In situ structure of the *Legionella* Dot/Icm type IV secretion system by electron cryotomography

Debnath Ghosal¹, Yi-Wei Chang¹, Kwangcheol C Jeong^{2,†}, Joseph P Vogel² & Grant J Jensen^{1,3,*}

Abstract

Type IV secretion systems (T4SSs) are large macromolecular machines that translocate protein and DNA and are involved in the pathogenesis of multiple human diseases. Here, using electron cryotomography (ECT), we report the *in situ* structure of the Dot/Icm type IVB secretion system (T4BSS) utilized by the human pathogen *Legionella pneumophila*. This is the first structure of a type IVB secretion system, and also the first structure of any T4SS *in situ*. While the Dot/Icm system shares almost no sequence similarity with type IVA secretion systems (T4ASSs), its overall structure is seen here to be remarkably similar to previously reported T4ASS structures (those encoded by the R388 plasmid in *Escherichia coli* and the *cag* pathogenicity island in *Helicobacter pylori*). This structural similarity suggests shared aspects of mechanism. However, compared to the negative-stain reconstruction of the purified T4ASS from the R388 plasmid, the *L. pneumophila* Dot/Icm system is approximately twice as long and wide and exhibits several additional large densities, reflecting type-specific elaborations and potentially better structural preservation *in situ*.

Keywords Dot/Icm; electron cryotomography; *Legionella pneumophila*; subtomogram averaging; Type IV secretion systems

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Structural Biology

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Introduction

Type IV secretion systems (T4SS) are frequently found in Gram-negative and Gram-positive bacteria as well as in some archaea [1]. They exchange genetic material within and across kingdoms and translocate virulence factors into host cells [2]. T4SSs have been classified into two major groups, type IVA and type IVB [3,4]. Representative examples of T4ASS include those encoded by the conjugative plasmids F, RP4, R388, and pKM101 and the VirB T4SS (VirB1–11 and VirD4) encoded by the plant pathogen *Agrobacterium*

tumefaciens [5,6]. The VirB system is one of the best characterized T4ASSs and consists of a lytic transglycosylase (VirB1), pilins (VirB2 and VirB5), inner-membrane proteins (VirB3, VirB6, VirB8), ATPases (VirB4, VirB11, and VirD4), and three factors (VirB7, VirB9, and VirB10) that span the inner and outer membranes [2].

The designation of a type B class was based on major differences in composition and sequence [4]. T4BSSs include those encoded by the IncI conjugative plasmids R64 and ColIb-P9 and the Dot/Icm (defective in organelle trafficking/intracellular multiplication) system of the pathogens *Legionella pneumophila*, *Coxiella burnetii*, and *Rickettsiella grylli* [2,5,7]. In the case of *L. pneumophila*, the Dot/Icm system translocates more than 300 effector proteins into host cells [8], thereby allowing the pathogen to survive and replicate within phagocytic host cells [9,10]. The Dot/Icm T4BSS is more complex than most T4ASSs as it has ~27 components versus 12. The only clear sequence similarity between T4ASS and T4BSS components occurs within the C-terminus of DotG, which matches part of the VirB10 sequence [11]. The Dot ATPases (DotB, DotL, DotO) are also of the same general classes of proteins as the *A. tumefaciens* ATPases (VirB11, VirD4, VirB4). Based on relationships between ATPases, T4SSs have recently been reclassified into eight classes, with the IncI class being one of the most distinct [12]. How similar the structures and functions of different T4SS are remains unclear.

Great efforts have been invested into structurally characterizing different T4ASSs using an impressive array of biochemistry, crystallography, and electron microscopy (EM) [2,13–17]. The most notable achievements include a crystal structure of parts of VirB7, VirB9, and VirB10 from the pKM101 system (3JQO) [13], two cryo-EM structures of the same complex [14], and a negative-stained EM reconstruction of a recombinantly purified VirB_{3–10} complex from the related R388 system [15]. The features of the VirB_{3–10} reconstruction were described as consisting of a periplasmic complex (cap, outer layer, inner layer), linked by a relatively thin stalk to an inner-membrane complex (upper tier, middle tier, and a lower tier), with the latter forming two barrel-shaped densities that correspond to the VirB4 ATPase extending into the cytoplasm. However, to date, no structure has been reported for a T4BSS nor has an *in situ* structure been described for any T4SSs. Considering their distinct genetic organization and composition, whether and how the T4ASS and T4BSS are structurally related remains unclear.

