Recent advances in structural studies of the \textit{Legionella pneumophila} Dot/Icm type IV secretion system

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\section*{INTRODUCTION}

\textit{Legionella pneumophila} is an intracellular Gram-negative bacterium that causes acute pneumonia known as legionellosis.\textsuperscript{3} This bacterium was first identified in an outbreak among people who participated in the American Legion convention held in Philadelphia, USA in 1976. A year later, \textit{L. pneumophila} was clinically isolated as the causative pathogen.\textsuperscript{4} It took two more years until a method was established to stably cultivate \textit{L. pneumophila} using a medium in the laboratory.\textsuperscript{5} \textit{L. pneumophila} is primarily an environmental bacterium that resides in protozoan hosts, such as \textit{Acanthamoeba}. Humans have invented aerosol-producing devices, such as humidifiers, showers, and recirculating baths, facilitating human infection, which occurs by aerosol inhalation. To date, more than 60 different \textit{Legionella} species have been identified.\textsuperscript{4} \textit{L. pneumophila} serogroup 1 is the most prevalent pathogen among patients with severe symptoms.\textsuperscript{1} Human-to-human transmission of \textit{Legionella} can occur, even though it is very rare,\textsuperscript{5,6} and legionellosis morbidity is increasing annually worldwide.\textsuperscript{7}

Once \textit{L. pneumophila} is internalized by macrophages, the bacterial phagosome is converted into an endoplasmic reticulum (ER)-like compartment called the \textit{Legionella}-containing vacuole (LCV), which serves as a replicative niche. For LCV biogenesis, \textit{L. pneumophila} intercepts the early secretory pathway between the ER and the Golgi apparatus.\textsuperscript{8-12} In this process, \textit{L. pneumophila} uses effector proteins.\textsuperscript{13} A recent large-scale genomic analysis has...
revealed that the genus *Legionella* has acquired a wide variety of proteins from all domains of biology to hijack and manipulate host cellular systems during infection.\(^{14}\)

To translocate numerous effector proteins into host cells, *L. pneumophila* utilizes the Dot/Icm type IV secretion system (T4SS\(^{\text{Dot/Icm}}\)).\(^{15-19}\) The genes required for the survival of *L. pneumophila* in the host cell were originally identified in the late 1990s by two independent research groups led by Howard Shuman\(^{20}\) and Ralph Isberg.\(^{21}\) These genes are named *intracellular multiplication* (*icm*) or *defect in organelle trafficking* (*dot*), respectively,\(^{22-26}\) including those encoding the proteins composing T4SS\(^{\text{Dot/Icm}}\). Therefore, some T4SS\(^{\text{Dot/Icm}}\) components were assigned two distinct names. The *dot/icm* genes are arranged at two loci on the chromosome. The loci are highly conserved among *L. pneumophila* genomes,\(^{27}\) whereas the genes encoding effector proteins are distributed throughout the genome.\(^{23,26}\)

T4SS\(^{\text{Dot/Icm}}\) is classified as a type IVB secretion system (T4BSS). The T4BSS is evolutionarily closely related to I-type conjugation systems such as the self-transmissible IncI plasmids,\(^{27,29-31}\) and it is distinct from the type IVA secretion system (T4ASS), which is related to the VirB/D4 system of the plant pathogen *Agrobacterium tumefaciens*. Interestingly, although the T4ASSs and the T4BSSs function similarly by transporting either nucleic acids or proteins directly into the cytosol of recipient cells, the components and architecture of the T4BSSs are much more complex than those of the T4ASSs. There are more than 20 T4BSS components, whereas the T4ASS includes approximately 12 components. The structure and assembly process of T4ASSs have been analyzed at the atomic level,\(^{17,18}\) however, the exact mechanisms of transport have not been fully elucidated. In contrast, the structure of the T4BSSs remains to be fully elucidated due to its complexity. In the past few years, however, remarkable progress has been made in this field, facilitated by an improvement in cryo-electron microscopy (cryo-EM). This review focuses on recent findings in the structural biology of the *L. pneumophila* T4SS\(^{\text{Dot/Icm}}\).

