A unique cytoplasmic ATPase complex defines the *Legionella pneumophila* type IV secretion channel

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Type IV secretion systems (T4SSs) are complex machines used by bacteria to deliver protein and DNA complexes into target host cells\(^{1,5}\). Conserved ATPases are essential for T4SS function, but how they coordinate their activities to promote substrate transfer remains poorly understood. Here, we show that the DotB ATPase associates with the Dot–Icm T4SS at the *Legionella* cell pole through interactions with the DotO ATPase. The structure of the Dot–Icm apparatus was solved in situ by cryo-electron tomography at 3.5 nm resolution and the cytoplasmic complex was solved at 3.0 nm resolution. These structures revealed a cell envelope-spanning channel that connects to the cytoplasmic complex. Further analysis revealed a hexameric assembly of DotO dimers associated with the inner membrane complex, and a DotB hexamer associated with the base of this cytoplasmic complex. The assembly of a DotB–DotO energy complex creates a cytoplasmic channel that directs the translocation of substrates through the T4SS. These data define distinct stages in Dot–Icm machine biogenesis, advance our understanding of channel activation, and identify an envelope-spanning T4SS channel.

Bacteria use type IV secretion systems (T4SSs) to colonize and proliferate in various clinical and non-clinical settings\(^{1,5}\). The T4SSs are complex nanomachines that span the bacterial cell envelope and mediate the delivery of protein and DNA substrates to target cells, a process generally requiring direct cell-to-cell contact\(^1\). There are two phylogenetically distinct T4SSs, termed type IVA and IVB. The *Agrobacterium tumefaciens* VirB/VirD4 T4SS and *Escherichia coli* R388 and pKM101 plasmid conjugation systems are IVA systems composed of a dozen different proteins\(^1\). The IVB systems represented by the *L. pneumophila* Dot–Icm T4SS are more complex, requiring more than two dozen proteins\(^1\). Effector-translocating IVA and IVB systems have three highly conserved ATPases, the substrate receptor VirD4/DotL (also termed the type IV coupling protein or T4CP), VirB4/DotO and VirB11/DotB. How these ATPases are structurally arranged at the base of their respective T4SSs to coordinate substrate transfer remains to be determined.

The *L. pneumophila* Dot–Icm T4SS also is composed of an OMCC, originally described as a ring- or barrel-shaped structure reminiscent of the IVA OMCC, but with a much larger cross-section of ~400 Å\(^7\). Recently, a structure of the Dot–Icm T4SS was revealed in the native context of the *L. pneumophila* cell envelope by cryo-electron tomography\(^7\). The OMCC visualized in situ displayed two distinct curved layers, the larger just below the outer membrane and the smaller in the periplasm. This structure was designated as ‘WiFi-like’ and is composed of at least five subunits, DotC, DotD, DotE, DotG and DotH. An IMC was observed at lower resolution. A side-view image of the IMC identified four rod-like structures extending into the cytoplasm. Based on their overall dimensions and positions with respect to the inner membrane and OMCC, and by analogy to the IVA\(_{10}\) structure, these projections might represent the walls of two barrel-shaped hexamers of the DotO ATPase.

Fundamental questions remain about how the IVA and IVB systems are architecturally configured and translocate substrates, particularly across the cytoplasmic membrane. Here, fluorescence microscopy was combined with a high-throughput cryo-electron tomography pipeline to investigate the *L. pneumophila* Dot–Icm system. These data demonstrate the association of the DotB ATPase with the T4SS, and reveal that the cytoplasmic complex is composed of an ATPase subassembly. Remarkably, the cytoplasmic complex of this IVB system is highly symmetric with two hexamers of DotB and DotO stacked on one another and centrally positioned at the base of an inner membrane-spanning channel. Consequently, in contrast to the asymmetric IVA\(_{10}\) structure\(^7\), the IVB structure lends itself readily to the visualization of a translocation pathway by which substrates recruited to the base of the secretion apparatus are conveyed from the bacterial cytosol through a continuous channel to the cell surface.

**DotB association with the polar Dot–Icm machine.** The Dot–Icm system assembles at cell division sites and localizes at the bacterial poles\(^6\).
To investigate the requirements for polar positioning and characterize dynamic features associated with machine biogenesis, genes encoding superfolder green fluorescent protein (sfGFP) or mCherry were fused to dot and icm genes and recombined at their native positions on the chromosome. This approach preserved both the stoichiometry and timing of fusion protein production relative to the other Dot–Icm machine subunits.