1 California Institute of Technology, Pasadena, CA, USA

2 Washington University School of Medicine, St. Louis, MO, USA

3 Howard Hughes Medical Institute, Pasadena, CA, USA

*Corresponding author. Tel: +1 626 395 8827; E-mail: jensen@caltech.edu

†Present address: University of Florida, Gainesville, FL, USA

Results and Discussion

To generate the first three-dimensional structure of a T4BSS, here we used ECT to visualize *L. pneumophila* Dot/Icm machines directly in intact, frozen-hydrated bacteria cells. In our tomograms, we observed multiple dense, cone-shaped particles in the periplasm primarily near the cell poles (Fig 1A and B; Movie EV1). These structures exhibited the characteristic shape of a “Wi-Fi” symbol comprising two distinct curved layers, the larger just below the outer membrane and the smaller in the middle of the periplasm (Fig 1C). In some cases, the outer membrane bulged outward around the “Wi-Fi” particles, but not always. We also observed top views of these particles, which appeared to have two concentric rings (Fig 1D). Because similar ~40-nm-diameter rings were observed previously in EM images of portions of the Dot/Icm complex [18], and no “Wi-Fi” particles were observed in a

L. pneumophila strain lacking the *dot/icm* genes (Figs 1E and EV1), we hypothesized that the “Wi-Fi” particles were Dot/Icm complexes.

To further investigate the molecular architecture of these complexes, we generated a subtomogram average using 386 particles. In the initial average, substructures were resolved within the curved layers but details were lacking near the inner membrane (Fig EV2A–D). Given the previous observation of structural flexibility between the outer- and inner-membrane-associated parts of the VirB₃₋₁₀ complex [15], we used masks to align and average components near the outer membrane separately from the components near the inner membrane (Fig EV2A–D). A composite average was then constructed by juxtaposing the well-aligned regions of the outer- and inner-membrane averages. In the final composite average, many distinct densities were resolved including a hat, alpha, and beta densities near the outer membrane; a stem, stalk, and wing densities near the inner membrane; and rods near the inner membrane.

Figure 1. In situ structure of Dot/Icm T4BSS.

- A, B Tomographic slices through intact *Legionella pneumophila* cells. Black arrows point to Dot/Icm particles. Scale bar, 100 nm.
- C Enlarged view of Dot/Icm particles, outer membrane (OM), and inner membrane (IM). Scale bar, 20 nm.
- D Tomographic slices showing a top view of a Dot/Icm particle (white arrow head), enlarged in the inset. Scale bar, 100 nm.
- E Tomographic slice through a *L. pneumophila* cell lacking the *dot/icm* genes. Scale bar, 100 nm.
- F Subtomogram average of wild-type Dot/Icm particles. The subtomogram average was generated using 386 particles. OM, outer membrane; IM, inner membrane. Scale bar, 10 nm.
- G Schematic representation of the subtomogram average labeling the prominent densities.
- H Local resolution of the subtomogram average calculated by ResMap [29].

gamma densities in the periplasmic region; and weaker densities, which we call “wings”, extending from the inner membrane into the periplasm (Fig 1F and G). Although of lower resolution, multiple vertical rod-like densities also appeared below the inner membrane in the cytoplasm. We estimate the local resolution of our composite model to be 2.5–4.5 nm (Fig 1H), likely limited by inherent flexibility of the complex, as the resolution within the curves layers was the highest and the rods the lowest.