**T4SS\(^{\text{Dot/Icm}}\) TRANSPORT APPARATUS**

**Core complex**

A T4ASS sub-complex called the “core complex” was first visualized via cryo-EM images of a complex derived from the IncN plasmid pKM101 conjugation system by Waksman’s group in 2009.\(^{32}\) The T4ASS core complex consists of three proteins, the inner-membrane protein VirB10 and the outer-membrane proteins VirB7 and VirB9, and was initially thought to span both the inner and outer membranes. Regarding the T4SS\(^{\text{Dot/Icm}}\), Vogel et al. found five *L. pneumophila* proteins, DotC, DotD, DotH, DotG, and DotF, as putative components of the core complex in 2006.\(^{33}\) Kubori et al. first visualized the structure of the T4SS\(^{\text{Dot/Icm}}\) core
complex biochemically isolated from *L. pneumophila* in 2014, by using a method similar to the one used to visualize the needle complex of the *Salmonella* type III secretion system. The native T4SS\textsuperscript{Dot/Icm} core complex is ring-shaped with a central pore, and contains all five putative component proteins. Unexpectedly, it was found that DotG is dispensable for complex assembly; a ring-shaped complex with a larger pore was formed in the absence of DotG. At the sequence level, the C-terminal domain of DotG is highly homologous to that of T4ASS VirB10, a central channel component of the T4ASS core complex.

**In situ structure of the T4SS\textsuperscript{Dot/Icm} transport apparatus**

In 2017, the structure of the T4SS\textsuperscript{Dot/Icm} complex embedded in bacterial membranes was visualized by Ghosal et al. using electron cryotomography (ECT). This was the first *in situ* structure reported for all T4SS complexes. The structure has the characteristic shape of a "Wi-Fi symbol," consisting of two distinct curved layers, a larger layer just below the outer membrane and a smaller layer in the middle of the periplasm (Figure 1).

The same researchers further performed molecular dissection of the T4SS\textsuperscript{Dot/Icm} complex. They assigned T4SS\textsuperscript{Dot/Icm} component proteins to the electron densities of the images using a series of *L. pneumophila* mutants lacking T4SS\textsuperscript{Dot/Icm} components or strains expressing component proteins fused to the superfolder green fluorescent protein (sfGFP). The resulting model of the T4SS\textsuperscript{Dot/Icm} complex is shown in Figure 1a. The authors proposed that (i) DotC, DotD, and DotH form the beta and gamma densities and the elbow, (ii) DotG forms the hat and the channel, and (iii) DotF forms the wing. In addition to these proteins, DotK, IcmX, DotA, and IcmF were placed in the model. DotK (also known as LphA, see below), an outer membrane lipoprotein reported previously to be co-purified with the core complex, forms the alpha density. The soluble periplasmic protein IcmX forms a part of the plug, whose density decreased significantly in the ΔicmX mutant. The periplasmic domain of IcmF, a homolog of the type VI secretion system (T6SS) core component TssL, forms a central part of the plug density. Interestingly, the periplasmic domain of the inner membrane protein DotA, which is secreted via the T4SS\textsuperscript{Dot/Icm} to extracellular milieu, was positioned at the upper part of the stalk channel as a ring structure. DotK disruption did not affect the growth of HL-60 cells, but a strain lacking IcmF showed partial growth defects in U937 and HL-60 cells. This suggests that DotK and IcmF are required for maximal intracellular growth but are not essential for effector translocation per se, which substantiates the unusual plasticity of the functional T4SS\textsuperscript{Dot/Icm} transport apparatus composition. The *in situ* T4SS\textsuperscript{Dot/Icm} structure revealed a complex with 13-fold symmetries, while all known T4ASS complexes exhibit 14-fold symmetries.