The OMCC is composed of three outer membrane-associated proteins, the DotC and DotD lipoproteins and DotH, and two large proteins, DotF and DotG, that extend from the inner membrane across the periplasm to form contacts with the DotC/D/H subassembly (Fig. 1a)\(^1\). We confirmed that fluorescently tagged OMCC subunit DotG–sfGFP, the IMC subunit DotI–sfGFP, the T4CP subunit DotL–sfGFP, cytoplasmic secretion ATPase DotB–sfGFP and the secretion chaperone IcmS–sfGFP were produced as stable and functional components as monitored by immunoblot analysis and a Dot–Icm-dependent intracellular growth assay (Supplementary Fig. 1a,b). As predicted, DotG–sfGFP and DotB–sfGFP localized at the cell poles (Fig. 1b). By contrast, IcmS–sfGFP was distributed throughout the cytosol, and DotB–sfGFP was polar-localized only in a subset of cells (Fig. 1b). Polarity scores that indicate the ratio of fluorescence measured at the poles compared with...
fluorescence measured near the middle of the cell were determined for individual cells. The majority of cells producing DotB–sfGFP had a lower polarity score than cells producing either DotG–sfGFP or DotL–sfGFP (Fig. 1b; Supplementary Fig. 1c). Furthermore, time-lapse videos revealed that DotB–sfGFP displayed cytosolic movement in most bacterial cells (Supplementary Video 1), indicating that this ATPase is present in a dynamic cytosolic population but is also capable of associating with the polar Dot–Icm complex. Similar to DotG–sfGFP fluorescence, DotB–sfGFP fluorescence at the poles did not recover rapidly after photobleaching, whereas the fluorescence of cytosolic DotB–sfGFP and soluble sfGFP alone recovered rapidly (Fig. 1c,d). Thus, the assembled Dot–Icm machine creates a stable complex localized at the cell poles, and the DotB protein is present as a dynamic entity in the cytosol that is capable of polar association.

DotB mutations that confer defects on ATP binding or hydrolysis were used to determine how ATP-dependent movement of this energizing factor might impact DotB cellular localization (Fig. 1e)\(^{11}\). All but one of the mutations severely impaired polar recruitment of DotB, which indicated that ATP catalysis was important for polar positioning (Fig. 1f,g). The notable exception, DotB\(E_{191K}\), was previously reported to bind but not hydrolyse ATP \(^{11}\). The DotB\(E_{191K}\)-sfGFP variant localized almost exclusively at cell poles (Fig. 1f,g) and was significantly less dynamic than native DotB–sfGFP (Supplementary Fig. 1d and Video 1). This indicated that ATP-bound DotB protein associates stably with the Dot–Icm machine at cell poles (see also below).

**Polar-positioned DotO ATPase recruits DotB.** To define the requirements of the Dot–Icm machine for DotB polar localization, individual dot or icm genes were deleted to assess effects on DotB\(E_{191K}\)-sfGFP positioning (Fig. 2a,b). Deletions that eliminated production of essential OMCC subunits, DotC, DotD and DotG, but not the non-essential core subunit DotF or the periplasmic protein DotK, abolished polar localization of DotB\(E_{191K}\)-sfGFP. Similarly, deletions that eliminated production of most IMC components (DotI, IcmT, IcmV, IcmQ, DotL, DotU, DotE, DotA) strongly abrogated DotB\(E_{191K}\)-sfGFP polar localization. Elimination of the inner membrane subunits DotP or DotV had less severe effects on DotB\(E_{191K}\)-sfGFP localization, possibly because these subunits play ancillary roles in machine biogenesis. Among the cytosolic IVB components, only the AdotO mutation eliminated DotB\(E_{191K}\)-sfGFP polar localization, consistent with the DotO ATPase having an essential role in the docking of DotB to the IVB apparatus. By contrast, deletions of the T4CP DotL and components of the associated effector-binding adapter complex (DotM, DotN, IcmW, LvgA)\(^{12-14}\) did not have a dramatic effect on DotB\(E_{191K}\)-sfGFP polar localization, arguing against a role for this cytoplasmic subassembly in DotB recruitment. Deletions of the entire Dot–Icm system or of essential OMCC (DotG, DotD) or IMC (DotU, DotA) subunits abrogated polar positioning of native DotB (Supplementary Fig. 2a), confirming the importance of the Dot–Icm machine for recruitment of this ATPase.