To confirm that the “Wi-Fi” particles were the Dot/Icm system, we imaged a strain expressing DotC, DotD, DotF, DotG, and DotH (previously defined as the “core complex” [19]) in an otherwise *dot/icm* null mutant strain (Fig 2A). Western blot analysis showed all five proteins were expressed at similar levels to those in the wild-type strain (Fig EV1). The subtomogram average of this reconstituted complex revealed a strong similarity to the wild-type structure as it contained the hat, beta, and gamma densities and some of the stem, but there were also major densities missing (Fig 2A–C). Since the “Wi-Fi” particles were not observed in a strain lacking the *dot/icm* genes, and a portion but not all of the complex reappeared upon reintroduction of the five core Dot proteins, we conclude that these particles are the Dot/Icm system rather than some other membrane complex such as the *L. pneumophila* T2SS or a different T4SS.

In T4ASS, a “core complex” has been described consisting of three proteins with major domains in the periplasm, the inner-membrane protein VirB10, the outer-membrane protein VirB9, and a lipoprotein VirB7, which plays a role in the insertion of VirB9 [2]. In the *L. pneumophila* T4BSS, DotF and DotG are inner-membrane proteins, DotH is an outer membrane, and there are two lipoproteins, DotC and DotD, that function to insert DotH [19]. Markedly, and as mentioned above, among these proteins, there is only one domain

shared between the Dot and VirB systems: the C-terminus of DotG has clear sequence homology to VirB10 (Fig 3). Despite this paucity of sequence similarity between components, the *in situ* structure of the Dot/Icm T4BSS and the negative-stain reconstruction of the VirB₃₋₁₀ T4ASS complex clearly share key features. First, the size and shape of the hat density in the Dot/Icm apparatus match the VirB10 density from the crystal structure 3JQO, which contains parts of VirB7, VirB9, and VirB10 (Fig 4A–C, Movie EV2). This makes sense because the domain of VirB10 present in the crystal structure is the one with sequence homology to DotG (Fig 3B). Thus, it is not surprising that there would be a similar-shaped feature in the equivalent location of the Dot/Icm structure (as seen in the hat). Second, both the Dot/Icm and VirB₃₋₁₀ structures contain flexible stalks between the outer-membrane and inner-membrane complexes. Finally, the four rod-like densities in the Dot/Icm structure correspond well in size, shape, and position (with respect to both the inner membrane and stalk) to the walls of the two barrels seen in the VirB₃₋₁₀ complex, leading us to propose that there are two similar barrels present in the Dot/Icm complex, even though they are poorly resolved (Fig 4A, D, G and H). Our interpretation is that each pair of rods forms one barrel ~11 nm wide and ~15 nm long (Fig 4A, IM complex, double-headed arrows), just like the barrels seen in the VirB₃₋₁₀ complex. Thus, the basic architecture of the Dot/Icm system is strikingly similar to that of the VirB₃₋₁₀ complex: Each contains a hat, stalk, and two off-axis cytoplasmic barrels.

However, there are also major differences. First, the Dot/Icm complex is approximately twice as wide and long as the VirB₃₋₁₀ complex (Fig 4G and H). Second, there are no densities in the VirB₃₋₁₀ complex peripheral to the hat which might correspond to the alpha and beta densities in Dot/Icm structure. Third, it is not clear

Figure 2. Subtomogram average of a reconstituted subcomplex.

- A Subtomogram average of a reconstituted subcomplex consisting of DotC, DotD, DotF, DotG, and DotH in the *dot/icm* deletion mutant. The subtomogram average was generated using 261 particles. Dotted yellow lines indicate where the outer-membrane average is merged with the inner-membrane average to generate the composite images.
- B Difference map between the WT complex and the reconstituted core complex. Densities missing in the reconstituted complex are colored yellow, additional densities in red.
- C Outline of the core complex (orange dotted line) superimposed on the Dot/Icm structure. The same image is presented as in Fig 1F.
- Data information: OM, outer membrane; IM, inner membrane. Scale bars, 10 nm.

Figure 3. Genetic organization of T4ASSs and T4BSSs.

A Organization of the T4ASS (VirB type) and T4BSS (Dot/Icm type) genes. The T4BSS has a more elaborate and complex genetic organization. Genes colored in blue are ATPases.