**Atomic resolution structure of the T4SS\textsuperscript{Dot/Icm} transport apparatus**

Durie et al. reported the high-resolution structure of the T4SS\textsuperscript{Dot/Icm} outer membrane complex using single-particle cryo-EM. They purified the native core complex using essentially the same procedure as that used by Kubori et al. and reconstructed the atomic model of the complex. Combined with their follow-up study utilizing the new "3D variability analysis" technology, they built the model structure of the T4SS\textsuperscript{Dot/Icm} transport apparatus consisting of three distinctive structural modules: the dome, the outer membrane cap (OMC), and the periplasmic ring (PR), which showed 16-fold, 13-fold, and 18-fold symmetries, respectively, illuminating the unexpected symmetry mismatch in the complex (Figure 1b). The flexible and dynamic arrangement of the molecules at the interface between the structural modules is thought to be the key feature of the T4SS\textsuperscript{Dot/Icm}. The symmetry of the OMC matches that observed in the *in situ* structure reported by Ghosal et al. The core complex isolated from the ΔdotG mutant lacks the dome and the PR, which is consistent with the previous observation and suggests that these modules contain DotG. In the refined model, this research group assigned the C-terminal domain of DotG to the dome, the model of DotG positioning from the PR to the dome is consistent with the fact that DotG shares sequence homology with T4ASS VirB10, the central channel component of the T4ASS core complex, even revealing the unexpected copy number in the dome. Importantly, DotH was identified as the key protein that accommodates the symmetry mismatch by connecting the PR and OMC, revealing the distinctive apparent copy numbers between the modules. As for the OMC disk density, the core components DotC, DotD, and DotH can be fitted together with DotK and Dis1 (Lpg0657), which had been a hypothetical interactor with the Dot/Icm T4BSS. The stoichiometry of each component (DotD:DotC:DotH:DotK:Dis1) was estimated to be 2:1:1:1:1. Both DotK and Dis1 have an OmpA-like domain, which is known as a peptidoglycan-binding domain. Dis1 was shown to be required for maximum intracellular growth in *Acanthamoeba castellanii* and mouse macrophages. These observations, together with the model obtained from the *in situ* ECT, suggest that DotK and Dis1 are auxiliary components of the T4SS\textsuperscript{Dot/Icm} transport apparatus. They assigned two additional proteins outside the dot/icm locus, Dis2 (Lpg0823) and Dis3 (Lpg2847), which are associated with the OMC. They proposed a final stoichiometry of 31:26:18:18:13:13:13:13 (DotF: DotD: DotG: DotH: DotK: Dis1: Dis2: Dis3) for the T4SS\textsuperscript{Dot/Icm} transport apparatus.

**Cytoplasmic ATPases: DotO–DotB complex**

DotO and DotB are ATPases of the T4S\textsuperscript{Dot/Icm} essential for the T4SS function. DotO and DotB correspond to...
VirB4 and VirB11 in the T4ASS, respectively. Biochemical analyses showed that DotO was membrane-associated, while DotB was partially detected in the cytosolic fraction. However, these two ATPases contribute to the function of the T4SS remains to be clarified.

In 2018, Chetrit et al. examined the molecular assembly of the L. pneumophila T4SSDot/Icm complex including the two distinctive ATPases. Time-lapse images using fluorescence microscopy revealed that wild-type DotB moved back and forth between the two poles of a bacterial cell, while DotBE191K, which can bind ATP but is unable to hydrolyze it, was stably recruited to the cell pole. Using ECT analysis of the L. pneumophila strain expressing DotBE191K, they were able to capture the in situ structure of the entire complex, including DotO and DotB. The reconstruction revealed that a DotB hexamer was directly associated with DotO, which formed a cylindrical structure connected to the T4SSDot/Icm core complex. DotO also formed a hexamer composed of six dimers positioned at the base of the inner-membrane spanning channel. This molecular positioning is distinctive from the R388 T4ASS complex, which has two VirB4 hexamer "legs". In 2020, Park et al. demonstrated that docking the DotB hexamer to the DotO hexamer promotes conformational changes in the entire complex to open the channel in the inner membrane, supporting the hypothesis that DotB binding is essential for an early stage of substrate translocation by the T4SSDot/Icm machinery. Interestingly, the non-hydrolyzable DotB mutant protein does not localize at bacterial cell poles in the mutant strains lacking inner membrane proteins, such as DotJ, IcmT, IcmV, IcmQ, Dotl, DotU, DotE, and DotA. In addition, DotO localization at the cell poles was abrogated in the absence of Dotl and DotU. Considering that DotU is required for the polar localization of the T4SSDot/Icm complex (see Section Assembly pathway of the T4SSDot/Icm transport apparatus) and that Dotl is the counterpart of T4ASS VirB8, which is a part of a subcomplex connecting the T4ASS core complex and the cytoplasmic VirB4 legs, it is plausible that the DotO-DotB cytoplasmic complex is connected to the T4SSDot/Icm complex via inner membrane components such as Dotl and DotJ, a partial homolog of DotI.