The possible role of DotO in recruitment of DotB to the polar-localized Dot–Icm T4SS was evaluated further. As shown for DotB\(E_{191K}\)-sfGFP and in contrast to DotB–sfGFP, DotO–sfGFP was positioned at the cell poles and was static (Fig. 2c,d). The OMCC subunit DotG and the IMC subunits DotI or DotU were essential for polar recruitment of DotO–sfGFP or DotO–mCherry (Fig. 3a,b, Supplementary Fig. 2b). By contrast, deletion of the DotO or DotL ATPases had no effect on DotO–sfGFP polar positioning (Fig. 3a,b). Thus, DotO recruitment to cell poles requires a Dot–Icm platform containing the OMCC and IMC, but does not require the ATPases DotB or DotL (Fig. 3c). Additional studies supported a model where DotO recruits DotB to Dot–Icm machines at the cell pole. In cells coproducing DotO–mCherry and DotB\(E_{191K}\)-sfGFP, tagged DotO localized to the cell pole but tagged DotB\(E_{191K}\) did not (Fig. 3d,e). As expected from the above findings, DotL–mCherry had no effect on polar localization of tagged DotB\(E_{191K}\)-sfGFP (Fig. 3d,e). Thus, addition of mCherry specifically to the carboxy (C) terminus of DotO impeded DotB recruitment to the T4SS, suggestive of a steric effect in blocking a DotO–DotB interaction. Further evidence for a DotB–DotO interaction was gained by immunoprecipitation, in which GFP-specific antibodies co-precipitated DotO in a complex containing DotB–sfGFP or DotB\(E_{191K}\)-sfGFP (Supplementary Fig. 2c).

These studies support a biogenesis pathway in which the Dot–Icm T4SS assembles at the L. pneumoniae cell poles, first by formation of a stable OMCC/IMC substructure that spans the entire cell envelope, and then by recruitment of the DotO ATPase. When positioned, DotO recruits the ATP-bound form of DotB, which for native DotB is likely to be in response to channel-activating signals that are presently not specified.

**Dot–Icm structure revealed by cryo-electron tomography.** To define the spatial organization of the IVB-encoded ATPases relative to each other and the IVB channel, high-throughput cryo-electron tomography was used to visualize the intact Dot–Icm system in wild-type (WT) and mutant cells. A typical reconstruction of a L. pneumoniae cell revealed multiple cone-shaped complexes embedded in the cell envelope (Fig. 4a, Supplementary Video 2). These cone-shaped structures resemble the previously observed polar-localized Dot–Icm subassemblies\(^{10}\). We identified 3,314 cone-shaped complexes from 771 tomographic reconstructions of the cell tips. Subtomogram averaging was then utilized to determine the overall structure of the intact IVB machine at a resolution of 3.5 nm (Supplementary Fig. 3). In the resulting images, the envelope-spanning Dot–Icm machine was shown to consist of two large subassemblies, a distinct OMCC and a cytoplasmic complex (Fig. 4b).

The OMCC is approximately 42 nm in width and 31 nm in height, and further refinement revealed important details that were not apparent in previous structures\(^{8,9}\). The top portion of this subassembly, incorporated in the outer membrane, adopts a wheel-like structure with 13-fold symmetry (Fig. 4c and Supplementary Fig. 3). The entire wheel-like structure is embedded in the inner leaflet of the outer membrane and establishes intimate contacts with the outer leaflet of the outer membrane (Fig. 4c and Supplementary Fig. 3–j). Association of the wheel-like structure also appears to locally remodel the outer membrane (Fig. 4a,b). The wheel has a central pore of ~6 nm in diameter that projects across the outer leaflet of the outer membrane (Fig. 4c, Supplementary Fig. 3). The wheel extends into the periplasm to the peptidoglycan, where it is connected to a cylinder of ~20 nm in diameter and with a central channel of ~6 nm at the connection point (Fig. 4b–f). As the cylinder extends to the inner membrane, however, it abruptly narrows to a diameter of ~10 nm and a channel of ~3 nm (Fig. 4b, Supplementary Fig. 3). Another striking feature of the OMCC is the presence of a plug domain of ~25 nm in length that extends through the centre of the wheel beginning at the outer membrane and extending through the centre of the cylinder to the point of abrupt narrowing (Fig. 4b,c,f). A collar also surrounds the cylinder that extends from the inner membrane to the peptidoglycan layer (Fig. 4d,e). These features are displayed clearly in three-dimensional (3D) reconstructions: a side view of the intact T4SS (Fig. 4d), a cross-section view showing the architectural features (Fig. 4e) and an end-on view showing the 13-fold symmetry of the wheel and outer membrane pore with the interior plug possibly functioning as a regulatory gate (Fig. 4f).

