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Many substrates and functions of type II secretion: lessons learned from *Legionella pneumophila*

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Abstract

Type II secretion is one of six systems that exist in Gram-negative bacteria for the purpose of secreting proteins into the extracellular milieu and/or into host cells. This article will review the various recent studies of *Legionella pneumophila* that have increased our appreciation of the numbers, types and novelties of proteins that can be secreted via the type II system, as well as the many ways in which type II secretion can promote bacterial physiology, growth, ecology, intracellular infection and virulence. In this context, type II secretion represents a potentially important target for industrial and biomedical applications.

Gram-negative bacteria

intracellular infection; *Legionella pneumophila*; Legionnaires' disease; secreted enzyme; sliding motility; type II secretion; virulence

In Gram-negative bacteria, protein secretion is a complex process that involves the active transport of protein substrates across the inner membrane, periplasmic space and outer membrane. Gram-negative bacteria have six, and arguably eight, systems that permit the secretion of proteins from the interior of the bacterium to the extracellular milieu and/or into host cells; that is, types I–VIII [1]. The first system to be defined was type II secretion (T2S). This system has since proven to be common but not universal among the various types of Gram-negative bacteria [2,3]. T2S is a multistep process (Figure 1) [4,5]. Proteins destined for secretion are first translocated across the inner membrane by the Sec or Tat pathway. Upon delivery into the periplasm, unfolded proteins then assume their tertiary conformation. Finally, the proteins are translocated across the outer membrane by a multiprotein complex specifically dedicated to T2S; that is, the T2S apparatus or machinery. Evolutionarily related to type-IV pili, the T2S apparatus has 12 'core' proteins (Figure 1); a cytoplasmic ATPase (T2S E), three inner membrane proteins that create a platform and binding site for T2S E (T2S F, L and M), major and minor pseudopilins, which form a pilus-like structure that spans the periplasm (T2S G, H, I, J and K), an inner membrane peptidase that processes the pseudopilins prior to their integration into the apparatus (T2S O), an outer membrane 'secretin' that oligomerizes to form the secretion pore (T2S D), and a protein that appears to link inner and outer membrane

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components (T2S C) [2,4–8]. Overall, it is hypothesized that proteins destined for T2S are somehow recognized by the apparatus, perhaps by T2S D and T2S C, and then, using energy generated at the inner membrane, the pseudopilus acts like a piston to push the proteins through the outer membrane secretin pore (Figure 1) [9–14]. The molecular characteristic or signature that identifies a protein as a substrate for T2S is still not known but likely involves its tertiary structure and multiple separate contacts with the T2S components [4,15]. Functional studies indicate that T2S promotes the survival of many bacteria in the environment, as well as the virulence of a variety of human, animal and plant pathogens [2,3,16]. In many ways, though, observations made with the environmental pathogen *Legionella pneumophila* have given us the broadest appreciation of the biological significance of T2S.

Legionella pneumophila is a ubiquitous inhabitant of freshwater systems, where it survives free, within protozoan hosts and as part of biofilms [17,18]. However, it is most well known as the primary etiologic agent of Legionnaires' disease, a potentially fatal form of pneumonia that mainly afflicts immunocompromized individuals [19]. Disease occurs when inhaled bacteria, possibly including those still associated with protozoa, invade and grow within lung macrophages. Although many factors influence the ecology and pathogenesis of *L. pneumophila* [20–22], protein secretion stands out for its multifaceted significance [23–26]. *L. pneumophila* clearly expresses T2S and type IV secretion [27–29], and genome sequencing suggests the existence of type I and type V secretion [30,31]. The Dot/Icm type IVB system is unquestionably critical for intracellular infection of mammalian and protozoan hosts, with a large number of secreted effectors working to create the replicative phagosome [32–36]. However, as will be described in this review, T2S has been implicated in a remarkably broad array of *L. pneumophila* phenotypes.