B VirB10 and DotG show clear sequence similarity with both proteins having a conserved Trbl domain in their C-terminal region.

whether the Dot/Icm gamma density is part of what was described as the inner layer of the VirB₃₋₁₀ complex. Fourth, the Dot/Icm structure has periplasmic wings instead of the membrane-associated arches seen in the VirB₃₋₁₀ complex. While some of these differences are likely due to the additional factors present in the Dot/Icm system, others may reflect the loss or collapse of components in the VirB₃₋₁₀ complex upon purification and negative staining. The arches of the VirB₃₋₁₀ complex, for instance, may correspond to collapsed Dot/Icm wings, and the shorter and thinner stalk in the VirB₃₋₁₀ complex may also be a result of collapse (the distance between the outer and inner membranes in our cryotomograms of different species of intact bacterial cells is typically ~40 nm [20,21], twice as far as proposed in the VirB₃₋₁₀ complex structure).

Recently, two-dimensional class average images of a negatively stained *Helicobacter pylori* T4ASS comprising Cag3, CagM, CagT/VirB7, CagX/VirB9, and CagY/VirB10 were reported (Fig 4E and F) [22]. While the *H. pylori* T4ASS consists of approximately the same number of components as the *L. pneumophila* Dot/Icm T4BSS, the additional factors share no sequence similarity [22]. Despite also being purified, dried, and negatively stained like the R388 plasmid VirB₃₋₁₀ complex, the *H. pylori* structure has almost exactly the same size and overall shape as the periplasmic region of the *L. pneumophila* structure, with a large bulbous structure at one end and a stalk at the other (Fig 4A, E and F). Because the *H. pylori* images were of a purified subcomplex, it was impossible at the time to assign an orientation of the structure relative to the cell envelope [22]. Based on the similarities to our *in situ* T4BSS structure, we now predict that the convex surface of *H. pylori* T4ASS faces the outer membrane and the stalk points in the direction of the inner membrane.

In summary, we have revealed the first *in situ* structure of a T4SS and shown that despite very little sequence similarity between

representative T4ASSs and T4BSSs (Fig 3A and B), their basic architectures and therefore likely secretion mechanisms are remarkably similar. They are much more similar than different when compared to the structure of other secretion systems. Type III secretion systems, for example, consist of a series of rings in the inner membrane, periplasm, and outer membrane connected by a central channel that serves as the conduit for protein export [23]. In contrast, neither the T4ASS nor the T4BSS exhibits an obvious tube-like channel along the symmetry axis through which substrates might be transported. While already helping to clarify the relationship between T4ASS and T4BSS, the *in situ* Dot/Icm structure also sets the stage for future work identifying each protein in the complex and elucidating how this elaborate nanomachine assembles and functions.

Materials and Methods

Strains, growth conditions, and mutant generation

All experiments mentioned here were performed using the *L. pneumophila* Lp02 strain (*thyA hsdR rpsL*), which is a derivative of the clinical isolate *L. pneumophila* Philadelphia-1. *L. pneumophila* strains were grown on ACES [N-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal yeast extract agar (CYE) or in ACES-buffered yeast extract broth (AYE), each supplemented with ferric nitrate and cysteine hydrochloride. Since Lp02 is a thymidine auxotroph, cells were always grown in the presence of thymidine (100 µg/ml). JV5443 is a derivative of Lp02 lacking the *dot/icm* genes (JV5319) that was transformed with plasmid pJB4027, which expresses *dotD*, *dotC*, *dotH*, *dotG*, and *dotF*.

Figure 4. Comparison between T4ASSs and T4BSSs.

