DOTB: *LEGIONELLA PNEUMOPHILA'S* DOT/ICM

TYPE IV SECRETION SYSTEM'S AAA+ MOTOR

A Thesis presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

By

CIAIRRA RILEY

Dr. Mark Hannink, Thesis Supervisor

JULY 2023

The undersigned, appointed by the dean of the Graduate School, have examined

the thesis entitled

DOTB: *LEGIONELLA PNEUMOPHILA'S* DOT/ICM

TYPE IV SECRETION SYSTEM'S AAA+ MOTOR

presented by Ciairra Riley,

a candidate for the degree of master of science,

and hereby certify that, in their opinion, it is worthy of acceptance.

_____

Professor Mark Hannink

_____

Professor Charlotte Phillips

_____

Professor Stephanie Gates

ACKNOWLEDGEMENTS

I would like to thank Dr. Mark Hannink for being my supervisor and helping make sure I obtain my MS degree. I thank Dr. Mark Hannink, Dr. Charlotte Phillips, and Dr. Stephanie Gates for their help with thesis edits and feedback. Individual meetings with each committee member provided new perspectives into my project, how to better analyze primary literature, and communicate my research so I could grow into a respectable scientist. I would also like to thank other mentors, including my undergraduate PI Dr. Judy Brusslan, who was a key resource in providing me with resources and ideas for my thesis project.

TABLE OF CONTENTS

Chapter

   Legionella pneumophila: an adaptive Gram-negative bacterium

LIST OF ILLUSTRATIONS

Abbreviations

Dot/Icm T4SS: Defect in organelle trafficking/intracellular multiplication Type IV Secretion System

T4CC: Type IV Coupling Complex

AAA+: ATPases associated with diverse cellular activities

OMCC: Outer Membrane Core Complex

IMC: Inner Membrane Complex

LCV: *Legionella*-containing vacuole

cryo-EM: cryogenic electron microscopy

cryo-ET: cryogenic electron tomography

sf-GFP: super folded Green Fluorescent Protein

Abstract

The Gram-negative bacteria *Legionella pneumophila* is the causative agent of a severe form of pneumonia known as Legionnaire's Disease. *Legionella* utilizes its Defect in organelle trafficking/Intracellular multiplication Type IV Secretion System (Dot/Icm T4SS) to secrete over 300 effector proteins into the cytoplasm of alveolar macrophages for infection. The Dot/Icm T4SS has a Type IV Coupling Complex (T4CC) which has been identified to help recruit effector proteins for translocation during infection. However, not all effector proteins are translocated by the T4CC. DotB is a cytosolic ATPase from the pathogenic *Legionella pneumophila* and is suggested to mediate translocation of specific effector proteins during infection. DotB is a member of the ATPases associated with diverse cellular activities (AAA+) protein family. Characterization of DotB thus far has revealed its ability to form a complex with other T4SS-components and to function as an ATPase that plays a role with translocation. However, much remains unknown about the function of DotB. Earlier studies suggest that DotB may not only function to translocate proteins but can also help mediate other processes such as plasmid transfer and cytotoxicity. Thus, there is a need to further study and understand what proteins DotB can translocate. This review gives insight into the functions DotB may have within *Legionella* and suggests experimental approaches to further characterize DotB's role in the translocation of effector proteins.

1. *Legionella pneumophila*: an adaptive Gram-negative bacterium

1.1 Legionella pneumophila *infection and replication*

Legionnaire's Disease is a severe form of pneumonia caused by *Legionella pneumophila*, a Gram-negative bacteria.[1,2] *Legionella*'s primary hosts are amoeba and protozoa which are commonly found in fresh water, water reservoirs, or even moist soil.[3–6] Inhalation of aerosolized water droplets containing *Legionella* leads to the infection of *Legionella* within the lungs.[3,5] Alveolar macrophages detect the bacteria and secrete IL-1α, inducing chemokine secretion by alveolar epithelial cells, which recruits neutrophils and monocytes.[6] *Legionella* primarily infects alveolar macrophages but is able to infect epithelial cells, neutrophils, monocytes, endothelial cells and fibroblasts.[3–9] *Legionella* is engulfed by macrophages via receptor-mediated endocytosis[4] and utilizes its Defect in Organelle Trafficking/Intracellular Multiplication Type IV Secretion System (Dot/Icm T4SS) to secrete over 300 effector proteins into the cytoplasm of alveolar macrophages[10–13] in a time-dependent manner.[14] So far, only a third of the effector proteins have been characterized.[10] Within the first 8 hours of infection, some effector proteins hijack the vesicular trafficking pathway, forming a protective vacuole containing *Legionella*.[3,4] ER-derived vesicles are recruited to the *Legionella*-containing vacuole (LCV), transforming the LCV membrane to have ER-like shape and thickness.[3–5,7] Other effector proteins decorate the LCV with ribosomes, mitochondria, and host proteins like ubiquitinases, while inhibiting host cell defense mechanisms such as fusion of the LCV with the lysosome.[3–5] Within 8-12 hours,

