Use of chimeric type IV secretion systems to define contributions of outer membrane subassemblies for contact-dependent translocation

Jay E. Gordon,1 Tiago R. D. Costa,2 Roosheel S. Patel,1 Christian Gonzalez-Rivera,1 Mayukh K. Sarkar,1† Elena V. Orlova,2 Gabriel Waksman2 and Peter J. Christie1*

1Department of Microbiology and Molecular Genetics, McGovern Medical School, 6431 Fannin St, Houston, TX 77030, USA.
2Institute of Structural and Molecular Biology, University College London and Birkbeck, Malet Street, London WC1E 7HX, UK.

Summary

Recent studies have shown that conjugation systems of Gram-negative bacteria are composed of distinct inner and outer membrane core complexes (IMCs and OMCCs, respectively). Here, we characterized the OMCC by focusing first on a cap domain that forms a channel across the outer membrane. Strikingly, the OMCC caps of the Escherichia coli pKM101 Tra and Agrobacterium tumefaciens VirB/VirD4 systems are completely dispensable for substrate transfer, but required for formation of conjugative pili. The pKM101 OMCC cap and extended pilus also are dispensable for activation of a Pseudomonas aeruginosa type VI secretion system (T6SS). Chimeric conjugation systems composed of the IMCpKM101 joined to OMCCs from the A. tumefaciens VirB/VirD4, E. coli R388 Trw, and Bordetella pertussis Ptl systems support conjugative DNA transfer in E. coli and trigger P. aeruginosa T6SS killing, but not pilus production. The A. tumefaciens VirB/VirD4 OMCC, solved by transmission electron microscopy, adopts a cage structure similar to the pKM101 OMCC. The findings establish that OMCCs are highly structurally and functionally conserved – but also intrinsically conformationally flexible – scaffolds for translocation channels. Furthermore, the OMCC cap and a pilus tip protein coregulate pilus extension but are not required for channel assembly or function.

Introduction

The bacterial type IV secretion systems (T4SSs) are a highly versatile superfamily of macromolecular transporters (Costa et al., 2015; Christie, 2016). An early review highlighted this versatility by comparing three members of a subfamily of the T4SSs now termed the P-type or type IVa systems: (i) the Escherichia coli pKM101 Tra system, which mediates conjugative DNA transfer between bacteria, (ii) the Agrobacterium tumefaciens VirB/VirD4 system responsible for delivery of oncogenic T-DNA and effector proteins to plant cells and (iii) the Bordetella pertussis Ptl system that drives export of the multisubunit pertussis toxin (PT) across the B. pertussis outer membrane to the milieu (Winans et al., 1996). Each of these systems shares a common ancestry, subunit composition and, most likely, overall architecture (Christie and Vogel, 2000; Chandran Darbari and Waksman, 2015; Christie, 2016). In view of these commonalities, it is intriguing to ask how each of these systems was adapted during evolution to achieve such striking functional diversity.

Recent studies have provided a structural blueprint for the type IVa subfamily. The E. coli pKM101 Tra (designated TraPKM101) and closely related R388-encoded Trw (TrwR388) conjugation machines are composed of large (~1 Megadalton; MDa) similarly cage-shaped outer membrane (OM) core complexes (the OMCCs) (Chandran et al., 2009; Fronzes et al., 2009; Low et al., 2014). These OMCCs are composed of 14 copies each of VirB7- and VirB9-like subunits and of the C-terminal halves of VirB10-like subunits (we use here the A. tumefaciens VirB/VirD4 system, VirB/VirD4At, as a unifying nomenclature). The N-terminal halves of the VirB10 subunits extend to the inner membrane, where they form part of an even larger inner membrane complex (IMC; 2.5 MDa). Besides this portion of VirB10, the IMC is...
composed of 24 copies of VirB6 homologs, and 12 copies each of VirB3, VirB4 ATPase, VirB5 and VirB8 homologs (Low et al., 2014). Missing from the TrwR388 structure, which is designated as T4SS3-10 because it is composed of the VirB3 through VirB10 homologs, are VirB2-like pilins, the conjugative pilus and the VirD4- and VirB11-like ATPases. Recently, structural studies established that the F plasmid-encoded conjugative pilus is composed of a 5-start helical assembly. Strikingly, the F pilus consists of a stoichiometric complex of the TraA pilin subunit bound to a phospholipid (Costa et al., 2016), but it remains unknown how the pilus is mounted onto the T4SS IMC and OMCC.

