellanii (d) were infected with WT 130b and the nttC mutant NU425. H. vermiformis was also infected with the nttC mutant NU426 (b), and both H. vermiformis and A. castellanii were also infected with a complemented nttC mutant (nttC'/nttC+) (c, d). At the indicated times, the numbers of c.f.u. from the infected monolayers were determined. Data are the means and so for four infected wells and are representative of at least three independent experiments.
and 96 h ($P<0.01$) as well as that of the complemented mutant ($P<0.05$ at 24 h, and $P<0.01$ at 48, 72 and 96 h). Although the mutant eventually grew, the difference in bacterial numbers between the wild-type infection and the mutant infection was still >100-fold at days 3 and 4. These data confirm both that *L. pneumophila* infects *W. magna* and that T2S has a major role in infection of the willaertiae.

### T2S substrates PlaC, NttA and NttC are required for infection of *W. magna*

To begin to understand how T2S promotes infection of *W. magna*, we examined mutants of strain 130b that are specifically lacking individual T2S substrates (Table 1). Mutants lacking either the ProA metalloprotease, SrnA RNase, Map acid phosphatase, PlaA lysophospholipase, ChlA chitinase, LapA and LapB aminopeptidases, CelA endoglucanase, PlcA and PlcB phospholipases C, LipA and LipB lipases, GamA glucoamylase, LegP putative protease, or NttB novel protein grew as well as the parental wild-type strain 130b (Fig. S2), indicating that many of the T2S substrates are not required for optimal infection of *W. magna*. However, mutant NU367 lacking the acyltransferase PlaC mutant, mutant NU415 lacking the novel protein NttA and mutant NU425 lacking the novel protein NttC exhibited reduced recovery upon infection of the willaertiae. Although the three mutants behaved like the parental 130b during the first 48 h of incubation, each exhibited reduced recovery at both 72 and 96 h post-inoculation (Fig. 5; *$P<0.05$, **$P<0.01$*). For the *plaC* mutant and the *nttC* mutant, complementation was achieved utilizing plasmid copies of the gene, but for the *nttA* mutant, complementation was obtained when *nttA* was introduced into a neutral site on the chromosome (Table 1). This result confirmed that the mutant phenotypes were due to the mutations in the *plaC*, *nttA* and *nttC* genes as opposed to spontaneous second-site mutations. Taken together, these data indicate that PlaC, NttA and NttC are required for optimal infection of *W. magna*. The defects observed for the *plaC* mutant, *nttA* mutant and *nttC* mutant, when taken together, may still not account for the large defect exhibited by the *lspF* mutant (Table 3), and thus it is possible that a yet-to-be-identified T2S substrate(s) also facilitates intracellular infection of Willaertia amoebae.

### DISCUSSION

Our results confirm that *L. pneumophila* is capable of growing in *W. magna* amoebae. Indeed, all three strains of serogroup-1 that we tested, i.e. 130b, Philadelphia-1 and Paris, multiplied more than 100-fold in T5[S]44, an aquatic isolate of *W. magna*. In the only previous laboratory study
that examined the interaction between \textit{L. pneumophila} and \textit{W. magna}, strain Philadelphia-1 grew approximately 10- and 100-fold in the \textit{W. magna} c2c Maky strain and the Z503 strain, respectively (Dey et al., 2009). That study also reported that strain Paris does not replicate in c2c Maky and Z503 willaertiae (Dey et al., 2009). Thus, our results when combined with the earlier study’s findings indicate that \textit{W. magna} strains can differ significantly in terms of permissiveness for different strains of \textit{L. pneumophila}. Given these results and the fact that environmental sampling has documented the coexistence of Willaertia species and \textit{L. pneumophila} within both natural and man-made water systems (Declerck et al., 2007), further investigation into the molecular basis for the Legionella–Willaertia interaction is important for understanding the natural history of legionnaires’ disease.

With the incorporation of \textit{W. magna} into our analysis, T2S is now known to be critical for \textit{L. pneumophila} infection of four amoebal hosts. Indeed, the \textit{ispF} T2S mutant displayed a prolonged lag phase and a slowed increase in numbers when incubated with \textit{W. magna} T5[S]44, indicating a markedly impaired ability to resist intracellular killing and/or multiply in the intracellular compartment. This severe defect was analogous to what we had seen previously upon infection of \textit{A. castellanii}, \textit{H. vermiformis} and \textit{N. lovaniensis} with the 130b T2S mutant (Rossier et al., 2004; Tyson et al., 2013). That T2S is required for infection of four out of four amoebae tested suggests that T2S contributes to infection of many, if not all, amoebal hosts and is thus very important for \textit{Legionella} survival in the environment. Further work along these lines would involve infection of \textit{E. exudans} and \textit{V. jugosa}, the two remaining types of amoeba that are known to coexist with and be permissive for \textit{L. pneumophila} (Fields et al., 1989; Rowbotham, 1986). Yet, the host range of \textit{L. pneumophila} is remarkably broad. Species of \textit{Filoamoeba}, \textit{Cochliopodium}, \textit{Comandonia},