Legionella begins replicating in the LCV,[4] where effector proteins recruit host cell proteins for the import of necessary nutrients, such as amino acids and carbohydrates.[3,10] The replicative, or vegetative phase, of *Legionella* is characterized by reduced infectivity. As
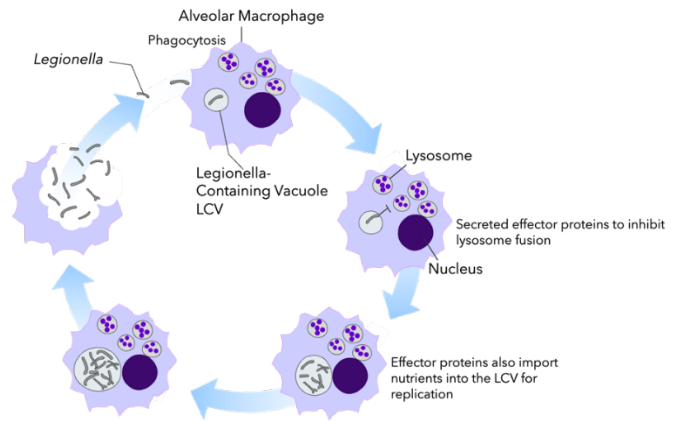

Figure 1. *Legionella pneumophila* **infection.**
Depiction of *Legionella pneumophila* infecting human alveolar macrophages.

replicating bacteria accumulate in the LCV, the bacteria change back into a mature form, characterized by smaller size, accumulation of intracytoplasmic vesicles and increased infectivity.[3] Once the LCV is filled with new bacteria, other effector proteins utilize osmotic lysis to open up pores in the plasma membrane of alveolar macrophages.[3–5,7,15] *Legionella* bacteria that are released will infect other alveolar macrophages and repeat this cycle (**Figure 1**). Cytotoxic release of mature *Legionella* causes tissue damage, leading to inflammation of the lungs and severe pneumonia symptoms.[5] If *Legionella* infiltrates lung endothelial cells (pulmonary blood vessels), *Legionella* is able to
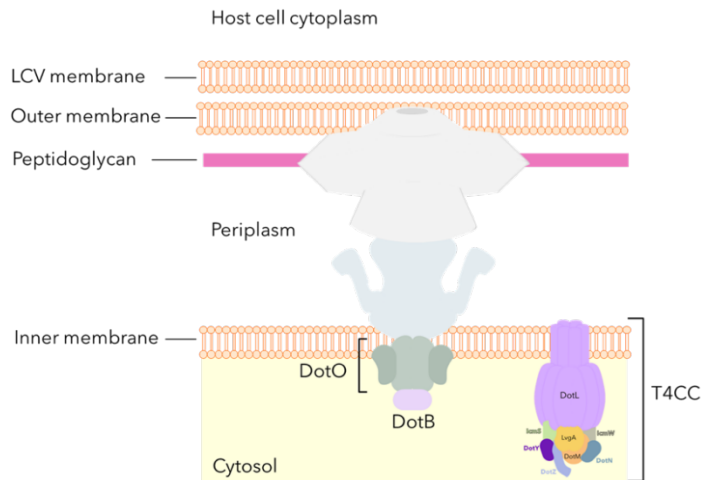

Figure 2. *Legionella pneumophila*'s **Dot/Icm T4SS.**
Depiction of *Legionella pneumophila*'s Dot/IcmT4SS spanning both membranes, the periplasm, and cytosol, with an emphasis on ATPases DotB, DotO and DotL. The type IV coupling complex (T4CC) responsible for effector protein recognition and recruitment is shown as well.

travel through the bloodstream and infect other organs,[7] causing more severe symptoms of Legionnaire's Dsease, including muscle aches, mental capabilities (confusion), and gastrointestinal issues[16]. *Legionella* infection is currently treated via antibiotics[2,16] which are nonspecific and kills many bacteria within the gut microbiome, leading to other health complications[17]. Effector proteins are crucial to *Legionella*'s survival within macrophages and have been studied as a possible target for the treatment of Legionnaire's Disease. Targeting a single effector protein is not an effective therapeutic approach as effector proteins often share similar or redundant functions.[5,10,11] However, as the Dot/Icm T4SS is required to translocate the effector proteins from the bacteria cytoplasm into the host cytoplasm, the Dot/Icm T4SS is an attractive therapeutic target.

1.2 Legionella pneumophila*'s Dot/Icm T4SS*

The Dot/Icm T4SS within *Legionella* spans both outer and inner membranes and has cytosolic proteins (**Figure 2**). The outer membrane core complex (OMCC) contains 10 proteins,[18] and the inner membrane complex (IMC) contains 9 proteins.[10] There are 3 ATPases in the cytosol, including DotL, DotO and DotB, which generate energy for Dot/Icm T4SS assembly and effector protein translocation.[10] The Dot/Icm T4SS also has 1 periplasmic protein, 2 inner-membrane (IM) proteins, and 1 cytosolic protein where the localization within the Dot/Icm complex is unknown[10] (**Table I**). DotO is associated with the inner membrane and forms a complex with DotB that has been suggested to participate

3

in the translocation of effector proteins.[19,20] DotL is an IM ATPase that forms a type IV coupling complex (T4CC) with 7 other proteins[10] (**Figure 2**). The T4CC recognizes and recruits effector proteins for translocation through the Dot/Icm T4SS.[10,11] There are two possible routes by which effector proteins in the bacterial cytoplasm can enter the T4SS: the T4CC or the DotO/DotB complex. It has not yet been determined if the T4CC and DotO/DotB complex alternate between localization to the Dot/Icm T4SS for translocation.