**DotG, a central channel subunit.** DotG extends from the inner membrane to the OMCC subassembly\(^{8,10}\). To determine the structural contribution of DotG to Dot–Icm machine architecture, 3D reconstructions of machines produced in a ΔdotG mutant strain were obtained. The AdotG mutation is non-polar on downstream...
gene expression, as shown by trans-complementation with the native dotG\(^{10}\). The ΔdotG mutant machines were detectable at L. pneumophila cell poles (Supplementary Fig. 4 and Video 2) and also retained the ability to assemble wheel-shaped OMCC complexes with 13-fold symmetry (Supplementary Fig. 4). However, the mutant machines differed from the WT machines in two pronounced ways. First, among the ΔdotG mutant machines examined (374 cone-shaped complexes from 96 tomographic reconstructions), the distance between outer membrane and inner membrane varied from 26 to 33 nm, in contrast to a more fixed dimension of ~30 nm observed for cells producing the WT machine (Supplementary Fig. 4). Second, the ΔdotG machines lacked most of the cylinder that in the WT machines extends from the outer membrane to the inner membrane (Supplementary Fig. 4). This was evidenced both by a lack of cylinder density in a side-view and an end-on view (Supplementary Fig. 4). These findings indicate that DotG physically couples the OMCC with the cytoplasmic complex by forming the portion of the central channel corresponding to the cylinder domain (see Fig. 4e).

A DotO–DotB ATPase subassembly. To characterize the cytoplasmic complex, the in situ structures of the WT and mutant T4SS machines lacking each of the 3 ATPases (DotO, DotB, DotL) were compared. In the WT machine, two ring-like discs with diameters of 16 nm and 27 nm were detected at the base of the inner membrane (Fig. 5a,g and Supplementary Fig. 5). Further refinement of the cytoplasmic complex at 3.0 nm resolution revealed inverted V-shaped structures of 32 nm in width and 24 nm in length connected to the inner membrane portion of the complex (Fig. 5b,h). The cytoplasmic complex consists of six V-shaped structures with the inner arms interacting to form a hexamer, and the outer arms splaying away from the hexamer in a rosette pattern (Fig. 5h,l,m). The apex of the V-shaped is associated with the inner membrane, and the distal tips of the inner arms are connected to a thin disc (Fig. 5b) that in the end-on view forms a central channel (Fig. 5m). Notably, in the side view the V-shaped structures formally resemble the rod-like appendages that were previously reported to extend into the cytoplasm at the base of the Dot–Icm machine\(^{8}\). Although these were interpreted to correspond to the sides of two hexamers of
Fig. 3 | The ATPase DotO is placed above DotB. a. Model summarizing the hierarchy of DotO and DotB recruitment to the poles. Both proteins require the Dot–Icm machine (OMCC and ICM) for polar recruitment. DotB requires DotO for polar recruitment. DotL is not required for recruitment of DotO or DotB to the polar complex. b. Real-time visualization of DotO–sfGFP expressed in strains having the indicated dot genes deleted. Scale bar, 3 μm. Arrowheads indicate regions magnified in insets. c. The polarity scores of DotO–sfGFP in strains having the indicated dot genes deleted. The black horizontal lines represent the medians of the polarity scores. The significance was calculated in comparison to DotO–sfGFP. d, Real-time visualization of DotBE191K–sfGFP co-expressed with DotO–mCherry or DotL–mCherry. Scale bar, 3 μm. Arrowheads indicate regions magnified in insets. e. The polarity scores of DotBE191K–sfGFP co-expressed with DotO–mCherry or DotL–mCherry. The black horizontal lines represent the medians of the polarity scores. The significance was calculated in comparison to DotBE191K–sfGFP. Samples sizes (n), significance values (P) and the type of statistical test used to calculate the significance in c and e are shown in Supplementary Table 2.

![Image of a model summarizing the hierarchy of DotO and DotB recruitment to the poles.](image)

The DotO ATPase by analogy to the type IVA VirB16 structure, data here clearly establish that six of these V-shaped structures indeed form a single concentric ring at the base of the Dot–Icm machine.

The ΔdotB mutant machines (1,571 cone-shaped complexes from 171 tomographic reconstructions) lacked the disc positioned at the base of the WT machine (compare Fig. 5b,h and Fig. 5d,j). To further evaluate whether DotB contributes directly to this disc, the structure of the cytoplasmic complex was determined using a ΔdotB strain producing DotB ΔE191K–sfGFP (2,290 cone-shaped complexes from 449 tomographic reconstructions). Remarkably, the density that was missing at the base of cytoplasmic complex in the ΔdotB mutant reappeared in the strain producing the DotB ΔE191K–sfGFP protein, and an extra density was detected that correlated with the additional mass of sfGFP (Fig. 5c,i). Thus, the disc positioned at the base of the cytoplasmic complex is a DotB hexamer (Fig. 5k).

