Agrobacterium type IV secretion system and its substrates form helical arrays around the circumference of virulence-induced cells

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The genetic transformation of plant cells by *Agrobacterium tumefaciens* results from the transfer of DNA and proteins via a specific virulence (*vir*)-induced type IV secretion system (T4SS). To better understand T4SS function, we analyzed the localization of its structural components and substrates by deconvolution fluorescence microscopy. GFP fusions to T4SS proteins with cytoplasmic tails, VirB8 and VirD4, or cytoplasmic T4SS substrate proteins, VirD2, VirE2, and VirF, localize in a helical pattern of fluorescent foci around the perimeter of the bacterial cell. All fusion proteins were expressed at native levels of *vir* induction. Importantly, most fusion proteins are functional and do not exhibit dominant-negative effects on DNA transfer to plant cells. Further, GFP-VirB8 complements a *virB8* deletion strain. We also detect native VirB8 localization as a helical array of foci by immunofluorescence microscopy. T4SS foci likely use an existing helical scaffold during their assembly. Indeed, the bacterial cytoskeletal component MinD colocalizes with GFP-VirB8. Helical arrays of foci are found at all times investigated between 12 and 48 h post *vir* induction at 19 °C. These data lead to a model with multiple T4SSs around the bacterial cell that likely facilitate host cell attachment and DNA transfer. In support, we find multiple T pili around *vir*-induced bacterial cells.

The soil bacterium *Agrobacterium tumefaciens* is a source of fundamental insights and is intensively used due to its ability to transfer any DNA of interest to plant cells (1). The transferred DNA is a single-stranded copy of the T-DNA region on its Ti plasmid. A separate region of the Ti plasmid, the *virulence* (*vir*) region, encodes proteins to produce the transferable DNA, and to form a membrane-spanning DNA transporter called the type IV secretion system (T4SS). Several Vir proteins, VirD2, VirE2, VirE3, and VirF, are also transported to plants by the *vir*-encoded T4SS (2).

The transfer of ssDNA to plants via the T4SS is highly evolutionarily conserved, best exemplified by T4SS-mediated conjugal ssDNA transfer between bacterial cells. Bacteria have evolved to use this ancient mechanism of DNA transfer between bacteria to also transfer DNA or protein toxins into eukaryotic host cells. Important human pathogens such as *Helicobacter pylori*, *Legionella pneumophila*, *Bordetella pertussis*, *Brucella suis*, *Bartonella henselae*, and *Rickettsia prowazekii* transport disease-causing factors via T4SSs (3).

T4SS components can be placed into subgroups based on function or location. VirB4, VirB11, and VirD4 have ATPase homology and ATP-binding motifs, which may energize substrate translocation (4–7) at the inner membrane. VirB3 interacts with VirB4 (5). VirB6 is an inner membrane protein and VirB8 spans the inner membrane, placing most of the protein in the periplasm (8, 9). Recent cryoelectron microscopy (10) and crystallography (11) reveal a high-resolution structure consisting of 14 copies each of the *Escherichia coli* pKM101 conjugation system VirB7, VirB9, and VirB10 homologs that form a double-chambered channel spanning the inner membrane, periplasm, and outer membrane forming the core of the T4SS. VirB2 is the major T4SS pilus component (12), and the minor component, VirB5 (13), localizes at its tip (14). VirB1 has two domains: the N terminus has homology to lytic transglycosylases and likely cleaves the pepti-

doglycan cell-wall layer to facilitate assembly of the T4SS (15–17), and the C-terminal processed portion, VirB1*, is secreted to the cell surface (18, 19) and is required for pilus formation (20).

To understand T4SS function it is critical to determine its localization in the bacterial cell. Several reports suggest that some T4SS proteins localize to one or a few predominantly polar sites (21–23); such localization is proposed to lead to polar substrate transport. However, the results are not entirely consistent, as some proteins localize to both poles, the midcell, and subpolar regions. Here we use deconvolution microscopy to provide high-resolution images of fluorescent fusions to T4SS components and its secretion substrates. The results reveal that the T4SS localizes to the poles and equally well as helically arranged foci around the perimeter of the bacterial cell. These data lead to a model where multiple T4SSs around the bacterial cell provide multiple sites for interaction with the host cell surface.