The first indication that *L. pneumophila* has T2S was the discovery of *pilD* in serogroup-1 strain 130b, which encodes the pseudopilin peptidase PilD (i.e., T2S O) [37]. Subsequently, it was shown that mutation of *pilD* abolished secretion, as evidenced by the loss of proteins in culture supernatants [38]. A study of strain Philadelphia-1 then revealed *lspFGHIJK*, predicted to encode the T2S F, G, H, I, J and K proteins [39]. Inactivation of *lspGH* also resulted in loss of protein secretion. In follow-up studies of strain 130b, the loci encoding T2S D and E (*lspDE*), C (*lspC*) and L and M (*lspLM*) were found, with mutational analysis of *lspDE* confirming their role in secretion [40,41]. The existence of a complete T2S system in *L. pneumophila* was confirmed when the genomes of serogroup-1 strains Philadelphia-1, Paris and Lens were published in late 2004 [2,30,42,43]. Sequencing has also confirmed that *L. pneumophila* has the genes encoding Sec and Tat translocation [30,42,44,45]. Based upon Southern hybridization analysis, T2S genes are found in the other serogroups of *L. pneumophila* as well as other species of *Legionella* [41].

By 2005, nine enzymes were determined to be substrates for *L. pneumophila* T2S, based upon the loss of distinct enzymatic activities in the culture supernatants of *lspDE*, *lspF*, *lspG*, *lspGH* or *pilD* mutants of strain 130b grown in buffered yeast extract broth at 37°C [2,38–41,46,47]. The enzymes identified were the tartrate-sensitive and tartrate-resistant acid phosphatases, phospholipase A, phospholipase C, lysophospholipase A, glycerophospholipid:cholesterol acyltransferase (GCAT), mono-, di- and triacylglycerol lipases, ribonuclease and metalloprotease (Table 1) [38–41,46–51]. Genes encoding some of the secreted activities were also identified; *map* (Map) for the tartrate-sensitive acid phosphatase [48], *plcA* (PlcA) for phospholipase C activity [49], *plaA* (PlaA) for the lysophospholipase A [51], *plcC* (PlcC) for GCAT [47], *lipA* (LipA) and *lipB* (LipB) for mono- and triacylglycerol lipases, respectively [49], and *proA/msp* (ProA/Msp) for the metalloprotease (Table 1) [38,39]. The analysis of supernatants from *proA* mutants indicated that some exoproteins are subject to cleavage and possibly activation by the T2S-dependent metalloprotease [47,51]. Based upon predicted signal sequences, the majority of the T2S

substrates are translocated across the inner membrane via Sec. However, PlcA of strain 130b is a Tat substrate, as evidenced by the twin-arginine in its signal peptide and the loss of phospholipase C activity in *tatB* mutant supernatants [45]. Since mutations in any one of these initially defined effectors did not completely abolish the corresponding activity, it was posited that *L. pneumophila* T2S mediates the secretion of more than nine proteins.