Assembly pathway of the T4SSDot/Icm transport apparatus

In the process of analyzing the in situ structure of the T4SSDot/Icm complex, Ghosal et al. reported that the densities corresponding to DotH, DotG, and DotF were drastically reduced in a ΔdotUΔicmF double mutant strain. This observation was consistent with previous results indicating that DotU and IcmF, which are homologs of T6SS TssL and TssM, respectively, play important roles in intracellular replication, effector translocation, and stabilization of the T4SSDot/Icm complex. In addition, Ghosal et al. observed a lower number of T4SSDot/Icm complexes at cell poles in the ΔdotUΔicmF mutant than in the wild-type strain, and found that the T4SSDot/Icm core components (DotC, DotD, DotF, DotG, and DotH) were unable to localize to the cell poles in the absence of DotU and IcmF. In contrast, DotU and IcmF could localize to cell poles in the absence of any other T4SSDot/Icm components. Based on these results, they proposed that DotU and IcmF are integral membrane proteins that can recruit other components to the bacterial cell poles.

In 2020, Park et al. reported structural heterogeneity of the T4SSDot/Icm complexes in each individual bacterial cell using ECT. Subtomogram averaging and classification of the T4SSDot/Icm complexes revealed that there were two distinct class averages: one had structures associated with the outer membrane but lacked cytoplasmic densities, and the other had intact structures containing components localized in the inner and outer membranes and cytoplasm. To further define potential subassemblies, they analyzed dotBE191K, ΔdotB, and Δdotl mutants and found five distinct subassembled intermediates. The smallest and least complex is called the outer membrane-embedded ring (OMER). Based on the identification of the distinctive subassembly intermediates, they proposed a pathway for hierarchal assembly initiated by the OMER. In summary, thus far, the precise T4SSDot/Icm assembly pathway remains unclear, and detailed structural analyses using T4SSDot/Icm component mutants are required for further elucidation.

T4SSDot/Icm COUPLING COMPLEX AND SUBSTRATE RECOGNITION

Type IV coupling proteins (T4CPs) are AAA-type hexameric ATPases associated with the bacterial inner membrane via N-terminal transmembrane segments. T4CPs are conserved in most, but not all, T4SSs. T4CPs are named after their functions of “coupling” translocating substrates and the transmembrane transport apparatus. DotL is a T4CP of the T4SSDot/Icm; thus, it is thought to play a central role in substrate recognition and recruitment to the transport apparatus. Several studies have reported that most T4SSDot/Icm substrate proteins harbor translocation signal sequences, which are rich in short polar, hydrophobic, or negatively charged amino acids at their C-terminus. However, early studies also demonstrated that mutant strains lacking icmW or icmS showed moderately defective phenotypes of intracellular growth and host cell cytotoxicity, suggesting that IcmS and IcmW are not essential for effector translocation per se, but are required for efficient translocation of an “IcmSW-dependent” subset of effector proteins. IcmS and IcmW, small acidic proteins that can form a heterodimer called IcmSW, have been proposed to serve as adaptors to recruit IcmSW-dependent substrate proteins to the transport apparatus. In 2012, Vincent et al. reported lines of evidence suggesting a T4SSDot/Icm subcomplex composed of the T4CP DotL, the apparatus proteins DotM and DotN, and the secretion adaptor proteins IcmS and IcmW. Furthermore, Sutherland et al. demonstrated that the DotL C-terminal extension region, which is prevalent...
among T4BSS, but not T4ASS coupling proteins, was able to bind directly to IcmSW. The DotL(671-783) segment was required for binding.75 Here, we describe the recent discoveries regarding the substrate recognition by the T4CP complex.