Results

We constructed GFP fusions to T4SS components and T4SS substrate proteins. As GFP loses its fluorescence when transported to the periplasm, we fused GFP to components that either reside at the cytoplasmic face of the T4SS channel, VirD4, or have predicted cytoplasmic tails, VirB8 and VirB10. Further, we fused GFP to the substrate proteins VirD2, VirE2, and VirF. As the latter proteins must target the T4SS for transport, their localization pattern should reflect the localization of the T4SS. The data presented are representative of all cells in a particular optical field, and we analyzed hundreds of cells for each construct; we explicitly state when fewer than 100% of the cells exhibit a particular pattern.

VirB8 Localizes in a Helical Pattern in *vir*-Induced *Agrobacterium*. We first assayed the localization pattern of GFP-VirB8. VirB8 has a cytoplasmic N-terminal region of ~40 amino acids, followed by a membrane-spanning domain of ~20 amino acids and a C-terminal domain of 177 amino acids in the periplasm (24). GFP was fused in-frame to the N terminus of VirB8, sequestering GFP in the cytoplasm, leaving the majority of VirB8 intact in the periplasm allowing for association with other proteins of the T4SS. GFP-VirB8 coding sequences were placed downstream of a *vir*-inducible promoter on the low-copy-number plasmid pDW029 (20). GFP-VirB8 was transferred into wild-type *Agrobacterium* or a deletion of *virB8* ($\Delta virB8$). Following induction of *vir* gene expression, GFP-fusion protein localization was monitored by fluorescence deconvolution microscopy.

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Fig. 2. GFP-VirB8 colocalizes with RFP-MinD. (A) A deconvolved stack of RFP-MinD, and three different orientations of a deconvolved 3D image. (B–D) GFP-VirB8 and RFP-MinD expressed in the same cells. (B) GFP fluorescence. (C) RFP fluorescence. (D) GFP and RFP colocalization. (E) Scatterplot of green (x axis) and red (y axis) pixel intensities used to determine the threshold for colocalization.

VirB8 is produced at the same level as native VirB8 in wild type (Fig. 1Q, lanes 1 and 2) and $\Delta virB8$ (Fig. 1Q, lane 3). GFP-VirB8 is stably expressed as antibodies to GFP to detect only the fusion protein and not lower-molecular-weight proteins (Fig. S1, lane 5).

Agrobacterium MinD Forms Helical Arrays. We compared the GFP-VirB8 localization pattern with a protein known to form helical arrays. Bacterial cytoskeletal components such as the actin homolog MreB form helical arrays (reviewed in ref. 25). However, *Agrobacterium* (and other members of the *Rhizobiaceae*) do not contain an MreB homolog (30). Another cytoskeletal protein, MinD, forms helical arrays (25). *Agrobacterium* contains a MinD homolog, and fusion to RFP results in helices similar to those observed for VirB8 (Fig. 1D–F).

We next tested whether RFP-MinD and GFP-VirB8 arrays colocalize. We cloned RFP-MinD into the plasmid carrying GFP-VirB8. Each fusion is under the control of a copy of the same *vir* promoter, so that RFP and GFP-fusion proteins should be expressed at the same level. Indeed, the levels of detection of GFP-VirB8 and RFP-MinD are similar (Fig. 2B–E). Interestingly, the two proteins colocalize, albeit at the resolution (~ 100 nm) of fluorescence microscopy. The images of RFP-MinD and GFP-VirB8 colocalization were obtained using a spinning-disk confocal microscope. These images reveal a more “banded” appearance of MinD and VirB8 than those obtained with the deconvolution microscope; such bands support the helical arrays of foci observed in Fig. 1D–L and below.