To define additional proteins secreted by *L. pneumophila* T2S, proteins in wild-type and *lsp* mutant supernatants were compared by 2D polyacrylamide gel electrophoresis (PAGE), and mass spectrometry was then used to obtain the identity of the secreted proteins [27]. A total of 20 proteins that contained a signal sequence and were present in the wild-type strain 130b but lacking in the isogenic mutant were identified (Table 1). Three of these were ProA, PlaA and Map, which had been previously defined as T2S substrates. A fourth was annotated as a T2 ribonuclease, and subsequent cloning and mutational analyses confirmed that the protein (SrnA) encodes the secreted ribonuclease activity that had been observed earlier [52]. A fifth was annotated as a chitinase, and additional cloning and mutational analysis confirmed the existence of a T2S-dependent chitinase (ChiA) [27]. Following a similar path of investigation, the next two exoproteins were defined as aminopeptidases, being active against leucine, phenylalanine and tyrosine aminopeptides (LapA), or lysine- and arginine-containing substrates (LapB) [53]. Three of the other type II-secreted proteins were annotated as hypothetical but showing weak similarity to bacterial enzymes, including an amidase, a cysteine protease and an endoglucanase [27]. Two others were most similar to eukaryotic proteins, with one having collagen-like repeats, and the other relatedness to astacin-like zinc proteinase [27]. Five others had no similarity to any known protein or domain in the database and thus may encode novel activities [27]. The final three proteins were IcmX, LvrE and a VirK-like protein. Whereas IcmX and LvrE are linked to the type IV secretion systems of *L. pneumophila* [54–57], VirK is associated with type IV secretion in *Agrobacterium tumefaciens* [58,59]. These data suggest a possible connection between type II and type IV secretion. Although the genes encoding IcmX, LvrE and the VirK-like protein are linked to type IV secretion genes, secretion of the proteins may occur by T2S. Interestingly, the eukaryotic-like zinc proteinase (LegP), which was defined as being T2S-dependent by proteomic analysis, was later found to be translocated by Dot/Icm type IV secretion when the legionellae were growing within macrophages [60]. Thus, it is possible that some effectors may be secreted via multiple pathways, with the environmental conditions potentially dictating which secretion pathway(s) is used. Taken together, the results of the proteomic analysis combined with the earlier assessments of culture supernatant enzymatic activities indicated that the number of proteins secreted by the T2S system of *L. pneumophila* strain 130b is 25 (Table 1). However, the output of this system was hypothesized to be even greater; for example, not every protein observed by 2D-PAGE was submitted for identification, low-level expression or degradation might have impaired detection of some proteins, the supernatants examined were from bacteria grown under a single growth condition and *in silico* analysis of the *L. pneumophila* genome revealed 60 proteins that contain a signal sequence and are predicted to be extracellular by at least one program [27]. Thus, it was hypothesized that the *L. pneumophila* T2S system processes between 25 and 60 substrates [27], a prediction that has gained support from the recent proteomic analysis of supernatants from strain JR-32 [61]. Even at 25, the experimentally defined catalog of *L. pneumophila* T2S effectors was the largest known in bacteria [2,27], with similar or slightly lower numbers obtained from the analysis of *Burkholderia glumae*, *Erwinia chrysanthemi*, *Pseudoalteromonas tunicata* and *Ralstonia solanacearum* [2,62–65].

The study of *L. pneumophila* has given new insight into the process of secretion – in particular the involvement of Mip, a surface-associated peptidylproline *cis*–*trans*-isomerase (PPIase), in the elaboration of secreted activity [66]. Mip mutants of strain 130b displayed a 40–70% reduction in secreted phospholipase C activity. When supernatants were examined by

chromatography, the secreted activity linked to Mip proved to be T2S-dependent but distinct from the previously defined PlcA, suggesting that Mip promotes the elaboration of a 'new' T2S-exoprotein (Table 1). On the one hand, Mip might be involved in the extracellular release of an active phospholipase C, by acting directly on the exoprotein or a protein(s) that forms part of the secretion pathway. On the other hand, Mip might associate with the newly secreted protein and cause changes that convert it from being inactive to active. These data represented the first case of a surface PPIase being linked to the secretion or activation of proteins beyond the outer membrane [66].

Recently, it was observed that *L. pneumophila* exhibits surface translocation when it is grown on media containing 0.5–1.0% agar [67]. The growing legionellae appear in an amorphous, lobed pattern that is most manifest at 25–30°C. *L. pneumophila* mutants lacking flagella and/or type IV pili behaved as wild-type did, indicating that the surface translocation is not swarming or twitching motility. A translucent film was visible atop the agar in front of the legionellae [67]. The film acted like a surfactant; for example, it dispersed water droplets and promoted the spreading of heterologous bacteria. A sample of the film rapidly dispersed when it was spotted onto plastic. Interestingly, *L. pneumophila* T2S mutants were defective for surface translocation and film [67]. When the mutants were spotted onto film made by wild-type, they spread, suggesting that T2S promotes the secretion of the surfactant. Thus, *L. pneumophila* exhibits surface translocation that is most similar to 'sliding motility' and uniquely dependent upon T2S. The simplest scenario is that T2S, directly or indirectly, mediates the release of surfactant, which in turn permits legionellae to passively slide over surfaces. The surfactant might represent another 'new' T2S effector that was not detected in the earlier studies using broth-grown legionellae (Table 1). Overall, these data represent a novel observation, linking bacterial sliding, surfactant and T2S [67].