Targeting sequences for the T4CC complex have been identified in some effector proteins. These targeting sequences include a short stretch of hydrophobic residues and a glutamate-rich motif near the C-terminus of the effector proteins.[10,11] Two chaperone proteins, IcmS and IcmW, recognize internal translocation signals.[10,11] Of the ~300 effector proteins that are translocated by the Dot/Icm T4SS, the majority contain hydrophobic translocation signals.[10,11,13,21] RalF is an effector protein containing a hydrophobic C-terminal translocation sequence, but is not recognized by the T4CC.[10,11,13,21] Thus, the subset of effector proteins that are translocated independent of the T4CC are likely translocated via the DotO/DotB complex.

1.3 DotB: the Dot/Icm AAA+ protein structure

DotB is part of the AAA+ protein family, <u>A</u>TPases <u>a</u>ssociated with diverse cellular activities.[22,23] These proteins form hexameric rings and perform a myriad of cellular

processes driven by ATP-hydrolysis, including ER-associated protein Degradation (ERAD), DNA replication, transcriptional regulation, and molecular transport.[24,25] AAA+ proteins have conserved ATP hydrolysis pockets with well characterized Walker A and Walker B motifs.[24,25] A subset of AAA+ proteins, termed Additional Strand Catalytic E (ASCE) proteins, contain both of these motifs in repeating α-helix/β-sheet units that form a P-loop when folded.[24,26] The Walker A motif is identified with a GXXXXGK[S/T] sequence, where X is any amino acid and is responsible for nucleotide binding.[24,25] The Walker B motif is denoted by a hhhh[D/E] sequence, where h is any hydrophobic residue and is critical for ATP hydrolysis.[24,25] DotB's nucleotide binding pocket (NBP) is suggested to fall within the αβα-fold of the P-loop, as observed in other ASCE proteins.[24,26] A DotB monomer also has an "Aspartate box" region (containing no Asp) and a "Histidine

Table I. Dot/Icm T4SS components.

Protein	Location	Function
DotC	Outer Membrane Core Complex	Complex formation
DotD	Outer Membrane Core Complex	Complex formation
DotH	Outer Membrane Core Complex	Complex formation
DotF	Inner Membrane; associates with OMCC	Integrates DotG into complex
DotG	Inner Membrane; associates with OMCC	Unknown
DotK	Outer Membrane Core Complex	Unknown
Dis 1	Outer Membrane Core Complex	Unknown
Dis 2	Outer Membrane Core Complex	Unknown
Dis 3	Outer Membrane Core Complex	Unknown
Unknown	Outer Membrane Core Complex	Unknown
IcmX	Periplasm	Unknown
DotU	Inner Membrane Complex	Stabilizes OMCC; recruits DotO and DotB
IcmF	Inner Membrane Complex	Stabilizes OMCC
DotI	Inner Membrane Complex	Recruits DotO
DotA	Inner Membrane Complex	Recruits DotB
IcmQ	Inner Membrane Complex	Recruits DotB
IcmV	Inner Membrane Complex	Recruits DotB
IcmT	Inner Membrane Complex	Recruits DotB
DotJ	Inner Membrane Complex	Recruits DotB
DotE	Inner Membrane Complex	Recruits DotB
DotV	Inner Membrane	Unknown
DotP	Inner Membrane	Unknown
DotL	Inner Membrane, Type IV Coupling Complex	C-term extensions form T4CC; recognition and recruitment of effector proteins
IcmS	Cytosolic chaperone; Type IV Coupling Complex	heterodimer with IcmW; recognize internal translocation signals
IcmW	Cytosolic chaperone; Type IV Coupling Complex	heterodimer with IcmS; recognize internal translocation signals
DotN	Cytosolic; Type IV Coupling Complex	Effector protein recruitment
LvgA	Cytosolic; Type IV Coupling Complex	Effector protein recruitment
DotM	Cytosolic, Inner Membrane associated; Type IV Coupling Complex	Effector protein recruitment
DotY	Cytosolic; Type IV Coupling Complex	Effector protein recruitment
DotZ	Cytosolic; Type IV Coupling Complex	Effector protein recruitment
DotO	Cytosolic, Inner Membrane associated	Recruits and forms a complex with DotB for effector protein translocation
DotB	Cytosolic	Forms complex with DotO for effector protein translocation
IcmR	Cytosolic	Unknown

box" (containing two His) (**Figure 3**). Visualization of DotB via Negative-Stain Electron Microscopy revealed that DotB forms homohexameric rings, with or without nucleotides present.[22] An x-ray crystallography structure of DotB, (PDB: 6GEB, **Figure 3**)[23] showed that DotB has two asymmetric subunits, α and β, such that the hexamer is a dimer of trimers. In the α subunit, the C-terminal domain is aligned with a β-strand in the center of the N-terminal domain. In the β subunit, the C-terminal domain is rotated towards the next β subunit (**Figure 3A**). When incubated with a non-hydrolysable ATP analog (AMPPNP), only a single phosphate is observed within each subunit. The nucleotide binding domain is composed of the Walker A, Walker B, Asp box, His box, and two arginine fingers (R104 and R123) in both αβ subunits, consistent with other AAA+ ATPase structures[24–28]. DotB also shares homology with AAA+ pilus biogenesis proteins (PilT, PilB, EspE, HP0525, and VirB11) found in different bacterial pathogens, but are not part of the ASCE protein clade.[23] Superposition of DotB's NBP with PilT, PilB, and VirB11 homologs in a nucleotide-bound state suggests that the observed phosphate is the β-phosphate of the nucleotide.[23] Superposition of PilT and HP0525 in a nucleotide-bound/unbound state revealed a change within the P-loop where the first α helix of the Walker A motif is unwound to provide space for nucleotide binding.[23]