T4SS Substrates VirD2, VirE2, and VirF also Localize in a Helical Pattern in *vir*-Induced *Agrobacterium*. Three T4SS substrate proteins, VirD2, VirE2, and VirF, contain C-terminal signal sequences essential for their transport through the T4SS to the plant cell (2). Thus, we made N-terminal GFP fusions to these proteins to preserve their targeting to the T4SS. All three proteins localize in a helical pattern of foci in *vir*-induced cells (Fig. 3A, B, D, E, G, and H). GFP-VirF helical arrays are observed in all *vir*-induced cells (Fig. 3D). GFP-VirD2 forms helical arrays in at least 50% of the cells (Fig. 3A), and GFP-VirE2 forms helical arrays in at least 30% of the cells (Fig. 3G).

The different degrees of helical pattern formation likely reflect the inherent properties of these substrate proteins and whether or not GFP interferes with their structure/function. As GFP-VirF does not interfere with tumor formation (Fig. 3F), its 100% helical localization likely represents wild-type VirF function. VirD2 interacts with the T strand via a conserved tyrosine at position 29 (31), so that an N-terminal GFP fusion may acutely

Fig. 3. T4SS substrates VirD2, VirF, and VirE2 localize to a helical array of foci in *vir*-induced *Agrobacterium*. (A and B) GFP-VirD2. (D and E) GFP-VirF. (G and H) GFP-VirE2. (J and K) VirD4-GFP. A, D, G, and J are widefield images of representative populations of *vir*-induced cells. B, E, H, and K are 3D deconvolved images. C, F, I, and L show tumor assays on carrot discs for each strain.

interfere with its localization. However, GFP-VirD2 does not interfere with long-term T4SS function and tumor formation (Fig. 3C). VirE2 is the most abundant Vir protein produced, forms homodimers, heterodimers with VirE1, and complex solenoids in complex with ssDNA (32). VirE2 dimers and solenoids are head to tail (N- to C-terminal), so an N-terminal GFP tag likely interferes with multimer formation, localization to T4SS helices, and tumor formation (Fig. 3I). Or, the abundance of VirE2, coupled with the size of the fusion protein, 95 kDa, may lead to plugging of the T4SS and reduction of tumor formation.

The VirD4-Coupling Protein Forms Helical Arrays. An important member of the T4SS is the VirD4-coupling protein that escorts the VirD2-bound T strand to the T4SS (reviewed in ref. 33). VirD4 association with VirB11 in the inner membrane is the first step in T-strand transport (34). The *E. coli* TrwB homolog of VirD4 forms a hexamer residing in the inner membrane with a C-terminal cytoplasmic tail (35). Thus, we made a C-terminal GFP fusion to VirD4. VirD4-GFP also localizes in a helical pattern in *vir*-induced cells (Fig. 3J and K), and allows tumor formation (Fig. 3L). These results support that the C terminus of VirD4 resides in the cytoplasm and show that fusion to GFP does not interfere with its hexameric structure.

Do GFP Fusions to Other T4SS Components Form Helical Arrays? As mentioned earlier, we also made a GFP fusion to the N terminus of VirB10, as it has a predicted ~30 amino acid cytoplasmic tail followed by a ~20 amino acid membrane-spanning domain and 327 amino acids in the periplasm (36). However, even though VirB10 has the same general topology as VirB8, it did not form foci (Fig. S2E). Instead, GFP-VirB10 forms a wide band of fluorescence at polar and subpolar sites around approximately one-third of the bacterial cell. This localization pattern is abnormal, as GFP-VirB10 is not functional and interferes with tumor formation (Fig. S2F).

Cryoelectron microscopy (10) and crystallographic studies (11) reveal that *E. coli* pKM101 homologs of VirB7, VirB9, and VirB10 are each found in 14 copies in a complex that spans the periplasm with its ends contacting the inner and outer membrane. Notably, even though VirB7, VirB8, VirB9, and VirB10 were expressed together, VirB8 was not purified along with the core complex (10). Hence, VirB8 appears not to associate tightly with the VirB7-VirB9-VirB10 core complex.

VirB8 may have more structural flexibility than VirB10, so the addition of GFP to VirB8 does not hinder its function and localization. Remarkably, the independence of VirB8 from the core complex allowed us to detect the formation of GFP-VirB8 helical arrays. The most logical explanation for the lack of helical array formation by GFP-VirB10 is that VirB10 is part of the T4SS multimeric core complex (10), and its fusion to GFP interferes with its ability to form the core complex.