At the distal end of the OMCC is an interesting feature termed the cap domain. An X-ray structure of the outer layer (O-layer) of the OMCC from TrapKM101 revealed that this cap domain is composed of a helix-loop–helix extension of VirB10, termed the antennae projection (AP). The AP helices from the 14 TraF monomers come together to form the cap with a diameter of ~100 Å and a central channel of ~10–30 Å (Chandran et al., 2009; Fronzes et al., 2009). The cap domain spans the OM where it is envisioned to participate in various surface phenomena including pilus biogenesis, substrate transfer across the OM, and formation of target cell contacts.

Here, we report results of mutational and domain swapping studies of the TrapKM101 and VirB/VirD4At model systems establishing the requirement for the OMCC cap for assembly of the conjugative pilus, but not productive mating junctions or even contact-dependent activation of a type VI secretion system (T6SS). We also show that chimeric T4SSs composed of the IMCpKM101 coupled to heterologous OMCCs from the TrwR388, VirB/VirD4At, and PtlBp systems support conjugative DNA transfer in E. coli and activate T6SS killing, but fail to elaborate detectable pili. Finally, we solved the structure of the A. tumefaciens VirB/VirD4 OMCC by transmission electron microscopy and negative staining, enabling comparisons with the other solved OMCCs. We discuss our findings in the context of a model for conjugation machines functioning in Gram-negative species in which the distal end of the OMCC, together with a pilus tip protein, coordinates a late-stage morphogenetic switch that alternatively directs pilus extension or intercellular substrate transfer.

Results

Genetic requirements for assembly of the pKM101 Tra T4SS

We initiated studies of the TrapKM101 T4SS by constructing a set of individual tra gene deletion mutations to define genetic requirements for machine assembly (Supporting Information Fig. S1). Eight of the 11 tra genes were precisely deleted from pKM101 by recombineering, but for unknown reasons we were unable to delete traL, traN and traO. We therefore created miniaturized versions of pKM101 consisting of the entire tra gene cluster and upstream regulatory sequences introduced into ColE1 or pACYC184 plasmid vectors or joined to pKM101’s oriV replication region along with a selectable Kanr gene. These mini-pKM101 plasmids encode fully functional Tra T4SSs (see below) and served as templates for further genetic manipulations of the tra cluster, including the construction of a complete set of Δtra mutations.

E. coli donors conjugatively transfer pKM101 at frequencies approaching 1 transconjugant per donor (Tc/D) in 2 h matings on solid-surfaces (Winans and Walker, 1985). In contrast to previous reports that IncN plasmids typically transfer poorly in liquid matings (Bradley et al., 1980; Jorgensen and Stenderup, 1982), MG1655 (pKM101) cells delivered the plasmid to recipients at frequencies of approximately 5 \times 10^{-4} and 10^{-1} Tc's/D in 2 h and overnight liquid matings (under constant agitation), respectively (Supporting Information Fig. S1). A donor carrying mini-pKM101 pRP100 mobilized transfer of pJG142, a plasmid that carries the entire traI/oriT/tral/onT mobilization (mob) region from pKM101 (Paternson et al., 1999), at frequencies slightly higher than observed for pKM101 in both solid surface and liquid matings (Fig. 1).

Most of the Δtra mutations abolished Trap T4SS function (Fig. 1 and Supporting Information Fig. S1), in agreement with previous findings for the A. tumefaciens VirB/VirD4 and E. coli TraR388 T4SS's (Berger and Christie, 1994; Larrea et al., 2013). Strains harboring the pKM101Δtra or pRP100Δtra mutant plasmids were complemented by trans-expression of the corresponding tra gene from the Pbad promoter (Fig. 1 and Supporting Information Fig. S1). In certain cases, complementation did not fully restore plasmid transfer to wild-type (WT) levels, possibly due to negative effects accompanying nonstoichiometric production on machine assembly or slight polar effects of the deletion mutations on downstream tra gene expression. Regardless, results of the complementation studies confirmed that 9 of the 11 Tra proteins are essential for elaboration of a functional TrapKM101 T4SS.

The virB1-like traL gene codes for a peptidoglycan (PG) hydrolase and its deletion had no discernible effect on plasmid transfer in either solid-surface or liquid matings (Fig. 1). This finding contrasts with previous reports documenting attenuating effects of PG hydrolase deletions on substrate transfer through related T4SSs (Berger and Christie, 1994; Bayer et al., 1995; Zahrl

© 2017 John Wiley & Sons Ltd, Molecular Microbiology, 105, 273–293
Complementation of the ΔtraL mutation with P\textit{BAD}:\textit{traL} conferred an apparent ~ 10-fold increase in transfer frequencies (Fig. 1). These cells, however, exhibited growth defects and enhanced cell lysis compared with cells expressing \textit{traL} from the native promoter (data not shown), as observed previously for the P19 hydrolase associated with the plasmid R1-encoded conjugation system (Bayer \textit{et al.}, 2001). The apparent increase in mating frequency (in Tc's/D) is therefore likely due to a reduction in donor cell viability accompanying TraL overproduction.