Significant questions surrounding DotB's contribution to effector protein translocation remain. For example, it is unknown which effector proteins DotB recognizes or how they are translocated by DotB through the Dot/Icm T4SS. Since

DotB shares structural similarity to AAA+ proteins, one hypothesis is that DotB could translocate unfolded protein substrate through the axial channel in a similar manner as has been shown for other AAA+ proteins (Hsp104, 26S proteasome, ClpB, Vps4, etc).[29] However this hypothesis remains to be tested.
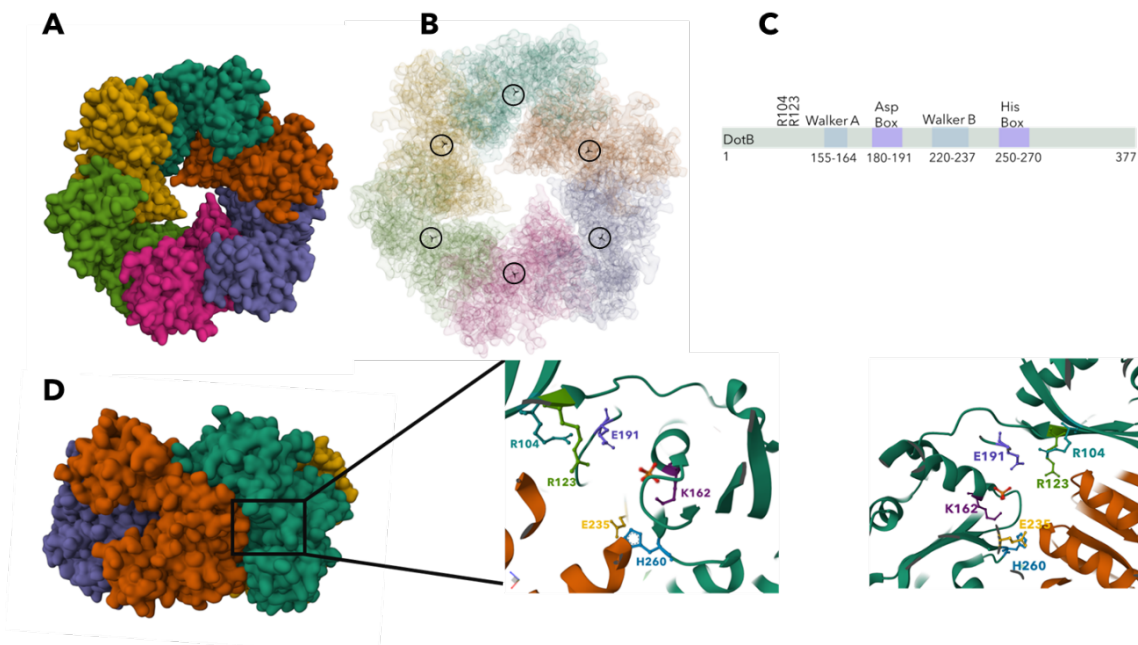

Figure 3. DotB's Nucleotide Binding Pocket.
(A) X-ray crystallography structure of DotB as a homohexamer with (B) a phosphate ion residing in the binding pocket for each subunit (black circles). (C) Schematic of the *dotB* gene with the Walker A and B motifs, Asp box, and His box. (D) Magnification of one nucleotide binding pocket with surrounding P-loop residues are shown in 90° rotations. PDB code: 6GEB.

AAA+ proteins have been widely studied for their function regarding critical cellular processes, with functional mutations within the Walker A and B motifs as a method to test for ATP binding and hydrolysis defects. As part of this family, DotB has been studied with mutations in both of these regions, which led to a broader understanding of its function within *Legionella*.[19,20,22,30] For that purpose, this review will focus on how DotB has been characterized and how further studies can improve our understanding of *Legionella pneumophila* infection within humans.

2. Using DotB's structure to support its translocation function

Since DotB is a AAA+ protein we can extrapolate about DotB's translocation mechanism. Studies of other AAA+ proteins demonstrated that nucleotide exchange in one subunit promotes ATP hydrolysis in the neighboring subunit,[27,28,31] which is likely to be the case for DotB. Cryogenic Electron Microscopy (cryo-EM) has been utilized to obtain structures of 1780 AAA+ proteins, some bound to a protein substrate, as found on the PDB website. All AAA+ motors bound to a polypeptide substrate adopt the same asymmetric "spiral-staircase" conformation.[29] Polypeptide substrates are unfolded within the axial channel of the AAA+ motor and interact with conserved aromatic residues (Tyr, Phe, and sometimes Trp), located on pore loops that extend into the axial channel. Since DotB does not contain pore loops that have aromatic residues, the unfolded polypeptide substrate might interact with other residues in the axial channel. Alternatively, the protein substrate may not interact with any residues within the axial channel of DotB. This could allow for the substrate to partially re-fold during translocation as it exits DotB's N-terminal face. From AAA+ structures it is predicted that translocation occurs in a "hand-over-hand" mechanism, in which subunits will sequentially bind to and translocate the substrate through the central channel in an ATP-hydrolysis dependent manner[29]. ASCE proteins are able to translocate substrate in this manner, or in an alternative mechanism depending on the specific function of the ATPase (interaction with metal ions, a "swinging" translocation motion, etc).[24] DotB forms an asymmetric hexamer where its N-