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig_3.png}
\caption{Infection of \textit{W. magna} and other amoebae by \textit{L. pneumophila} 130b. (a) \textit{W. magna}, (b) \textit{H. vermiformis}, (c) \textit{A. castellanii} and (d) \textit{N. lovaniensis} were infected with WT strain 130b at a bacteria to host cell ratio of 5, and at the indicated times, c.f.u. from the infected monolayers were determined. Data are the means and SD for four infected wells, and are representative of at least three independent experiments (a, b, d) and of two separate trials (c).}
\end{figure}
Neoparamoeba, Paratetramitus, Platymoeba, Saccamoeba, Thecamoeba, Vannella and Vexillera are also evident in Legionella-containing waters, although bacterial growth in these amoebae has not yet been documented (Breiman et al., 1990; Farhat et al., 2012; Grimm et al., 2001; Kuroki et al., 1998a, b; Paszko-Kolva et al., 1991; Steinert et al., 2002; Valster et al., 2010).

Table 3 illustrates how particular T2S substrates are necessary in some amoebal hosts but not others; i.e. the importance of the T2S substrates varies in a host cell-specific fashion. Each amoebal host undoubtedly represents a sufficiently distinct environment, and therefore to achieve a broad host range, L. pneumophila must depend on different exoproteins at different times. With the documentation of a role for NttA, NttC and PlaC in infection of

![Graph](image1)

**Fig. 4.** Infection of *W. magna* by various wild-type strains and a T2S mutant. (a) Amoebae were infected with WT strains 130b, Philadelphia-1 (Phil) and Paris (a) and with WT 130b, *IspF* mutant NU275 and the complemented *IspF* mutant (b), and then c.f.u. from the infected monolayers were determined. Data are the means and SD for four infected wells, and are representative of three independent experiments (a). The comparison between WT and the *IspF* mutant (b) is also representative of five trials, and examination of the complemented mutant was done twice with comparable results.

![Graph](image2)

![Graph](image3)

**Fig. 5.** Infection of *W. magna* by various T2S substrate mutants. Amoebae were infected with WT 130b (a–c), *plaC* mutant NU367 and its complemented derivative (a), *nttA* mutant NU415 and its complemented derivative (b), and *nttC* mutant NU425 and its complement (c), and then at the indicated times, the numbers of c.f.u. from the infected monolayers were determined. Data are the means and SD for four infected wells. The comparison between WT and the mutants is representative of at least five trials, and examination of the complemented mutants was done twice with comparable results. *P<0.05, **P<0.01 compared with WT.
W. magna, we have identified at least one T2S substrate that is required for infection of each of the four amoebal models used here. The newly defined NttC is notable for several more reasons. First, based on the magnitude of the nttC mutant’s defect, NttC appears to be more critical than the other four substrates. Second, it might actually be important in three types of amoebae, because our two independent nttC mutants did display impaired infection of A. castellanii, although complementation of that defect was not achieved at this time, perhaps because of a need for a different level of gene expression. Third, to our knowledge, NttC is the first example of a T2S substrate that is required for infection of H. vermiciformis but is not needed for infection of N. lovaniensis. Finally, NttC is the second ‘novel’ protein that we have found to be required for infection. The fact that NttC and NttA were confirmed as being important after our examining only four novel substrates (i.e. NttA, NttB, NttC and LegP) (Tyson et al., 2013) indicates that the exoproteins that are unique and specific to Legionella are especially critical for *L. pneumophila* growth and survival. Further characterization of the nttC mutant’s defects as well as the purification and structural determination of NttC might eventually reveal clues to the function of this T2S substrate. The newly found role of PlaC in *W. magna*, although modest, warrants additional comment. Past work has shown that while the ProA protease is needed to fully activate PlaC glycosphospholipid: cholesterol acyltransferase and phospholipase A activities, it is not required to activate PlaC lysophospholipase A activity (Banerji et al., 2005; Lang et al., 2013). As PlaC but not ProA promoted infection of *W. magna*, it is possible that the lysophospholipase A activity of PlaC is promoting infection of the willaertiae. Alternatively, a protease present within *W. magna* might be capable of activating PlaC, negating the need for ProA.

Our analysis of the genome databases revealed that NttC is present in most but not all other *Legionella* species examined. This is compatible with our previous studies in which other T2S substrates or T2S-dependent activities were present in some but not all *Legionella* species examined (Pearce et al., 2012; Söderberg et al., 2008; Stewart et al., 2009; Tyson et al., 2013). It is tempting to speculate that variations in T2S output might translate into different amoebal host ranges for the different *Legionella* species. Thus, the observations we have made concerning T2S in *L. pneumophila* may have broad implications and not only for other legionellae but also for the many unrelated bacteria that possess T2S systems (Cianciotto, 2005, 2009).

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