The last \textit{tra} gene, \textit{virB5}-like \textit{traC}, is postulated to encode a pilus tip protein, as deduced from studies of homologs in the VirB/VirD4\textsubscript{AT} and \textit{Helicobacter pylori} Cag T4SSs (Aly and Baron, 2007; Shaffer \textit{et al.}, 2011). Interestingly, ΔtraC mutant donors transferred DNA substrates at frequencies of \(5 \times 10^{-5}\) Tc's/D on solid surfaces, but were completely defective for plasmid transfer in liquid matings (Fig. 1 and Supporting Information Fig. S1). In the F plasmid transfer system, F pili extend to initiate distant contacts and then retract to promote formation of mating junctions, and it is thought that these dynamic rounds of extension and retraction account for the observed high-frequency transfer of F plasmids in liquid (Clarke \textit{et al.}, 2008). There is no evidence that the Tra\textsubscript{pKM101} pilus dynamically extends and retracts, yet its production might still facilitate target cell contacts enabling fairly efficient transfer in liquid.

**Fig. 1.** \textit{E. coli} pKM101 \textit{tra} gene deletion and complementation analyses. The \textit{E. coli} pKM101 \textit{tra} and \textit{A. tumefaciens virB} loci are similar in gene composition and order, as shown by color-coding of genes encoding homologs of the T4SS subunits. The pKM101 \textit{tra} genes expressed from pRP100 encode a fully functional Tra T4SS, as shown by efficient conjugative DNA transfer and IKe bacteriophage sensitivity. The schematic depicts effects of individual Δ\textit{tra} mutations (histogram, upper bars) and results of complementation studies (histogram, lower bars) in which corresponding genes were trans-expressed from the P\textit{BAD} promoter (black arrow) on conjugative transfer. Matings (2 h) were carried out on solid-surface (solid bars) and in liquid (stippled bars); pRP100-carrying donors also were mated overnight in liquid with constant agitation (light stippled bars). *, denotes transfer frequencies below the threshold of detection (< \(10^{-8}\) Tc's/D). Transfer frequencies are presented as transconjugants/donor (Tc's/D). IKe phage sensitivity (S, sensitive, R, resistant) for Δ\textit{tra} mutants and complemented strains is shown at the right.
pKM101-encoded pili are difficult to visualize microscopically, but pilus production can be assessed by susceptibility of plasmid-carrying cells to IKe, an M13-like filamentous phage that uses the pKM101 pilus as a receptor (Bradley, 1979). Among the Δtra strains, only the ΔtraL mutant exhibited IKe sensitivity suggesting that TraL is dispensable for pilus production. TraC, however, was required for IKe infection and plasmid transfer in liquid, supporting the notion that TraC plays an important role in pilus biogenesis but not elaboration of the translocation channel.

The OMCC cap is dispensable for substrate transfer but required for pilus biogenesis

With a pKM101 molecular ‘toolkit’ in hand, we sought to define the T4SS machine requirements for productive engagement with recipient cells. Although conjugative pili clearly play a role in initiating donor–target cell interactions, mutations have been isolated that block detectable pilus production (Pil*) without affecting substrate transfer (Tra*) (Sagulenko et al., 2001; Jakubowski et al., 2003, 2005; Haase et al., 1995). In the absence of extended pili, these systems must elaborate a surface feature capable of promoting donor–target cell contacts and formation of productive mating junctions.

We sought to define the functional importance of the OMCC cap, which to date is the only identified surface-exposed domain of the type IVa T4SSs other than the pilus. As mentioned above, this cap is assembled from the 14 AP domains of VirB10-like subunits. In the TrapKM101 O-layer structure, the α2 and α3 helices of the AP domains of TraF form the OM-spanning channel while the intervening AP loops (APLs) project from the cell surface (Fig. 2A) (Chandran et al., 2009). We introduced deletion or substitution mutations in the AP of TraF_{pKM101}, which spans residues 307–355. Interestingly, substitution of the APL with 5 Gly residues (5xGly) or introduction of a FLAG epitope in TraF's APL had no detectable effects on TraF protein accumulation (Fig. 2C) or donor-directed plasmid transfer (Fig. 2B). Deletion of the entire AP domain also did not affect TraF abundance and conferred only a modest reduction in DNA transfer in solid-surface matings compared with donors producing native TraF (Fig. 2B and C). The ΔAP mutant donors, however, failed to transfer the plasmid substrate at detectable levels in liquid matings (see below).