terminal face is ~45 Å in diameter and the C-terminal face is ~13-28 Å in diameter, as measured on the PDB site. It is possible that DotB also unfolds protein substrate while translocating it, driven by multiple rounds of ATP-hydrolysis, however this has not yet been determined. Thus, another possibility is that DotB may interact with another chaperone protein within *Legionella*, which directs unfolded protein substrate to DotB for translocation. It is then suggested that after translocation, the effector protein can refold while traveling through the larger C-terminal pore of DotO (65 Å after formation of the DotO/DotB complex), and then travels through the IMC and OMCC to enter the host cell for infection.

3. DotB oligomerization and characterization

The Vogel lab[22,30] studied the effect of nucleotide binding on hexamerization with a Walker A mutation, substituting a lysine to a glutamine at position 162 (K162Q). They demonstrated that this mutant could not bind or hydrolyze ATP but can still form stable hexamers in solution. DotB$_{K162Q}$ completely impaired *Legionella*'s ability to grow intracellularly in macrophages, indicating that ATP binding and hydrolysis by DotB is important for effector protein translocation. In another study, they performed a genetic analysis of DotB's function.[30] DotL is required for *Legionella* survival, but deletion of DotL can be suppressed by loss-of-function DotB mutants, further suggesting DotB's role with effector protein translocation. A random mutagenesis screen identified 30 different mutations located throughout DotB that were able to act in a dominant-negative manner, affecting WT DotB's function

within *Legionella*. The 30 dominant-negative mutations are found in the N-terminus, active sites, and C-terminus of DotB (**Figure 4**). The N-terminal mutations, some His box mutations, and the C-terminal deletion interacts with other subunits within the hexamer. The Walker A, Walker B, Asp box, and other His box mutations are in the active site of DotB. These mutations were then stably expressed and purified to determine the effects on hexamerization, ATP-binding, and *Legionella* function[30] (**Table II**).

Mutations in the N-terminus, active site, and a C-terminal deletion (C110R, L111P, K162Q, K162N, S163L, K182E, and Δ33 aa) could not bind ATP, while one mutation in the Asp box (E191K) exhibited enhanced ATP-binding. Four mutations in the N-terminus and His box (D35N, S83P, R270C, and R270H) that bind ATP were partially or unable to form hexamers, indicating another oligomeric state, since these mutations interact with the neighboring subunits in DotB. Other oligomeric states were further supported by another study,[23] where two Arg finger mutations were characterized. One Arg finger (R104)

Figure 4. Location of DotB mutations.
(A) Highlighted in green are all the DotB mutations listed in this paper. Mutations are seen in the monomer for better visualization. (B) mutations in the monomer along with their interactions with neighboring subunits in the hexamer.

Table II. Characterization of DotB mutations.

Mutation	ATP-binding	ATP-Hydrolysis	Hexamerization	Cytotoxicity	Intracellular Replication
D35N	Yes	N/A	Partial	Partial	Partial
N76Y	Yes	N/A	Yes	No	Partial
S83P	Yes	N/A	Partial	No	No
R104G	Yes	N/A	Yes	No	No
R104W	Yes	N/A	Yes	No	No
R104E	N/A	No	No	N/A	N/A
C110R	No	N/A	No	No	No
L111P	No	N/A	No	No	No
R123E	N/A	No	No	N/A	N/A
K162Q	No	No	Yes	No	No
K162N	No	N/A	Partial	No	No
S163L	No	N/A	Yes	No	No
N180I	Yes	N/A	Yes	No	Partial
K182E	No	N/A	No	No	No
E191K	Yes	N/A	Yes	No	No
G234D	Yes	N/A	Yes	Partial	Partial
H253R	Yes	N/A	Yes	Partial	Partial
H260R	Yes	N/A	Yes	No	No
H260Y	Yes	N/A	Yes	No	No
T267A	Yes	N/A	Yes	No	No
R270C	Yes	N/A	Partial	Partial	Partial
R270H	Yes	N/A	Partial	No	No
Δ33aa	No	N/A	No	No	No

interacts with the Asp box within the DotB monomer, while another Arg finger (R123) interacts with the C-term residues (**Figure 3D**). Through size exclusion chromatographic analyses, it was demonstrated that both mutations could not form hexamers and instead formed either a monomer or dimer, and were unstable in solution, indicating a disruption with hexamerization.