Significantly, the ΔdotO mutant machines (1,513 cone-shaped complexes from 251 tomographic reconstructions) completely lacked the V-shaped structures and associated central disc shown above to correspond to DotB (Fig. 5f, Supplementary Fig. 5). These findings, coupled with the imaging studies showing that DotO recruits DotB to IVB machines at the pole, strongly implicate DotO as the membrane proximal component of the cytoplasmic complex. Previous studies have shown that VirB4-like subunits assemble both as dimers and hexamers and that their amino (N)-proximal domains mediate contacts with the inner membrane15–18. Thus, the V-shaped structures most likely correspond to two DotO subunits connected to each other and to the inner membrane platform via their N-terminal domains. In the 3D model, the C-terminal domains of DotO extend into the cytoplasm (Fig. 5k). In support of this configuration, the atomic structures of a C-terminal domain of a VirB4 homologue19 fit well within the densities comprising the proximal halves of the V arms (Fig. 5l,m). An interesting and unique feature of this structure is that the presumptive DotO subunits comprising the V inner arms interact laterally to form a central hexamer. Accordingly, we present the central channel positioned at the base of the Dot–Icm T4SS as a DotO hexamer comprising six dimers. The cytoplasmic complex therefore consists of two stacked hexamers, one of DotO assembled as a hexamer of dimers and the second of DotB, positioned at the entrance to the Dot–Icm channel (Supplementary Video 2).

No density corresponding to the large DotL–adaptor complex14 was apparent in this solved structure. To further evaluate whether the DotL–adaptor complex contributes to assembly of the cytoplasmic complex, we visualized machines in a ΔdotL mutant. Deletion of dotL is normally toxic to L. pneumophila, but this toxicity can be suppressed by loss-of-function mutations in other dot or icm genes20. Accordingly, the ΔdotL mutation was constructed in the dotB–gfp strain producing the non-functional DotB ΔE191K–sfGFP protein. The ΔdotL mutant machines (864 cone-shaped complexes from 160 tomographic reconstructions) showed no discernible differences in the cytoplasmic complex compared with the functional Dot–Icm machines in strains producing DotB–sfGFP (compare Fig. 5e with Fig. 5f).
and Fig. 5c). These findings confirm that the DotL–adaptor complex is neither a stable component nor necessary for assembly of the cytoplasmic structure. The association of the DotL–adaptor complex with the Dot–Icm machine might be transient and mediated by effector binding or other intracellular or extracellular signals.

**Summary.** Here, complementary cell imaging and cryo-electron tomography were used to define the dynamics and architecture of the DotB–DotO energy complex at the base of the Dot–Icm channel. Our cell imaging data identified a late-stage assembly reaction in which the spatially dynamic DotB ATPase is recruited to the polar-localized Dot–Icm T4SS (Supplementary Video 1), presumably to activate the T4SS for transport. The 3D structure of the intact Dot–Icm system revealed striking details about the OMCC and also identified a cytoplasmic complex comprised of hexamers of DotB and DotO (Supplementary Video 2). The OMCC has 13-fold symmetry, an outer membrane pore, and a central channel that extends along its length to the inner membrane platform. In line with functions assigned to other VirB10-like subunits, DotG links the OMCC to the cytoplasmic complex and may also comprise part of the central channel in the periplasm named here as the cylinder. The cytoplasmic complex displayed 6-fold symmetry because of the stacked configuration of the DotO and DotB hexamers. Significantly, despite the mismatch in OMCC and cytoplasmic symmetries (Supplementary Fig. 6), the Dot–Icm IVB structure overall is highly symmetrical. This is in contrast to the solved IVA structure of the VirB3,10 complex, whose IMC is asymmetric and marked by the presence of two side-by-side VirB4 hexamers and whose connection to the symmetrical OMCC is structurally undefined (Supplementary Fig. 6). Our structure therefore offers for the first time an architectural rendering of the route by which substrates are conveyed through a T4SS, beginning with the recruitment of substrates to the DotB–DotO energy complex at the channel entrance and ending with their passage across the outer membrane through a pore formed by the distal wheel of the OMCC (Supplementary Fig. 7).

T4SSs translocate substrates in response to activating signals conveyed upon contact with target cells. Certain architectural features of the central channel, for example constrictions within the hexameric ATPases and the periplasmic cylinder and the large plug domain extending across the wheel–cylinder interface (Figs. 4,5), thus might undergo signal-activated conformational transitions to regulate substrate passage through the T4SS. Activating signals are also likely to stimulate recruitment of the DotB ATPase to the Dot–Icm T4SS and the recently described DotL–adaptor complex that was not detected in the in situ structure. In the activated Dot–Icm machine, the DotL–adaptor complex might position adjacent to the Dot–Icm T4SS where its long, C-terminal extension acts as a flexible arm to feed substrates to the DotB–DotO channel entrance (Supplementary Fig. 7).