To evaluate the generality of these findings, we inserted duplicate (2×) or triplicate (3×) FLAG tags at several positions within the AP domain of A. tumefaciens VirB10 (Supporting Information Fig. S2A). These insertions did not affect VirB10 protein accumulation or function as deduced by the appearance of morphologically wild-type plant tumors resulting from A. tumefaciens-mediated delivery of oncogenic T-DNA into plant cells (Supporting Information Fig. S2B,C). The AP domains of TraF and VirB10 share only 26% identity (Fig. 3A and Supporting Information Fig. S3), yet reciprocal swaps of these domains yielded stable (Fig. 3C and Supporting Information Fig. S2C) and fully functional TraF/AP_{VirB10} and VirB10/AP_{TraF} chimeric proteins as shown by robust transfer of a pKM101 substrate in E. coli matings (Fig. 3B) and oncogenic T-DNA in A. tumefaciens infection assays (Supporting Information Fig. S2B). Together, these findings support a conclusion that the OMCC caps of the TrapKM101 and VirB/VirD4A4, T4SSs enhance, but are not required for, substrate transfer to target cells.

Complete deletions of the APs from TraF and VirB10, however, abolished pilus production, as evidenced in E. coli by a failure of the ΔAP mutant to transfer the pKM101 substrate in liquid and their resistance to IKe phage infection (Fig. 2B). In A. tumefaciens, the ΔAP mutant lacked detectable VirB2 pilin on the cell surface, which serves as a convenient assay for T pilus production by the VirB/VirD4A4, T4SS (Supporting Information Fig. S2B) (Sagulenko et al., 2001; Kerr and Christie, 2010). Further mutational analyses established the importance of the AP membrane-spanning α-helices, but not the APLs, for pilus biogenesis. For example, strains producing TraF with a 5xGly replacement of the APL, or a FLAG insertion in this domain, transferred their plasmid substrates in liquid matings and were susceptible to infection by IKe (Fig. 2B). Similarly, A. tumefaciens strains producing VirB10 variants with FLAG insertions in the APL accumulated abundant amounts of VirB2 pilin on the cell surface, whereas strains with FLAG insertions in VirB10's α2 or α3 helices had comparatively lower levels of surface pilin (Supporting Information Fig. S2B).

The C-terminal (CT) domain, but not the lever arm, is critical for TraF function

The C termini of TraF and VirB10 are highly related (72% identity), and both domains possess an RDLDf motif that is also highly conserved among the VirB10 family members (Fig. 3A and Supporting Information Fig. S3C) (Jakubowski et al., 2009). As shown by the TrapKM101 O-layer structure, TraF's C-terminal (CT)
domain forms a β-strand that extends along the β-barrel domain (see Fig. 2A and Supporting Information Fig. S3) (Chandran et al., 2009). Deletions of TraF’s AP and CT domains or just the CT domain were strongly destabilizing, as evidenced by low abundance of the mutant proteins and accumulation of presumptive proteolytic breakdown products (Fig. 2C). Deletion of the RDLDF motif or of the 9 C-terminal residues did not affect TraF’s abundance, but completely eliminated function as monitored by substrate transfer and IKe phage infection (Fig. 2B and C). Next, we substituted VirB10’s AP and CT domains or just the CT domain for those of TraF. The respective TraF/AP-CTVirB10 and TraF/CTVirB10 chimeric proteins supported plasmid transfer on solid surfaces, but not transfer in liquid or IKe phage infection (Fig. 3B). Interestingly, VirB10’s CT domain extends 11 residues beyond that of TraF, and a chimera (TraF/CTΔ11VirB10) deleted of this extension was stably produced and supported substrate transfer and IKe phage infection (Fig. 3B and C). In A. tumefaciens, a VirB10 chimera bearing

**Fig. 2.** Substitution and deletion mutational analysis of the outer membrane cap of the pKM101 Tra T4SS.

A. Ribbon diagram of the O-layer of the pKM101 outer membrane core complex (OMCC). VirB7-like TraN and VirB9-like TraO are color-coded magenta and cyan respectively. The α-helical antennae projection (AP) forming the OM-spanning cap and the C-terminal (CT) domain of TraF are color-coded red, and the β-barrel domain of TraF is color-coded yellow. At right, ribbon diagram of a TraF monomer depicting the β-barrel, AP and CT domains in same color-coding. Domain junctions (residues from N terminus) and positions of deletion or substitution mutations are indicated.