In the presence of wildtype (WT) DotB (WT *Legionella* strain with DotB mutant insertion), DotB active site mutants (R104G, R104W, E191K, H260R, H260Y, and T267A) completely inhibited intracellular replication of *Legionella*, confirming that ATP binding and hydrolysis are crucial for DotB's function. DotB mutants were also tested for their effect on *Legionella*'s ability to grow intracellularly in monocytes within a Δ*dotB Legionella* strain. Six mutations in the N-terminus and active site (D35N, N76Y, N180I, G234D, H253R, and R270C) partially restored *Legionella*'s

ability to replicate within macrophages, suggesting their role with protein translocation. It is known that *Legionella* exhibits contact-dependent cytotoxicity in which pores form in the plasma membranes of host cells.[15] Mutations in the N-terminus and active site (D35N, G234D, H253R, R270C) exhibited partial cytotoxicity to macrophages. DotB mutations exhibiting these biological effects suggest an interference with effector protein translocation. These results indicate that DotB's ability to bind and hydrolyze ATP in its hexameric form is required to translocate effector proteins through the Dot/Icm T4SS.

4. DotB localization and complex formation with other T4SS proteins

Current knowledge is that DotB solely forms a complex with DotO,[19,20] in preparation for translocation by *Legionella*, and it is unknown if DotB interacts with other proteins for effector protein translocation. To determine DotB localization to the Dot/Icm T4SS, the Liu group utilized WT and mutant DotB tagged with a superfolded GFP (sfGFP) and performed fluorescence microscopy to observe the localization of DotB within *Legionella*.[19] The polarity of DotB was quantified and revealed that WT DotB is localized to the cell poles with the Dot/Icm T4SS, whereas in comparison, an enhanced ATP-binding mutant (E191K) was predominantly localized to the cell poles. This indicates that DotB requires ATP binding to be recruited by DotO for complex formation in preparation for effector protein translocation, which was confirmed through immunoprecipation.

It is known that the IMC recruits DotO to the inner membrane, which then recruits DotB for complex formation[19] (**Table I**), which is crucial to begin effector protein translocation via the Dot/Icm T4SS. Deletion of the IMC and DotO prevented DotB$_{E191K}$'s ability to localize at the cell pole, confirming their role in recruitment of DotB for protein translocation. Deletion of the T4CC had no effect on DotB's localization, suggesting they do not need to interact for effector proteins to be translocated. A co-localization assay with DotB$_{E191K}$-sfGFP and DotO-mCherry or DotL-mCherry was also performed. Upon merging of the fluorescence signals, no co-localization was observed between DotB$_{E191K}$ and DotO or DotL. No co-localization between DotO and DotB supports that DotB interacts with DotO's C-terminal side for complex formation and substrate translocation, which was further supported by a cryogenic Electron Tomography (cryo-ET) structure of the DotO/B complex.[19,20] They saw that the DotO/B complex formation elicits a conformational change in DotO and the IMC. Upon association with DotB, DotO's C-terminal pore widens from 35Å to 65Å, and the IMC opens in a "pre-active" state for effector protein translocation through the Dot/Icm T4SS.[20] DotB is a cytosolic protein,[19,20,22,23,30] and a western blot demonstrated that deletion of DotB did not affect production of other Dot/Icm proteins, nor did their deletion affect DotB production within *Legionella*.[32] Some Dot/Icm proteins can only be produced in the presence of each other, due to providing stabilizing forces for T4SS complex formation[10,11,19,20] (**Table I**). Thus, Dot/Icm proteins do not affect DotB translation

but are required for DotB to associate with the Dot/Icm T4SS and perform its role with protein translocation.

Closing Remarks: Where do we go from here?

Studying the mutations spanning DotB in its entirety provided a broader understanding of DotB's function within *Legionella*. We learned that (1) DotB assembles into a homo-hexameric complex that is competent for binding and hydrolysis of ATP; (2) DotB's ability to bind and hydrolyze ATP is important for its biological function(s); (3) DotB-dependent translocation of effector proteins contributes to intracellular replication and cytotoxicity of *Legionella*; (4) DotB, through binding to DotO, may regulate effector protein translocation through the T4SS. Among the questions that remain unanswered is how *Legionella* can secrete such a large repertoire of proteins during infection. One approach to answer this would be an *in-situ* pull-down with DotB to determine all the proteins it interacts with during different growth stages in *Legionella*. Elucidating the effector proteins that are dependent on DotB for translocation will provide knowledge of how many of the ~300 substrates are translocated independent of the T4CC. In addition, cryo-EM has been utilized to capture structures of AAA+ motors interacting with polypeptide substrates.[27,28] The DotB hexamer is ~260 kDa and is a perfect candidate for cryo-EM. Cryo-EM could be used to capture how effector proteins can be translocated by DotB, which may utilize a different mechanism than what is characterized for other AAA+ proteins.

In summary, understanding DotB's explicit role with protein translocation may pave the way for new drug targets, and therapeutic/vaccine development for treating Legionnaire's Disease without the use of antibiotics that kill crucial bacteria within our gut microbiome. These studies further elucidate DotB as the perfect candidate for anti-virulent studies. For anti-virulent therapies, one method for targeted treatment of Legionnaires' Disease includes directly targeting DotB's function. A similar study was done in *Helicobacter pylori*'s Cag T4SS, which is responsible for gastritis.[33] A promising drug candidate affected Cagα's ability to hexamerize, to bind and hydrolyze ATP, and inhibited secretion of effector proteins necessary for infection of gastric cells when incubated with *H. pylori*. Thus, there are a multitude of ways to progress forward in the field and deepen our understanding of DotB and the role it plays within *Legionella pneumophila*, which will pave the way for treatments for Legionnaires Disease.