We also predicted that not all T4SS proteins would fold or function correctly when tagged with GFP. Indeed, GFP fusions to VirB4, VirB6, VirB7, VirB9, and VirB11 do not form helical foci, and all exhibit dominant-negative effects on tumor formation (Fig. S2). The lack of foci formation for GFP fusions to VirB7 and VirB9 is expected, as these proteins are entirely periplasmic (10). VirB4 and VirB11 are hexameric ATPases that associate with the inner membrane, and GFP fusion may interfere with their assembly and function. Whereas VirD4 is also a hexameric ATPase, it has a structurally distinct C-terminal cytoplasmic tail (35), and GFP fusion did not impair its function or localization. VirB6 is highly hydrophobic with six membrane-spanning regions, and GFP may interfere with its folding and function.

The lack of distinct foci formation cannot be explained by degradation of the fusion proteins; whereas VirB4, VirB6, VirB7, VirB9, and VirB11 fusions display some breakdown products, the expected GFP fusions represent at least 50% of

Fig. 4. Time course of T4SS and T4SS substrate localization. GFP-VirB8 was localized in wild-type C58 *Agrobacterium* and $\Delta virB8$. GFP-VirB10 and GFP-VirF were assayed in wild-type C58 *Agrobacterium*. The cells shown are representative of the localization patterns of fields of cells at 12, 18, 24, and 48 h post *vir* induction. All images are widefield fluorescence. (A) GFP-VirB8 in C58. (B) GFP-VirB8 in CB1008. (C) GFP-VirB10 in C58. (D) GFP-VirF in C58.

their GFP signals (Fig. S1, lanes 2–4 and 6–7, respectively), and whereas VirD4-GFP displays significant breakdown (Fig. S1, lane 9), it still forms distinct helical arrays of foci (Fig. 3K).

Time Course of Helical Localization of the T4SS. The images above (Figs. 1–3) were taken following 48 h of *vir* induction at 19 °C. To determine whether these images are representative of T4SS localization at different times following *vir* induction, we performed a time course of GFP-VirB8, GFP-VirB10, and GFP-VirF localization representing a functional helically localized T4SS component, a nonfunctional GFP fusion that failed to localize in a helical array, and a functional helically localized T4SS substrate, respectively. We also compared the time course of localization of GFP-VirB8 in wild-type C58 versus a $\Delta virB8$ strain. GFP-VirB8 and GFP-VirF form helical arrays at 12, 18, 24, and 48 h after *vir* gene induction (Fig. 4A, B, and D). The time course of helical localization of GFP-VirB8 is identical in wild type and $\Delta virB8$. We have observed such helical arrays as early as 6 h post *vir* induction. GFP-VirB10 does not form distinct foci at any time (Fig. 4C).

T Pili Localize Around the Circumference of *vir*-Induced *Agrobacterium*. The T4SS is required for the elaboration of an extracellular T pilus. Thus, it is expected that the T4SS and the T pilus colocalize.

Fig. 5. Detection of T pili in *vir*-induced *Agrobacterium* and during attachment to the plant cell surface. (A) Arrows indicate multiple T pili around the circumference of *vir*-induced *Agrobacterium*. Top right shows flagellum for comparison (arrowhead). (B) Multiple T pili of the *vir*-induced bald strain of *Agrobacterium* contact the surface of a tobacco leaf cell.

alize. If there are multiple T4SSs around the circumference of *vir*-induced *Agrobacterium*, there should be multiple T pili on the surface of *vir*-induced *Agrobacterium*. Indeed, transmission and scanning electron microscopy reveal multiple T pili around *vir*-induced *Agrobacterium* (Fig. 5 A and B, respectively). We analyzed hundreds of *vir*-induced cells and always observed multiple T pili around each cell. In the absence of *vir* induction, T pili are not produced (20). As T pili are extremely fragile, the T pili observed likely represent a minimal number. We also observe multiple T pili attaching to the surface of a plant leaf cell (Fig. 5B). For the latter studies, we used the so-called bald strain of *Agrobacterium* that lacks flagella (37) so that all projections from the bacterial surface are T pili. The plant leaf cell surface removed of its cell wall was obtained following peeling away of the epidermal cell layer from a *Nicotiana benthamiana* leaf, thus presenting an optimal site for high-resolution imaging of *Agrobacterium* attachment. *Agrobacterium* was added to the leaf surface immediately after peeling the epidermal layer.