B. Schematic depicts TraF domain architecture with junctions (in residues) indicated. Mutations in the AP or CT are listed at left, and effects of the mutations on plasmid transfer (transconjugants per donor, Tc’s/D) and IKe phage infection (S, sensitive; R, resistant).

C. Levels of His-TraF and mutant proteins in total cell extracts, as monitored by immunostaining with α-His antibodies. RNA polymerase β-subunit (α-RNAP) served as a loading control.
TraF’s AP-CT (VirB10/AP-CT TraF) also supported WT levels of substrate transfer to plants but not detectable T pilus production (Supporting Information Fig. S2). The CT domains thus promote stabilization of the VirB10 proteins and also mediate intra- or intersubunit contacts necessary for channel formation and pilus production. The 11 C-terminal residues of VirB10 are required for T pilus production by the VirB/VirD4At T4SS, but poison pilus production when appended to TraF in the TrapKM101 system. Interestingly, however, both TraF and VirB10

**Fig. 3.** Domain swapping reveals compositional flexibility of TraF’s β-barrel, antennae projection (AP) and C terminus (CT).

A. Sequence alignment of the AP and C-terminal (CT) domains of TraF and VirB10, with identical (red) and nonidentical (black) residues shown. Numbers correspond to domain junctions (residues from N terminus). Sequences comprising the α2 – loop (APL) – α3 regions of AP domains and the highly conserved RDLF motifs are highlighted.

B. Schematics depicting domains of TraF and VirB10, with junctions (residues from N terminus) indicated: Cyto, cytoplasmic; TM, transmembrane domain; Pro-Rich, proline-rich-region; β-Barrel; AP, antennae projection; CT, C-terminal domain. Schematics of the TraF/VirB10 chimeras depict the VirB10 domain(s) swapped for the equivalent domain(s) of TraF. Strains producing the TraF/VirB10 chimeras supported plasmid transfer in 2 h solid-surface matings at the frequencies shown in transconjugants per donor (Tc’s/D), and exhibited sensitivity (S) or resistance (R) to IKε infection.

C. Levels of His-TraF and chimeric proteins in total cell extracts, as monitored by immunostaining with α-His antibodies. RNA polymerase β-subunit (α-RNAP) served as a loading control.

TraF’s AP-CT (VirB10/AP-CT_TraF) also supported WT levels of substrate transfer to plants but not detectable T pilus production (Supporting Information Fig. S2). The CT domains thus promote stabilization of the VirB10 proteins and also mediate intra- or intersubunit contacts necessary for channel formation and pilus production. The 11 C-terminal residues of VirB10 are required for T pilus production by the VirB/VirD4At T4SS, but poison pilus production when appended to TraF in the TrapKM101 system. Interestingly, however, both TraF and VirB10

© 2017 John Wiley & Sons Ltd, Molecular Microbiology, 105, 273–293
accommodate C-terminal epitope tags without effects on function (Fig. 2B and C, and see below).

In the TrapKM101 O-layer crystal structure, a domain of TraF designated as the lever arm extends laterally from one TraF monomer to form a network of contacts with 3 adjacent TraF monomers. This results in a tetradecameric complex in which the 14 lever arms form a continuous inner shelf at the base of the OMCC (Chandran et al., 2009). Notably, TraF’s CT domain, and more specifically β-strand β7c containing the RDLDF motif, interacts with β-strand β3,1 in the lever arm of an adjacent TraF monomer (Fig. 2 and Supporting Information Fig. S3) (Chandran et al., 2009). To evaluate the functional importance of this putative CT domain – lever arm interaction, we constructed a variant of TraF deleted of the lever arm. In contrast to native TraF, the TraFΔlever mutant migrated in SDS-polyacrylamide gels as multiple, presumptive degradation products (Supporting Information Fig. S4A).

Strikingly, however, donors producing the TraFΔlever mutant protein were proficient for DNA transfer and also exhibited IKe phage sensitivity (Supporting Information Fig. S4B). Despite an apparent stabilizing effect of TraF’s lever arm, therefore, the network of lateral contacts forming the lever arm shelf at the base of the OMCC is dispensable for elaboration of the translocation channel and pilus.