**DotLN-IcmSW-LvgA complex**

In 2017, Kwak et al.79 reported the crystal structures of DotL (656-783)-IcmSW, DotL(590-659)-DotN, and DotL(656-783)-IcmSW-LvgA. Based on the individual structures, they reconstituted DotL(590-783)-DotN-IcmSW-LvgA (Figure 2). In the complex, the C-terminal segments of DotL bound to DotN or IcmSW adopted an unfolded conformation. Thus, the structure of DotL segments is likely unstable in the absence of interacting partners, which is consistent with the chemical instability of DotL in the absence of DotN or IcmSW, as reported by Vincent et al.70 Taking advantage of the well-established properties of structure-solved T4CPs, such as R388 TrwB, which forms a ring-shaped hexamer,70 Kwak et al.79 constructed the model for the "T4CP holocomplex" consisting of a DotLN-IcmSW-LvgA hexamer. The T4CP holocomplex resembled an elongated bell-shaped architecture, consisting of the membrane-proximal ATPase hexamer and the membrane-distal assemblies containing the DotL C-terminal segment. One important caveat was that this "holocomplex" did not contain DotM (see Section DotM and Glu-rich/E-block-containing effectors).

LvgA was discovered as a virulence factor of *L. pneumophila* using signature-tagged mutagenesis and a guinea-pig infection model; however, its molecular role remains unknown.81 Kwak et al.79 found that IcmSW-dependent effector proteins Vpdb, SetA, and PieA are able to interact with DotLN-IcmSW-LvgA, but not with DotLN-IcmSW, suggesting that LvgA may induce a conformational change in IcmSW for substrate recognition, or that effectors may interact with LvgA, but not with IcmSW. Kim et al.82 further expanded the structural analysis of the interaction between the T4CP complex and the effector proteins. First, they found that the C-terminal region of Vpdb is required for binding to DotL (656-783)-IcmSW-LvgA and then solved the crystal structure of DotL(656-783)-IcmSW-LvgA-Vpdb(461-590). The results demonstrated that Vpdb directly interacts with LvgA, but not with IcmSW, indicating that IcmSW serves as an adaptor connecting the T4CP and LvgA-substrate complexes. Consistently, the translocation of all these effector proteins is partially abrogated by the loss of LvgA; however, the extent of the defects does not exceed the loss of IcmSW. Phe476 of Vpdb is important for the interaction with the hydrophobic pocket composed of four residues (Phe149, Ile153, Pro166, Tyr173) of LvgA. They found the LvgA binding motif (FxxxLxxxK) in many IcmSW-dependent effectors including Vpdb and SidH, but not in PieA and SetA. Of note, Vpdb also has a Glu-rich/E-block signal sequence28 near the C-terminus, which may interact with DotM (see Section DotM and Glu-rich/E-block-containing effectors).

Xu et al.83 also reported the crystal structure of DotL (661-773)-IcmSW and provided evidence for the existence of the DotLN-IcmSW-LvgA complex in 2017. They also explored the detailed mechanism of substrate recognition by IcmSW, but not in the complex with DotL. They determined the binding surface of IcmSW to an IcmSW-dependent effector SidF using photocrosslinking assays and found that the effector-binding surface of IcmSW overlaps with the DotL-binding surface.