This work describes dynamic features of T4SS machine assembly, presents an in situ structure of a T4SS in unprecedented detail, and facilitates a better understanding of the substrate translocation route through a T4SS. Our findings form a solid foundation for further studies aimed at defining further mechanistic and structural details of type IV secretion. The T4SSs are widely used not just for inter-kingdom transfer of effector proteins, but also for dissemination of genetic material including antibiotic resistance genes between bacteria. The dynamic and structural features of the Dot–Icm system identified here thus represent viable targets for the development of intervention strategies aimed at suppressing pathogenic potential as well as the spread of antibiotic resistance.
**Methods**

**Bacterial strains and plasmids.** Bacterial strains used in this study are derived from *L. pneumophila* strains L-P01 and L-P02 and are listed in Supplementary Table 1. *L. pneumophila* was grown on charcoal yeast extract (CYE) plates in 37°C as described previously. *L. pneumophila* plasmids were constructed by standard recombinant DNA and allelic exchange procedures using the plasmid pSR47S as described previously. sfGFP or mCherry were inserted downstream the Dot–Icm genes and separated by a DNA linker encoding Arg-Thr-Gly-Gly-Ala-Ala.

**Fluorescent microscopy imaging and processing.** Imaging of *L. pneumophila* expressing Dot–Icm fluorescent fusions was carried out by resuspension of 2-day heavy patches in water, after which they were spotted on a thin pad of 1% agarose, covered with a cover slip and immediately imaged at room temperature. Fluorescence micrographs were captured using a Nikon Eclipse TE2000-S inverted microscope equipped with a Spectra X light engine from Lumencor, CoolSNAP EZ 20 MHz digital monochrome camera from Photometrics and a Nikon Plan Apo100x objective lens (1.4 numerical aperture) under the control of SlideBook 6.0 (Intelligent Imaging Innovations). Samples were imaged using a 196 mW 485 nm or a 60 mW 560 nm LED lights, with typical exposure times of 100–400 ms and 2–4×2 binnning. Time lapses of DotB–sfGFP were acquired using continuous illumination. Polarity scores were calculated with SlideBook by measuring the ratio between the variance and the mean of the fluorescence signal at region of interest located between the pole and the cell centre. DotB–sfGFP and DotO–sfGFP time lapse micrographs (Fig. 2C) were acquired using a Nikon TE2000 spinning disc confocal microscope equipped with a Nikon Plan Apo100x objective (1.4 numerical aperture) and restored by the Nearest Neighbors deconvolution algorithm of SlideBook. Fluorescent recovery after photobleaching (FRAP) was performed on a Nikon TiE by fibre coupling a 405 nm solid state laser into the FRAP arm of a TIRF/FRAP illuminator, which focuses the laser to a single diffraction-limited spot on the sample. Imaging was accomplished in wide-field by illuminating the sample with the 470/24 line of a SpectraX solid-state light source laser delivering a wavelength of 405 nm. The photobleached area was determined with SlideBook software; bleaching was achieved with minimal intensities to avoid possible phototoxic effects. Fluorescence intensity measurements were corrected for non-specific photobleaching. The premise for the correction is that the overall intensity of non-bleached areas (of different cells in the same field) should remain constant over time. Intensity measurements were multiplied by the inverse of the ratio of fluorescence at a given time point over fluorescence at the initial time point.

**Intracellular growth assay.** Replication of *L. pneumophila* in RAW 264.7 macrophage-like cells was determined over 2 days using a standard assay that has been described previously. Briefly, RAW 264.7 macrophage-like cells in 24 well tissue culture dishes were infected with *L. pneumophila* at a multiplicity of infection of 1. One hour after infection the wells were washed with PBS and fresh tissue culture medium was added to each wells. Colony-forming units were determined from individual wells after the PBS washes and at 2-days to measure intracellular replication.

**Immunoblot analysis and co-immunoprecipitation.** For immunoblot analysis, *L. pneumophila* cultures were grown for 48 h at 37°C on CYE plates, resuspended in water and adjusted to an *A*<sub>600</sub> of 1.2. Cells from 300 µl were collected by centrifugation at 21,000g for 1 min and resuspended in 300 µl Laemmli sample buffer and boiled for 10 min. A 15 µl bolus was loaded for western blot analysis using a primary polyclonal anti GFP antibody (GenScript) followed by incubation with secondary antibody conjugated to horseradish peroxidase (Sigma). Proteins were visualized by using an ECL detection kit (Amersham Biosciences). For co-immunoprecipitation analysis, *L. pneumophila* cultures were grown for 48 h at 37°C on CYE plates. Cultures were resuspended in water and adjusted to an *A*<sub>600</sub> of 6. Cells from 10 ml were collected by centrifugation at 11,000g for 10 min, re-suspended in 1.5 ml of in lysis buffer (50 mM Tris-HCl pH 8) with protease inhibitor cocktail (Roche Diagnostics), EDTA (2 mM) and lysozyme (0.2 mg ml<sup>−1</sup>, Sigma) and incubated for 1 h on ice prior to sonication. Lysates were centrifuged 10 min at 10,000g and filtered to remove unlysed cells before the supernatant was incubated for 1 h at 4°C with a polyclonal anti GFP antibody (GenScript). Samples were then incubated over night with protein A magnetic beads (Novex) and washed ten times with the lysis buffer. Finally, beads were resuspended in 30 µl Laemmli sample buffer and boiled for 10 min. A 10 µl sample was loaded for western blot analysis using a primary polyclonal anti DotO antibody (provided by Ralph Isberg).