**TraF chimeras with substituted OMCCs support substrate transfer**

VirB10 subunits are unique among known Gram-negative bacterial proteins in spanning the entire cell envelope (Jakubowski et al., 2009; Chandran et al., 2009). To determine if TraF could tolerate substitutions of domains other than the AP and CT, we constructed additional TraF/VirB10 chimeras (Fig. 3B). Chimeras in which VirB10’s cytoplasmic (Cyto), transmembrane (TM) or periplasmic Proline-Rich Region (PRR) were substituted for the equivalent regions of TraF accumulated at detectable levels but failed to support substrate transfer or pilus production (Fig. 3B and C). These findings are in general agreement with results of previous studies showing that the N-proximal regions of the VirB10-like proteins form extensive interactions with cognate IMC components and VirD4 coupling proteins (Das and Xie, 2000; Llosa et al., 2003; Atmakuri et al., 2004; Rivera-Calzada et al., 2013).

By contrast, chimeras consisting of TraF’s N-terminal half joined to VirB10’s β-barrel domain with or without swaps of the AP and CT domains accumulated at low levels yet supported substrate transfer at frequencies of $10^{-5}$ to $10^{-6}$ Tc’s/D (Fig. 3B). The functionality of these chimeras was particularly striking in view of the low sequence identities of TraF and VirB10 across these domains (~19%; Fig. 3A and Supporting Information Fig. S3) and the TrapKM101 O-layer X-ray structure showing that TraF’s β-barrel forms extensive contacts with its partner subunit TraO (Supporting Information Fig. S3C) (Chandran et al., 2009). The OMCCs are intrinsically stable subassemblies (Fronzes et al., 2009). We therefore asked whether the TraFβ-CTVirB10 chimera would function more efficiently if the VirB7 and VirB9 subunits of the VirB/VirD4OMCC also were substituted for their TrapKM101 counterparts, essentially creating an IMCTra::OMCCVirB chimera (designated Tra::VirB; Fig. 4A).

To ensure temporal and stoichiometric synthesis of the IMC and OMCC subassemblies, we substituted codon-optimized virB7, virB9 and the traFβ-CTVirB10 chimera for traN, traO and traF within the tra region of the functional mini-pKM101 plasmid pCGR108 (see Fig. 4B). Interestingly, donors producing the Tra::VirB chimeric T4SS transferred the plasmid substrate in solid-surface matings, although at frequencies comparable to donors producing the TraFβ-CTVirB10 chimeric protein (compare Figs. 3B and 4B). The Tra::VirB-producing donors were transfer deficient in liquid matings and were insensitive to IKe phage infection, indicative of a lack of pilus production.

We engineered two more chimeric T4SSs, the first bearing an OMCC from the *E. coli* TrwR388 conjugation machine. The TrwR388 system is closely related phylogenetically and functionally to the TrapKM101, and evidence has been presented for the interchangeability of some constituent subunits between these systems (Liosa et al., 2003; De Paz et al., 2005). As expected, the chimera composed of pKM101’s IMC and the OMCC from TrwR388 supported transfer of the pKM101 substrate at a moderately high frequency of $10^{-3}$ Tc’s/D (Fig. 4B). The PtlΔp T4SS, by contrast, is highly divergent from the *E. coli* conjugation systems both in primary sequence (Supporting Information Fig. S3B) and in its function as a contact-independent PT export system (Locht et al., 2011). Surprisingly, the Tra::Ptl chimera also supported transfer, albeit at a lower level ($10^{-7}$ Tc’s/D) than either the Tra::VirB or Tra::Trw systems (Fig. 4B). Together, these findings confirm the modular and interchangeable nature of OMCCs from phylogenetically diverse type IVa systems.

**T4SS requirements for triggering of type VI-mediated killing**

One striking outcome from the above studies is that OMCCs from the *A. tumefaciens* and *B. pertussis* systems mediate formation of productive mating junctions with *E. coli* recipients, in spite of the fact that the former delivers substrates to plant cells and the latter functions...
as a contact-independent toxin exporter. These findings suggest that surface-exposed structures elaborated by the T4SSs can direct the formation of cell contacts with diverse target cells in the absence of pilus production or possibly even substrate transfer. We further defined the nature of T4SS surface structures mediating cell-cell contacts with a T6SS killing assay. It was shown previously that pKM101-carrying E. coli cells convey a signal across the P. aeruginosa cell envelope that triggers production of the H1-T6SS. In turn, P. aeruginosa cells kill...
the activating *E. coli* cells through transfer of toxic effectors (Ho et al., 2013). To identify T4SS surface features responsible for propagating the contact-dependent signal, we incubated *E. coli* donors producing WT or mutant T4SSs with *P. aeruginosa* strain PAO-1 and assayed for T6SS-mediating killing by serial dilution on media selective for *E. coli*.