Discussion

The data reveal that induction of *vir* gene expression in *Agrobacterium* results in the production of multiple T4SSs that form a helical array of foci around the periphery of the bacterial cell. The helical pattern was observed by fluorescence deconvolution microscopy of GFP fusions to components of the T4SS (VirB8, VirD4) as well as to substrate proteins (VirD2, VirE2, VirF) that are transported to plant cells via the T4SS. The helical arrays arise as early as 6–12 h and remain until at least 48 h post *vir* induction. The T4SS helical arrays resemble helical arrays previously observed for bacterial cytoskeletal proteins such as MreB (25) and MinD (26), as well as LytE (27), RNaseE (28), and SecA (29). We also show that *Agrobacterium* MinD localizes to helical arrays that colocalize with VirB8 in *vir*-induced cells. Thus, the T4SS likely associates with an underlying helical bacterial scaffold.

We suggest two major reasons for previous observations of polar localization of T4SS components and substrates. First, previous studies used standard fluorescence microscopy, whereas our results largely were obtained by deconvolution microscopy. The ability to take at least 10 optical sections through a single bacterial cell by deconvolution microscopy allows the cell to be in continuous focus, versus a single image with a limited depth of field. Second, overexpression of bacterial fusions to GFP likely leads to nonspecific sequestration to the bacterial poles as inclusion body aggregates (38). GFP fusions were generated to all open reading frames of *E. coli*, and images of cells harboring these plasmids are reported at http://sal.cs.purdue.edu:8097/GB7/GFP/gfp_top.jsp. Amazingly, over 10% of the GFP fusions show polar localization due to the formation of inclusion bodies and are unlikely to have biological meaning. Reports of polar localized GFP fusions to VirD4, VirE2, and VirC1 (21, 22) may result from overexpression from IncP plasmids with copy numbers five times higher than the Ti plasmid. In contrast, we employed a plasmid present at only one to two copies per cell (like the Ti plasmid) containing a *vir*-inducible promoter active at wild-type levels of expression (20). Indeed, GFP-VirB8 is produced in equal quantities compared to the endogenous wild-type VirB8.

Importantly, previous reports support multiple foci and helical arrays of the T4SS. First, immunogold detection revealed VirB8 around the circumference of the bacterial cell in cross-section, and at numerous locations along the length of the cell in longitudinal sections (24). Second, studies of native VirB8, VirB9, and VirB10 detected by fluorescent secondary antibodies revealed striking images of several “foci” arranged in a helical pattern (8). In support for the localization of VirB8 independent of the core VirB7-VirB9-VirB10 complex, VirB8 foci formed in the absence of VirB9 and VirB10, but not vice versa (8).

The helical pattern of T4SS localization leads to a model for T4SS localization and function following attachment to plant

Fig. 6. Model for T4SS localization and function during binding to plant cells. *Agrobacterium* with polar and helically localized T4SSs and T pili make initial contact with a plant cell membrane (thick curved line) (A), and following T pili retraction multiple T4SSs attach to the plant cell surface (B). The T4SS associates with an endogenous helix in the cell.

cells. Figure 6A shows the T4SS plus associated T pili around the bacterial cell on a scaffold of an endogenous helically arranged protein complex. T pili likely facilitate interaction with the host plant cell. By analogy with bacterial conjugation where F pili retract upon recipient cell contact (39) (reviewed in ref. 40), T pili may retract to tether *Agrobacterium* to the plant cell surface. This leads to multiple T4SSs in close association with the plant cell surface, thereby maximizing the possibility for effective contacts between bacteria and the plant cell surface for subsequent DNA and protein transfer. In support, conjugating bacteria associate along their lengths and produce electron-dense tight junctions spanning contacts between donor and recipient cells (41), and our initial data suggest multiple T pili associate with the plant cell surface, supporting our model for longitudinal binding of *Agrobacterium* to plant cells.