**DotM and Glu-rich/E-block-containing effectors**

DotM is supposed to be a T4CP complex component, but it was not included in the structural model described above.79,82 Similar to the T4CP DotL, DotM has membrane-spanning segments at its N-terminus.70 The crystal structures of the cytoplasmic domains of DotM have been independently reported by two groups.79,84 Meir et al.84 found a positively charged DotM surface composed of arginine and lysine residues, which is responsible for binding to the negatively charged Glu-rich/E-block motif located at the C-terminus of a subset of effector proteins.28 The *L. pneumophila* mutant strains carrying the amino acid substitutions in DotM (R196E/R197E or R217E), which alter the charge of the substrate recognition surface of DotM, showed intracellular growth defects in mouse J774A.1 cells and A. castellanii. The mutant strains also showed subtle but significant defects in translocation of effectors carrying the Glu-rich/E-block motif, but not of those lacking the motif.37 Thus, DotM plays a role in substrate recognition of Glu-rich/E-block-containing effector proteins.
DotLMNYZ hetero-pentameric complex

Meir et al. conducted a cryo-EM single particle analysis of the DotL-containing complex purified from *L. pneumophila* membrane fractions solubilized with detergent. The purified complex contained all known T4CP complex components: DotL, DotM, DotN, IcmS, IcmW, and LvgA, and two additional uncharacterized proteins, DotY (Lpg0294) and DotZ (Lpg1549). The assessed mass of the complex was ~300 kDa, which is consistent with a complex that may contain one copy each of the eight proteins. The loss of DotY, DotZ, or both resulted in a one-fold reduction in the translation of both IcmSW-dependent and IcmSW-independent effector proteins, suggesting that these uncharacterized proteins play a role in T4SS transport. The cytoplasmic domains of DotL and DotM, DotN, DotZ, and the first 77 N-terminal residues of DotY (DotLMNYZ hetero-pentameric complex) were assigned to the structure of the purified complex. However, IcmSW and LvgA were not assigned to the model structure. The flexibility of the DotL region connecting the DotLMNYZ hetero-pentameric complex and the DotL-IcmSW-LvgA complex explains why IcmSW-LvgA is invisible in the structure. They also constructed a hexamer model of the DotLMNYZ hetero-pentameric complex, similar to the DotLN-IcmSW-LvgA complex of Kwak et al. Intriguingly, the acidic surface of DotM faces outside the hexameric model of hetero-pentameric complex. Therefore, if DotM-dependent effectors are transported through the channel formed by the DotL hexamer, their Glu-rich/E-block segment must pass through the cavity formed by DotM, DotN, and DotZ to interact with DotM.

Integrating the findings described in this section, we propose possible models of substrate recognition and recruitment, as shown in Figure 2, where the substrate proteins are recruited to the T4CP holocomplex in the bacterial cytoplasm through interaction with IcmSW, LvgA, and/or DotM. These three pathways may not be mutually exclusive. Thereafter, substrate proteins associated with the T4CP complex may be transferred to the T4SS transport apparatus without going through the DotL channel (Route 1). Alternatively, the substrates might be placed under the channel formed by the DotL hexamer. Then, the substrates, being unfolded somehow, may traverse the central channel (Route 2). However, if this is the case, the exact connection of the DotL channel to the central conduit of the T4SS transport apparatus has not been addressed yet.

CONCLUSION AND PERSPECTIVES

In this review, we describe the recent discoveries regarding the structural biology of the T4SS transport apparatus and the coupling complexes. Needless to say, the huge technical advances in structural biology, such as cryo-EM, have promoted these seminal studies. Furthermore, the determination of atomic-level structure explaining the LvgA- and DotM-dependent substrate recognitions expanded our knowledge of how the T4SS recognizes numerous effector proteins with distinct types of signal sequences. However, these findings have raised new questions. To date, the in situ structure of the T4SS coupl complex has not been elucidated. How the coupling complex and the transport apparatus interact each other and how the substrate proteins are actually transported using this machinery remains unclear. We also do not know whether the known structures of the T4SS machinery reflect the active form of transport during infection. Understanding the structure and mechanism of the T4SS during *L. pneumophila* infection will be a major challenge for future research.

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DISCLOSURE

The authors declare that there are no conflicts of interest.

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