Clearly, *L. pneumophila* secretes a variety of factors via T2S (Table 1). Several of the types of enzymes observed, including proteolytic and lipolytic enzymes, chitinases and phosphatases, are also secreted by other T2S systems [2,53]. In some cases, the effectors of *L. pneumophila* are closely related to the exoproteins of others; for example, PlcA is highly related to a *Pseudomonas* phospholipase C [49]. However, the output of *L. pneumophila* uniquely also includes proteins that show their greatest similarity to eukaryotic proteins; for example, the Map acid phosphatase and LegP endopeptidase [27,48]. *L. pneumophila* is also unique, at least thus far, in its T2S of an RNase and a surfactant. Overall, the type of proteins most represented in the T2S repertoire are proteases and peptidases, a finding that is consistent with the fact that amino acids are the main carbon and energy source for broth-grown *L. pneumophila* [68]. However, the presence of a chitinase and putative endoglucanase suggests that *L. pneumophila* is also capable of degrading and utilizing complex carbohydrates. Perhaps most significantly, several of the *Legionella* exoproteins do not bear any similarity to known proteins, raising the possibility of there being entirely new types of effectors secreted by T2S. Based on the number of proteins uncovered and the types of factors detected (Table 1), the analysis of *L. pneumophila* highlights more than ever the impact that T2S has on bacterial secretion and function.

Although T2S mutants of *L. pneumophila* replicate normally in standard liquid and solid media at 30–37°C, they are greatly impaired for growth in bacteriological media at 25, 17 and 12°C [69]. In experiments that mimic aquatic habitats, the mutants also show reduced survival in tap water incubated at 25, 17, 12 and 4°C [70]. The mutants grew better at the lower temperatures when they were plated next to wild-type or wild-type supernatants, suggesting that a secreted factor promotes low-temperature growth [69,70]. In support of this hypothesis, 2D-PAGE comparisons of supernatants obtained from bacteria grown at 37°C versus 17 or 12°C identified a Sec-dependent protein that is hyper-expressed at low temperatures and predicted to have PPIase activity (Table 1) [71]. Another secreted PPIase that is also distinct from Mip but may

not require the T2S system is an important promoter of low-temperature growth [71]. Thus, akin to what has been observed in *Vibrio cholerae*, T2S stimulates the extracellular growth of *L. pneumophila* specifically at low temperatures [72,73]. In the case of *L. pneumophila*, secreted proteins account, at least in part, for the importance of T2S at low temperatures, and PPIases may help other secreted proteins to achieve a conformation that is most compatible with functioning at low temperatures. Finally, these data also help establish T2S as a major factor in enabling *Legionella* persistence in the environment.

Legionella pneumophila T2S mutants are highly attenuated for intracellular growth in protozoa, including *Hartmannella vermiformis* and *Acanthamoeba castellanii* [38–41,70,74]. Indeed, the mutants show little, if any, evidence of growth in the amoebae. Thus, T2S is critical for intracellular infection of protozoa. Among the various T2S effectors, the ProA metalloprotease and SrnA ribonuclease have proven to be required for optimal infection of amoebae (Table 1) [52,53]. This suggests that the infection defects of *lsp* mutants are due to the loss of secreted effectors as opposed to being simply due to potential changes in the bacterial cell (envelope). Double mutants lacking both ProA and SrnA exhibited an infectivity defect that was even greater than the corresponding single mutants, indicating that the role of T2S in intracellular infection is due to the combined effect of multiple secreted effectors [52]. The metalloprotease and ribonuclease may facilitate intracellular growth by helping bacteria to generate amino acids, nucleotides and/or phosphate as part of nutrient acquisition. By contrast, ProA and SrnA might degrade host proteins and RNA that can influence intracellular growth. Whether T2S effectors exit the *Legionella* phagosome and traffic into the host cytoplasm and/or whether host factors move into the phagosome is a key question. Relevant to that point, ProA has been observed within the cytoplasm of infected guinea pig macrophages [75]. Interestingly, *proA* exhibited differential importance among the amoebae tested, suggesting that *L. pneumophila* might have evolved some of its factors to specifically target certain protozoan hosts [53]. Thus, for *L. pneumophila*, T2S is not solely dedicated to extracellular growth (e.g., secretion into culture media) but rather, like the organism's type IV secretion system, it contributes to intracellular growth, albeit in different ways. These studies represent the first documentation of a role for T2S and a secreted ribonuclease in intracellular infection. Because of the role of protozoa in *L. pneumophila* survival in water, these data further establish T2S as a major factor in *Legionella* persistence in the environment. Since infected amoebae may be part of the infective dose that initiates lung infection [76,77], they also provided an early indication of the relevance of T2S in *L. pneumophila* pathogenesis.