It will be interesting to determine the bases for the colocalization between VirB8 and MinD. VirB8 may be the founding member for T4SS assembly (8, 42, 43), and its association with MinD may initiate the assembly of the VirB7-VirB9-VirB10 core complex. Besides MinD, does the T4SS colocalize with other helically arranged replication proteins (reviewed in ref. 44)? Finally, components of the T4SS may colocalize with the Sec secretion system found in helical arrays in other bacteria (45).

Methods

Strains and Growth Conditions. Wild-type *Agrobacterium tumefaciens* strain C58 contains the nopaline pTiC58. CB1008 is a nonpolar deletion in pTiC58 *virB8* (46). The bald strain is flagella minus (37). For *vir* induction, an overnight culture was diluted to an A_{600} of 0.1 in minimal AB medium (pH 5.5) and grown for 5 h at 19 °C (20). Cultures were then plated on AB agar medium containing antibiotics and 200 μ M acetosyringone at 19 °C.

GFP sequences with 5' BspHI and 3' HindIII restriction sites were inserted into pDW029 (20) to create pJZGFP. Oligonucleotide primers introduced an AvrII restriction site for N-terminal GFP fusions and a BspHI restriction site for C-terminal GFP fusions in pJZGFP. All genes were PCR-amplified from pTiC58. Constructs were confirmed by sequencing and transformed into *Agrobacterium* C58.

Imaging. A microscope slide was covered with a thin layer of 1% agarose; then 0.6–1 μ L of cells [in 50 mM potassium phosphate buffer (pH 5.5) following resuspension from plates at A_{600} 2.0] were placed on the agarose pad and covered with a coverslip. Bacteria were viewed with an Applied Precision Deltavision Spectris DV4 deconvolution microscope. Stacks of 10–20 optical sections were collected every 0.2 μ m on the z-axis. Data (200 iterations) were deconvolved using Huygens software (Scientific Volume Imaging). Three-dimensional structures were modeled using Imaris (Bitplane Scientific Software). Colocalization of GFP-VirB8 and RFP-MinD was detected using a Leica DMI6000B inverted microscope equipped with a Yokogawa CSU-10 spinning-disk confocal attachment (Yokogawa Electric) and a Photometrics 5125C EM-CCD camera (Photometrics). Image acquisition was via MetaMorph software (Molecular Devices) and data were analyzed with an Imaris colocalization module.

For immunofluorescence, cells from induction plates were resuspended to A₆₀₀ 2.0 in AB medium and fixed in 2.7% paraformaldehyde, 0.01% glutaraldehyde for 15 min at RT and 30 min on ice. Cells were collected, washed with PBS, and stored overnight in 50 mM glucose, 20 mM Tris (pH 7.5), 10 mM EDTA. After a PBS wash, cells were permeabilized with PBS containing 2 mg/mL lysozyme, 5 mM EDTA for 45 min at RT. Cells were washed three times with PBS, and then incubated with rabbit anti-VirB8 antibodies in PBS containing 0.1% BSA for 1 h at 37 °C. Following two washes with PBS containing 0.05% Tween-20, secondary antibody incubation was performed with AlexaFluor 546 conjugated to goat anti-rabbit IgG (Invitrogen) in PBS containing 0.1% BSA for 1 h at 37 °C. Cells were washed three times with PBS containing 0.05% Tween-20, once with PBS, and resuspended in 200 μ L PBS. Cells were imaged as described for GFP fluorescence.

Virulence Assay. Carrots sterilized with 20% bleach for 30 min were sliced into 6–10 mm sections and the apical surface was placed on water agar (1.5%) medium. Slices were inoculated with 10 μ L (10⁹ cells) of *Agrobacterium* harvested from a logarithmic-phase culture and suspended in 50 mM PBS. Slices were placed at 19 °C for 48 h and then transferred to 24 °C. Tumors were photographed 3–4 weeks after inoculation. All tumor assays are representative of at least three independent assays.

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