BIBLIOGRAPHY

(1) McDade, J. E.; Shepard, C. C.; Fraser, D. W.; Tsai, T. R.; Redus, M. A.; Dowdle, W. R. Legionnaires' Disease. *N Engl J Med* **1977**, *297* (22), 1197–1203. https://doi.org/10.1056/NEJM197712012972202.

(2) *Legionnaires Disease Diagnosis and Treatment | CDC*. https://www.cdc.gov/legionella/about/diagnosis.html (accessed 2023-02-28).

(3) Hoffman, P. S. Invasion of Eukaryotic Cells by Legionella Pneumophila: A Common Strategy for All Hosts? *Can J Infect Dis* **1997**, *8* (3), 139–146.

(4) Wiater, L. A.; Dunn, K.; Maxfield, F. R.; Shuman, H. A. Early Events in Phagosome Establishment Are Required for Intracellular Survival of Legionella Pneumophila. *Infection and Immunity* **1998**, *66* (9), 4450–4460. https://doi.org/10.1128/IAI.66.9.4450-4460.1998.

(5) Xu, L.; Luo, Z.-Q. Cell Biology of Infection by Legionella Pneumophila. *Microbes Infect* **2013**, *15* (2), 157–167. https://doi.org/10.1016/j.micinf.2012.11.001.

(6) Ziltener, P.; Reinheckel, T.; Oxenius, A. Neutrophil and Alveolar Macrophage-Mediated Innate Immune Control of Legionella Pneumophila Lung Infection via TNF and ROS. *PLOS Pathogens* **2016**, *12* (4), e1005591. https://doi.org/10.1371/journal.ppat.1005591.

(7) Chiaraviglio, L.; Brown, D. A.; Kirby, J. E. Infection of Cultured Human Endothelial Cells by Legionella Pneumophila. *PLOS ONE* **2008**, *3* (4), e2012. https://doi.org/10.1371/journal.pone.0002012.

(8) Hoppe, J.; Ünal, C. M.; Thiem, S.; Grimpe, L.; Goldmann, T.; Gaßler, N.; Richter, M.; Shevchuk, O.; Steinert, M. PilY1 Promotes Legionella Pneumophila Infection of Human Lung Tissue Explants and Contributes to Bacterial Adhesion, Host Cell Invasion, and Twitching Motility. *Frontiers in Cellular and Infection Microbiology* **2017**, *7*.

(9) Horwitz, M. A.; Silverstein, S. C. Legionnaires' Disease Bacterium (Legionella Pneumophila) Multiplies Intracellularly in Human Monocytes. *Journal of Clinical Investigation* **1980**, *66* (3), 441. https://doi.org/10.1172/JCI109874.

(10) Lockwood, D. C.; Amin, H.; Costa, T. R. D.; Schroeder, G. N. Y. 2022. The Legionella Pneumophila Dot/Icm Type IV Secretion System and Its Effectors. *Microbiology 168* (5), 001187. https://doi.org/10.1099/mic.0.001187.

(11) Voth, D. E.; Broederdorf, L. J.; Graham, J. G. Bacterial Type IV Secretion Systems: Versatile Virulence Machines. *Future Microbiol* **2012**, *7* (2), 241–257. https://doi.org/10.2217/fmb.11.150.

(12) Belyi, Y. Targeting Eukaryotic MRNA Translation by Legionella Pneumophila. *Front Mol Biosci* **2020**, *7*, 80. https://doi.org/10.3389/fmolb.2020.00080.

(13) Nagai, H.; Kubori, T. Type IVB Secretion Systems of Legionella and Other Gram-Negative Bacteria. *Front. Microbiol.* **2011**, *2*. https://doi.org/10.3389/fmicb.2011.00136.

(14) *Deciphering Legionella effector delivery by Icm/Dot secretion system reveals a new role for c-diGMP signaling | bioRxiv*.

https://www.biorxiv.org/content/10.1101/754762v2.full (accessed 2023-05-07).

(15) Kirby, J. E.; Vogel, J. P.; Andrews, H. L.; Isberg, R. R. Evidence for Pore-Forming Ability by Legionella Pneumophila. *Molecular Microbiology* **1998**, *27* (2), 323–336. https://doi.org/10.1046/j.1365-2958.1998.00680.x.

(16) *Legionnaires' disease - Symptoms and causes*. Mayo Clinic. https://www.mayoclinic.org/diseases-conditions/legionnaires-disease/symptoms-causes/syc-20351747 (accessed 2022-10-31).

(17) Ramirez, J.; Guarner, F.; Bustos Fernandez, L.; Maruy, A.; Sdepanian, V. L.; Cohen, H. Antibiotics as Major Disruptors of Gut Microbiota. *Front Cell Infect Microbiol* **2020**, *10*, 572912. https://doi.org/10.3389/fcimb.2020.572912.

(18) Sheedlo, M. J.; Durie, C. L.; Chung, J. M.; Chang, L.; Roberts, J.; Swanson, M.; Lacy, D. B.; Ohi, M. D. Cryo-EM Reveals New Species-Specific Proteins and Symmetry Elements in the Legionella Pneumophila Dot/Icm T4SS. *eLife* **2021**, *10*, e70427. https://doi.org/10.7554/eLife.70427.