Importantly, T2S mutants of *L. pneumophila* strain 130b are also very defective in an animal model of Legionnaires' disease [41]. Upon intra-tracheal inoculation into A/J mice, *lspDE*, *lspF* and *pilD* mutants display a reduced ability to grow in the lung, as measured by competition assays. The *lspF* mutant was further tested in an *in vivo* growth kinetics assay; whereas wild-type increased at least tenfold in the lungs, the T2S mutant gave no evidence of replication. The examination of wild-type-infected sera revealed that type II-secreted proteins are expressed *in vivo* [41]. Therefore, T2S is an important contributor to *L. pneumophila* virulence. *L. pneumophila* T2S mutants are also impaired for intracellular infection of macrophages *in vitro* [38,41,74]. Thus, T2S promotes lung infection at least in part by facilitating bacterial growth in resident alveolar macrophages. Because mutant numbers do not increase in the lungs, whereas they do, although not optimally, in macrophages *in vitro*, *L. pneumophila* T2S likely also promotes extra-macrophage processes, such as infection of epithelial cells, subversion of innate immunity and extracellular survival. These studies were the first direct assessment and documentation of a role for T2S in a mammalian model of disease. Furthermore, they highlight the multiple ways in which T2S can promote bacterial virulence.

Legionella pneumophila mutants that lack particular T2S effectors have also been tested for alterations in infection of mammalian hosts. The *map*, *plcA*, *plaA*, *plaC*, *lipA*, *lipB*, *proA/msp*,

lapA, *lapB*, *srnA* and *chiA* mutants all grew normally in macrophages *in vitro*, indicating that the proteins encoded by these genes are not required for macrophage infection [27,47–49,51–53]. These data indicate that the T2S system secretes a yet-to-be-defined factor that is necessary for macrophage infection. On the other hand, there might be redundancy in the effectors, such that one secreted factor can compensate for the loss of another. Interestingly, ChiA chitinase mutants were impaired fourfold when tested in the mouse model of Legionnaires' disease [27]. Western blot analysis using antisera from animals inoculated with wild-type showed that ChiA is expressed *in vivo*. These data indicate that ChiA is, directly or indirectly, required for optimal survival of *L. pneumophila* in the lung. ChiA is the first T2S effector to be implicated in *Legionella* virulence; other effector mutants tested displayed no defect in the mice (Table 1) [27,52,53]. Since the *chiA* mutant grew normally in macrophages *in vitro* and since its reduced survival in the lung was only manifest in the later stages of infection, ChiA likely promotes persistence versus initial replication. Since mammals do not have chitin, these data lead to the hypothesis that there is a chitin-like factor in the lung whose degradation aids bacterial persistence. Alternately, ChiA could be a bifunctional enzyme that has another substrate. That a protein having chitinase activity can promote the survival of a pathogen in a mammalian host has not been previously seen [27]. Thus, factors that are traditionally viewed as only being important in the environment may actually have great relevance in disease.