- A** Structure of the Dot/Icm complex with the outlines of existing structures of T4ASS subcomplexes (e.g., outline of the VirB10 part from the crystal structure 3JQO (red), outline of a cryo-EM single-particle reconstruction of purified VirB4 ATPase (yellow), and outline of a 2D average of purified *Helicobacter pylori* T4ASS core complex (white)) superimposed. Double-headed arrows indicate width of each barrel. OM, outer membrane; IM, inner membrane. Scale bar, 10 nm.
- B** Surface representation (left) and central cross section (right) of crystal structure 3JQO, an outer-membrane complex of parts of VirB7, VirB9, and VirB10 from the T4ASS encoded by plasmid pKM101. Red colored part of the cross section is density for VirB10, and light-pink color is combined density for VirB7 and VirB9. The outline of VirB10 density matches the hat density of the Dot/Icm structure (red dotted line in panel A, see also Movie EV2).
- C** Crystal structure 3JQO fit into the VirB₃₋₁₀ negative-stain single-particle reconstruction, showing its location with respect to the outer membrane [13,15].
- D** Isosurface of (left) and central cross section through (right) a single-particle reconstruction of purified VirB4 ATPase (EMD-5505) (Pena *et al* [16]). Because it is a hexameric barrel-shaped structure, its cross section is two parallel rod-like densities similar to the IM-associated densities found in purified VirB₃₋₁₀ complex from the R388 plasmid (panel H) and cytoplasmic densities found in the Dot/Icm structure *in situ* (panel A).
- E** 2D class average images of purified *H. pylori* T4ASS subcomplex comprising Cag3, CagM, CagT/VirB7, CagX/VirB9, CagY/VirB10 in top and side views (reproduced from [22]).
- F** Same side view as in (E) but rotated and enlarged to the same scale as the Dot/Icm structure. Outline marked in white dotted line and superimposed on the Dot/Icm structure in panel (A).
- G, H** Schematic representations of the *Legionella pneumophila* Dot/Icm T4BSS (G) and the *Escherichia coli* R388-encoded T4ASS (H, adapted from [15]) showing dimensions, underlying structural similarities, and differences.

Data information: Scale bars for all panels except (E), 10 nm. Scale bar for panel (E), 25 nm.

Sample preparation for electron cryotomography

Legionella pneumophila Lp02 cells were harvested at early stationary phase (OD₆₀₀ of ~3.0), mixed with 10-nm colloidal gold beads (Sigma-Aldrich, St. Louis, MO, USA) precoated with bovine serum albumin, and applied onto freshly glow-discharged copper R2/2 200 Quantifoil holey carbon grids (Quantifoil Micro Tools GmbH, Jena, Germany). Grids were then blotted and plunge-frozen in a liquid ethane/propane mixture [24] using an FEI Vitrobot Mark IV and stored in liquid nitrogen for subsequent imaging.

Electron tomography and subtomogram averaging

Tilt-series were recorded of frozen *L. pneumophila* Lp02 cells in an FEI Titan Krios 300 kV field emission gun transmission electron microscope (FEI Company, Hillsboro, OR, USA) equipped with a Gatan imaging filter (Gatan, Pleasanton, CA, USA) and a K2 Summit direct detector in counting mode (Gatan) using the UCSF Tomography software [25] and a total dose of ~100 e/A² per tilt-series and target defocus of ~6 µm underfocus. Images were aligned, contrast transfer function corrected, and reconstructed using IMOD [26]. SIRT reconstructions were produced using TOMO3D [27], and subtomogram averaging was performed using PEET [28]. Finally, the local resolution was calculated by ResMap [29]. As the Dot/Icm subtomogram average exhibited a gross twofold symmetry around the central midline in the periplasm, we applied twofold symmetry in those regions to produce the 2D figures shown, but no symmetry was applied to the cytoplasmic densities due to their poor resolution.

Accession numbers

Subtomogram averages reported in this study have been deposited in the EMDBank (EMDB) with accession numbers EMD-8566, EMD-8567, EMD-8568, and EMD-8569.

Expanded View for this article is available online.

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Author contributions

DG and GJJ conceived and designed the study. DG collected and processed ECT data. YWC helped with data processing and analysis. JPV and KCJ constructed and characterized strains. DG, GJJ, and JPV wrote the manuscript. All authors contributed to data analysis and manuscript writing.

Conflict of interest

The authors declare that they have no conflict of interest.

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