**Preparation of frozen-hydrated specimens.** Bacterial cultures were grown 48 h at 37°C on CYE agar plates. Bacteria were removed from the plates and
suspended in water, then mixed with 10 nm colloidal gold particles (used as fiducial markers in image alignment) and deposited onto freshly glow-discharged, holey carbon grids for 1 min. The grids were blotted with filter paper and rapidly frozen in liquid ethane, using a gravity-driven plunger apparatus as described previously24,25.

Cryo-electron tomography data collection and 3D reconstructions. The frozen-hydrated specimens were imaged at −170°C using a Polara G2 electron microscope (FEI Company) equipped with a field emission gun and a direct detection device (Gatan K2 Summit). The microscope was operated at 300 kV with a magnification of ×15,500, resulting in an effective pixel size of 2.5 Å at the specimen level. We used SerialEM17 to collect low-dose, single-axis tilt series with dose fractionation mode at about 5 μm defocus and a cumulative dose of −50 e−/Å2 distributed over 35 stacks. Each stack contains approximately eight images. Over 2,060 tilt series were collected from −51° to 51° with increments of 3°. We used Tomoauto26 to facilitate data processing which includes drift correction of dose-fractionated data using Motioncor27 and assembly of corrected sums into tilt series, automatic fiducial seed model generation, alignment and contrast transfer function correction of tilt series by IMOD28, and reconstruction of tilt series into tomograms by TomoDI3. Each tomographic reconstruction is 3,716 × 3,838 × 2,400 pixels and ~130 Gb in size. In total, 2,062 tomographic reconstructions from seven different strains were generated (Supplementary Table 2).

Subtomogram averaging and correspondence analysis. We used tomographic package29 (0.9.9) for subtomogram analysis as described previously14,23. A total of 10,291 type IVB secretion machines (400 × 400 × 400 voxels) were visually identified and then extracted from 2,062 cryo-tomographic reconstructions. Two of the three Euler angles of each type IVB secretion machine were estimated based on the orientation of each particle in the cell envelope. To accelerate image analysis, 4 × 4 × 4 binned subtomograms (100 × 100 × 100 voxels) were used for initial alignment and classification. The alignment proceeds iteratively with each iteration consisting of three parts in which references and classification masks are generated, subtomograms are aligned and classified, and finally class averages are aligned to each other. Classification focusing on core complex showed 13-fold symmetry feature, so in the following processing a 13-fold symmetry was imposed to assist the subtomograms alignment. Further classification focusing on the cytoplasmic complex showed a hexagonal structure in four different classes. After multiple cycles of alignment and classification for 4 × 4 × 4 binned subtomograms, we used 2 × 2 × 2 binned subtomograms for refinement. Fourier shell correlation (FSC) between the two independent reconstructions was used to estimate the resolution of the averaged structures (Supplementary Fig. 4).

3D visualization and molecular modelling. We used IMOD to visualize the maps and also to generate 3-D surface rendering of L. pneumophila cell and UCSF Chimera30 to visualize subtomogram averages in 3D and molecular modelling. The pseudo DotO C-terminal structure was modelled using VirB4 (PDB 4AGS) as template, each of DotO C-terminal structures was manually fitted onto the each of distal half of 12 rods. Since no DotB structure is available, we used package I3 (0.9.9) for subtomogram analysis as described previously31. A total 147–152 (2015).

Chetrit, D. et al. Negative regulation of the endocytic adaptor disabled-2 (Dab2) in mitosis. J. Biol. Chem. 286, 5392–5403 (2011).

Zuckman, D. M., Hung, J. B. & Roy, C. R. Pore-forming activity is not sufficient for Legionella pneumophila phagosome trafficking and intracellular growth. Mol. Microbiol. 32, 990–1001 (1999).

Hu, B., Lara-Tejero, M., Kong, Q., Galan, J. E. & Liu, J. In situ molecular architecture of the Salmonella type III secretion machine. Cell 168, 1065–1074 (2017).

Morado, D. R., Hu, B. & Liu, J. Using Tomoauto: A protocol for high-throughput automated cryo-electron tomography. J. Vis. Exp. 107, e53608 (2016).

Mastronarde, D. N. Automated electron microscope tomography using robust prediction of specimen movements. J. Struct. Biol. 152, 36–51 (2005).