(19) Chetrit, D.; Hu, B.; Christie, P. J.; Roy, C. R.; Liu, J. A Unique Cytoplasmic ATPase Complex Defines the Legionella Pneumophila Type IV Secretion Channel. *Nat Microbiol* **2018**, *3* (6), 678–686. https://doi.org/10.1038/s41564-018-0165-z.

(20) Park, D.; Chetrit, D.; Hu, B.; Roy, C. R.; Liu, J. Analysis of Dot/Icm Type IVB Secretion System Subassemblies by Cryoelectron Tomography Reveals Conformational Changes Induced by DotB Binding. *mBio* **2020**, *11* (1), e03328-19. https://doi.org/10.1128/mBio.03328-19.

(21) Nagai, H.; Cambronne, E. D.; Kagan, J. C.; Amor, J. C.; Kahn, R. A.; Roy, C. R. A C-Terminal Translocation Signal Required for Dot/Icm-Dependent Delivery of the Legionella RalF Protein to Host Cells. *Proceedings of the National Academy of Sciences* **2005**, *102* (3), 826–831. https://doi.org/10.1073/pnas.0406239101.

(22) Sexton, J. A.; Pinkner, J. S.; Roth, R.; Heuser, J. E.; Hultgren, S. J.; Vogel, J. P. The Legionella Pneumophila PilT Homologue DotB Exhibits ATPase Activity That Is Critical for Intracellular Growth. *J Bacteriol* **2004**, *186* (6), 1658–1666. https://doi.org/10.1128/JB.186.6.1658-1666.2004.

(23) Prevost, M. S.; Waksman, G. X-Ray Crystal Structures of the Type IVb Secretion System DotB ATPases. *Protein Science* **2018**, *27* (8), 1464–1475. https://doi.org/10.1002/pro.3439.

(24) Snider, J.; Thibault, G.; Houry, W. A. The AAA+ Superfamily of Functionally Diverse Proteins. *Genome Biol* **2008**, *9* (4), 216. https://doi.org/10.1186/gb-2008-9-4-216.

(25) Pathak, E.; Atri, N.; Mishra, R. Analysis of P-Loop and Its Flanking Region Subsequence of Diverse NTPases Reveals Evolutionary Selected Residues. *Bioinformation* **2014**, *10* (4), 216–220. https://doi.org/10.6026/97320630010216.

(26) Erzberger, J. P.; Berger, J. M. Evolutionary Relationships and Structural Mechanisms of Aaa+ Proteins. *Annual Review of Biophysics and*

Biomolecular Structure **2006**, *35* (1), 93–114.
https://doi.org/10.1146/annurev.biophys.35.040405.101933.

(27) Gates, S. N.; Yokom, A. L.; Lin, J.; Jackrel, M. E.; Rizo, A. N.; Kendsersky, N. M.; Buell, C. E.; Sweeny, E. A.; Mack, K. L.; Chuang, E.; Torrente, M. P.; Su, M.; Shorter, J.; Southworth, D. R. Ratchet-like Polypeptide Translocation Mechanism of the AAA+ Disaggregase Hsp104. *Science* **2017**, *357* (6348), 273–279. https://doi.org/10.1126/science.aan1052.

(28) de la Peña, A. H.; Goodall, E. A.; Gates, S. N.; Lander, G. C.; Martin, A. Substrate-Engaged 26S Proteasome Structures Reveal Mechanisms for ATP-Hydrolysis–Driven Translocation. *Science* **2018**, *362* (6418), eaav0725. https://doi.org/10.1126/science.aav0725.

(29) Gates, S. N.; Martin, A. Stairway to Translocation: AAA+ Motor Structures Reveal the Mechanisms of ATP-Dependent Substrate Translocation. *Protein Science* **2020**, *29* (2), 407–419. https://doi.org/10.1002/pro.3743.

(30) Sexton, J. A.; Yeo, H.-J.; Vogel, J. P. Genetic Analysis of the Legionella Pneumophila DotB ATPase Reveals a Role in Type IV Secretion System Protein Export. *Molecular Microbiology* **2005**, *57* (1), 70–84. https://doi.org/10.1111/j.1365-2958.2005.04667.x.

(31) Walker, J. E.; Saraste, M.; Runswick, M. J.; Gay, N. J. Distantly Related Sequences in the Alpha- and Beta-Subunits of ATP Synthase, Myosin, Kinases and Other ATP-Requiring Enzymes and a Common Nucleotide Binding Fold. *EMBO J* **1982**, *1* (8), 945–951.

(32) Vincent, C. D.; Friedman, J. R.; Jeong, K. C.; Buford, E. C.; Miller, J. L.; Vogel, J. P. Identification of the Core Transmembrane Complex of the Legionella Dot/Icm Type IV Secretion System. *Molecular Microbiology* **2006**, *62* (5), 1278–1291. https://doi.org/10.1111/j.1365-2958.2006.05446.x.

(33) Yuan, X.-Y.; Wang, Y.; Wang, M.-Y. The Type IV Secretion System in *Helicobacter Pylori*. *Future Microbiology* **2018**, *13* (9), 1041–1054. https://doi.org/10.2217/fmb-2018-0038.