*E. coli* cells lacking pKM101 exhibit comparable growth in the presence or absence of *P. aeruginosa* (Fig. 4C), whereas pKM101-carrying cells exhibit a ~2-log reduction in colony forming units (CFUs) when incubated in the presence versus the absence of *P. aeruginosa* PAO-1 or the presence of a T6SS\(^{-}\) (vipA) mutant (Fig. 4C) (Ho et al., 2013). Strains separately producing the IMC\(_{pKM101}\) or OMCC\(_{pKM101}\) subassemblies were not killed by *P. aeruginosa*, confirming the importance of an intact Tra\(_{pKM101}\) T4SS for activation of the T6SS. Interestingly, however, *E. coli* strains engineered to produce any of the chimeric T4SSs (Tra::Trw, Tra::VirB, Tra::Pti) triggered T6SS killing at levels comparable to the native Tra\(_{pKM101}\) T4SS (Fig. 4C). These chimeric systems thus efficiently transmit an activating signal to *P. aeruginosa* target cells, despite wide variations in substrate transfer and the absence of pilus production (Fig. 4B).

As expected from the above findings and our earlier phenotypic analyses (see Fig. 1), the \(\Delta traL\) mutant activated the *P. aeruginosa* T6SS while other \(\Delta tra\) mutants failed to trigger killing (Supporting Information Fig. S5). All complemented strains potentiated the killing response (Supporting Information Fig. S5), confirming the importance of an intact T4SS for signal transmission to *P. aeruginosa*. To further evaluate the requirements for contact-dependent signaling, *E. coli* strains harboring TraF mutations in the AP or CT domains were analyzed for T6SS activation. Interestingly, strains harboring TraF AP deletion (confers a Tra\(^{-}\), Pii\(^{-}\) phenotype) or CT mutations (Tra\(^{-}\), Pii\(^{-}\)) efficiently triggered T6SS killing (Fig. 5A). These findings establish that the Tra\(_{pKM101}\) T4SS transduces an activating signal to target cells even in the absence of DNA transfer, elaboration of the OMCC cap, or formation of the WT pilus.

**Structure of the *A. tumefaciens* VirB/VirD4 OMCC and comparisons with the *pKM101* OMCC**

Finally, to allow for further structural comparisons of OMCC subassemblies shown here to be functionally interchangeable, we purified and solved the *A. tumefaciens* VirB/VirD4 OMCC structure by negative-stain electron microscopy (see Supporting Information Fig. S6). This OMCC has dimensions of 180 Å in diameter and 155 Å in height, closely resembling the pKM101- and R388-encoded OMCCs (Chandran et al., 2009; Fronzes et al., 2009; Low et al., 2014). The VirB OMCC also has 14-fold symmetry with openings at its proximal and distal ends whose dimensions are similar to those of the other OMCCs (Fig. 6). The VirB OMCC is more cylindrically-shaped than its Tra\(_{pKM101}\) and Trw\(_{R388}\) counterparts, and its OM-spanning cap is also broader with a notable cup presumptively exposed to the extracellular milieu. It is not yet possible to assess the significance of these structural differences due to the low resolution achieved for the VirB OMCC by negative staining. Nevertheless, the VirB structure adds to an accumulating body of evidence that OMCCs from the type IVA T4SSs exhibit strong similarities in their overall architectures. These findings also provide a structural basis for understanding the interchangeability of the heterologous OMCCs in supporting conjugal DNA transfer through the Tra\(_{pKM101}\).

**Discussion**

*E. coli* pKM101 is widely known for its mutagenic and protective properties in cells exposed to UV irradiation and other DNA-damaging agents (Ames et al., 1975). pKM101 also encodes a highly-efficient T4SS (Winans and Walker, 1985; Paterson et al., 1999). In this study, we capitalized on the construction of a pKM101
‘molecular toolkit’ to address a central question in the type IV secretion field, namely, how do these machines establish productive contacts with target cells? Guided in part by solved structures of T4SS subassemblies, we evaluated the contributions of the pKM101 Tra subunits and OMCC domains of special interest to pilus production, formation of donor–target cell contacts, and intercellular substrate transfer. Importantly, we determined that T4SSs lacking identified surface features, including the OM-spanning cap domain and the TraC pilus tip protein, display the Tra$^+$, Pil$^-$ ‘uncoupling’ phenotype. Results of these mutational studies strongly implicate the OMCC cap and TraC as pilus-specificity factors and support a model in which the Tra pKM101 T4SS alternatively functions as a pilus-assembly machine or an active translocation channel. We also determined that heterologous OMCC subassemblies could be substituted for the Tra$^{pKM101}$ OMCC. From a structural perspective, these latter findings underscore the broad importance of a conserved OMCC scaffold for elaboration of T4SS translocation channels. From a mechanistic perspective, however, the functionality of these chimeric T4SSs is remarkable, first, because two of the swapped OMCCs were derived from systems adapted for substrate trafficking to eukaryotic cells either by contact-dependent or -independent mechanisms. Second, as discussed further below, T4SSs are known to initiate substrate transfer in response to transduction of intracellular and extracellular signals across the cell envelope. The Tra$^{pKM101}$ IMC must therefore not only physically interact with heterologous OMCCs, but also convey energizing signals to the OMCC for channel activation.