Future perspective

Studies of *L. pneumophila* clearly illustrate the many ways in which T2S can influence a bacterium's secretion, physiology, ecology and pathogenesis. For *L. pneumophila*, T2S is a key factor in situations ranging from extracellular growth at low temperatures to surface translocation, intracellular infection of protozoan and mammalian host cells and lung infection. We can also now see that the substrate output of a T2S system, in terms of both the number of proteins and their range of activities, is greater than previously indicated. With this expanded catalog of effectors in hand, comparisons between the many effectors may help uncover structural commonalities, which might in turn help explain how substrates are recognized by the T2S machinery. By continuing to define the enzymatic activities and expression patterns of the effectors, we will better understand how T2S influences growth and infection as well as what sets it apart from the other secretion systems of *L. pneumophila*. Although it is likely that some aspects of *L. pneumophila* T2S are a reflection of the organism's unique environmental and intracellular niches, it is also anticipated that many of the lessons learned from work done in *L. pneumophila* will have implications for understanding various other environmentally and/or medically important bacteria. Given its broad significance, T2S systems should be considered as potential targets for industrial application as well as disease diagnosis, control and prevention.

Executive summary

- Type II secretion (T2S) is one of at least six systems that operate in Gram-negative bacteria for the purpose of transporting proteins from the cytoplasm to the extracellular milieu and/or into target host cells.
- T2S systems can mediate the secretion of more than 25 proteins, encoding many different enzymes, some with potentially novel activities.
- T2S can promote bacterial growth under a very wide variety of conditions, ranging, in the case of *Legionella pneumophila*, from low-temperature waters to inside amoebae and the mammalian lung.

- T2S is critical for bacterial ecology and pathogenesis and therefore may represent an important target for industrial and biomedical applications.

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Figure 1. Model of type II secretion

Following translocation across the IM via Sec (or Tat, not shown) and periplasmic processing, substrates are recognized by the type II secretion (T2S) apparatus. Then, using energy generated at the IM, an extendable (and retractable) 'pilus' that is formed by G, H, I, J and K acts to push substrates through D in the OM. Whereas T2S I, J and K are believed to form the tip of the pseudopilus, and T2S G the shaft, the interactions of T2S H are presently unknown. O cleaves and N-methylates the pseudopilins prior to their integration into the T2S apparatus. See the text for further discussion and references.

C–M: Core constituents of the T2S system; D: Dodecameric secretin; G: Major pseudopilin; H–K: Minor pseudopilins; O: Prepilin peptidase.

Table 1

Secreted proteins and activities that are type II secretion-dependent in *Legionella pneumophila**.

Secreted substrate	Associated activity or predicted activity
ProA (MspA)	Zinc metalloprotease, promotes amoebal infection
Map	Tartrate-sensitive acid phosphatase
γ [‡]	Tartrate-resistant acid phosphatase
PlaA	Lysophospholipase A
PlaC	Glycerophospholipid:cholesterol transferase [§]
PlcA	Phospholipase C
?	Mip-dependent phospholipase C
LipA	Mono- and triacylglycerol lipase
?	Diacylglycerol lipase
LipB	Triacylglycerol lipase
SrnA	Type 2 ribonuclease, promotes amoebal infection
ChiA	Chitinase, promotes lung infection
LapA	Leucine, phenylalanine and tyrosine aminopeptidase
LapB	Lysine and arginine aminopeptidase
Lpg0264 [¶]	Weakly similar to bacterial amidases
Lpg2622	Weakly similar to bacterial cysteine proteases
Lpg1918	Weakly similar to bacterial endoglucanases
LegP (Lpg2999)	Predicted astacin-like zinc endopeptidase
Lpg2644	Some similarity to collagen-like protein
Lpg1809	No similarities; predicted novel activity
Lpg1385	No similarities; predicted novel activity
Lpg0873	No similarities; predicted novel activity
Lpg0189	No similarities; predicted novel activity
Lpg0956	No similarities; predicted novel activity
IcmX	Linked to Dot/Icm type IV secretion genes
LvrE	Linked to Dot/Icm type IV secretion genes
Lpg1832	Weakly similar to VirK
Lpg1962	Predicted low-temperature, cyclophilin-like peptidylproline <i>cis-trans</i> -isomerase
?	Surfactant

* As discussed in the text, this is probably an incomplete listing.

[‡]This indicates that the effector(s) encoding this activity has not yet been identified.

[§]PlaC also possesses lysophospholipase A and phospholipase A activity.

[¶]Proteins that have not been previously named or confirmed as possessing a particular (predicted) activity are referred to by the Lpg designations that exist within the Philadelphia-1 genome database.