Li, X. et al. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. Nat. Methods 10, 553–559 (2013).

Kremer, J. R., Mastronarde, D. N. & McIntosh, J. R. Computer visualization of three-dimensional image data using IMOD. J. Struct. Biol. 116, 71–76 (1995).

Agulleiro, J. I. & Fernandez, J. J. Tomo3D 2.0—exploitation of advanced vector extensions (AVX) for 3D reconstruction. J. Struct. Biol. 189, 147–152 (2015).
31. Hu, B. et al. Visualization of the type III secretion sorting platform of *Shigella flexneri*. *Proc. Natl Acad. Sci. USA* **112**, 1047–1052 (2015).

32. Petersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612 (2004).

33. Agard, D. A., Hiraoka, Y., Shaw, P. & Sedat, J. W. Fluorescence microscopy in three dimensions. *Methods Cell Biol.* **30**, 353–377 (1989).

34. Frick-Cheng, A. E. et al. Molecular and structural analysis of the *Helicobacter pylori* cag type IV secretion system core complex. *mBio* **7**, e02001–e02015 (2016).

35. Berger, K. H. & Isberg, R. R. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol. Microbiol.* **7**, 7–19 (1993).

36. Andrews, H. L., Vogel, J. P. & Isberg, R. R. Identification of linked *Legionella pneumophila* genes essential for intracellular growth and evasion of the endocytic pathway. *Infect. Immun.* **66**, 950–958 (1998).

37. Vogel, J. P., Andrews, H. L., Wong, S. K. & Isberg, R. R. Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science* **279**, 873–876 (1998).

38. Finan, T. M., Kunkel, B., De Vos, G. F. & Signer, E. R. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J. Bacteriol.* **167**, 66–72 (1986).

**Acknowledgements**

B.H. and J.L. were supported by the National Institutes of Health (R01AI087946 and R01GM107629) and the Welch Foundation (AU-1714). D.C. and C.R. were supported by the NIH (R37AI041699 and R21AI130671). P.C. was supported by the NIH (R01GM48476). We are grateful to S.S. Ivanov (Louisiana State University) for insightful suggestions and critique; H. Nagai (Gifu University) for the *L. pneumophila ΔT4SS* strain; R.R. Isberg (Tufts University) for the antibody to DotO; E.H. Rego (Yale University) for technical assistance with FRAP.

**Author contributions**

B.H., C.R., D.C. and J.L. designed research. D.C. constructed the *L. pneumophila* expression plasmids and strains. B.H. and D.C. collected and together with C.R., J.L. and P.C. analysed the data. B.H., C.R., D.C., J.L. and P.C. wrote the paper.

**Competing Interests**

The authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41564-018-0165-z.

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Experimental design

1. Sample size
   Describe how sample size was determined.
   Sample size was determined by selecting number of cells above saturation of medians and averages. For polarity scores, saturation was usually between 80-100 cells and sample size was roughly 200 cells.

2. Data exclusions
   Describe any data exclusions.
   No data was excluded

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All attempts at replication were successful

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   In each set of experiments data was collected randomly

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   The researchers were not blinded to sample identity because image collection was conducted using cells from randomly selected fields and raw data collected from the randomly selected fields was used for the analysis.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a  Confirmed
  ☐  x  The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
  ☐ x  A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   ☐  x  A statement indicating how many times each experiment was replicated
   ☐  x  The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   ☐  x  A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   ☐ x  The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   ☐  x  A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   ☐  x  Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software
Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Fluorescence signal intensity was calculated with SlideBook™ 6.0. Tomograms were generated by using IMOD 4.9.6 and tomo3D 2.0. Subtomograms were analyzed by tomography package i3 0.9.9. Averaged structures were visualized by UCSF Chimera 1.11.1.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents
Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials are available for distribution

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

1. Rabbit Anti GFP (GenScript, Catalog number A01388-40, diluted to 1 μg/ml) was validated by comparing a sample that did not contained GFP to samples that contained GFP and GFP fusions.

2. Rabbit anti DotO antibody was validated and published by Watarai M. et al. (Formation of a fibrous structure on the surface of Legionella pneumophila associated with exposure of DotH and DotO proteins after intracellular growth. Mol Microbiol. 2001).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

RAW 264.7 macrophage-like cells were from ATCC

b. Describe the method of cell line authentication used.

Identification was based by being purchased from a verified source (ATCC), by displaying morphological and cultural characteristics that are typical to this cell line and by being phagocytic.

c. Report whether the cell lines were tested for mycoplasma contamination.

The cell line was validated as being negative by ATCC and there was no morphological indication of mycoplasma contamination upon staining with DAPI

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used

Animals and human research participants
Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animals were used in this study

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participant