We defined the functional importance of TraC and the other Tra subunits through systematic $tra$ deletion/complementation analyses using native pKM101 and functional mini-pKM101 plasmids that were more amenable to genetic manipulation (Fig. 1 and Supporting Information Fig. S1). These studies established the essentiality of 9 of the 11 $tra$ genes and also confirmed an early report that TraC is not required for pKM101 transfer on solid surfaces (Winans and Walker, 1985). Interestingly, however, we also found that pKM101 transfers at
moderate frequencies \((5 \times 10^{-4} \text{ Tc's/D})\) in 2 h liquid matings and that TraC is required for such transfer events. These traC mating phenotypes, coupled with the requirement of TraC for infection by the male-specific phage iKe (Fig. 1 and Supporting Information Fig. S1) (Yeo et al., 2003), strongly indicate that TraC is critical for pilus production but not a functional transfer channel. In their early study, Wins and Walker (1985) supplied genetic evidence that TraC is surface-exposed and might even be transmitted intercellularly. In a phenomenon termed ‘extracellular complementation’, a TraC-producing (but non-conjugative) ‘helper’ strain restores mating proficiency of a ΔtraC mutant when the two strains are mixed with a third, plasmid-free recipient strain. These investigators postulated that the ‘helper’ strain delivers surface-localized TraC to the ΔtraC mutant to fulfill a requisite function for pilus assembly and mating pair formation.

Studies have since confirmed that TraC is indeed surface-localized even on cells lacking the Tra<sub>pKM101</sub> T4SS (Schmidt-Eisenlohr et al., 1999), although there is still no direct evidence for its cell-to-cell transmission. Further supporting the notion that the VirB5-like subunits are selectively involved in pilus production, <i>B. pertussis</i> Pil<sub>B</sub> systems function as an exporter even in the absence of a VirB5 homolog and detectable pilus production (Farizzo et al., 2000; Locht et al., 2011).

Mutations of virB5/traC-like genes do, however, abolish substrate transfer by some T4SSs (Berger and Christie, 1994; Fischer et al., 2001; De Paz et al., 2005; Larrea et al., 2013). In such systems, the VirB5-like subunits appear to have appropriated novel functions relating to target cell recognition. In <i>H. pylori</i>, for example, an RGD (Arg–Gly–Asp) motif and other motifs carried on VirB5-like CagL mediate binding of the Cag T4SS to β integrin receptors on human host cells (Kwok et al., 2007; Barned and Niemann, 2015). CagL also displays extensive sequence variation, thought to arise from evolutionary selection pressures within the human host, which allows for immune evasion or altered host cell binding by infecting <i>H. pylori</i> strains (Olbermann et al., 2010; Gorrell et al., 2016). In <i>A. tumefaciens</i>, VirB5 also carries an RGD motif (Backert et al., 2008), which similarly might contribute to establishment of productive contacts with susceptible plant cells (Lacroix and Citovsky, 2011). Finally, in <i>Bartonella henselae</i>, variant forms of surface-located VirB5 subunits are thought to determine host-specificity of erythrocyte parasitism (Dehio, 2008). CagL<sub>Hs</sub> and VirB5<sub>At</sub> localize at the tips of pili produced by the respective T4SSs, further supporting the idea that VirB5 subunits have evolved multiple functions relating to pilus nucleation and target cell binding (Aly and Baron, 2007; Kwok et al., 2007; Barned and Niemann, 2015).

The OM-spanning caps of the Tra<sub>pKM101</sub> and VirB/VirD<sub>4</sub> T4SSs were surprisingly permissive to mutation with respect to substrate transfer (Fig. 2 and Supporting Information Fig. S2), suggesting that the translocation channel assembles across the OM even without an intact cap domain. The nature of this channel is not yet defined, but a growing body of evidence suggests that it consists of pilin monomers most probably in the form of a short pilus extending from the inner membrane to the cell surface. In earlier crossing studies, we showed that the VirB<sub>2At</sub> pilin forms formaldehyde (FA)-crosslinkable contacts with DNA substrates during their transit through the <i>A. tumefaciens</i> VirB/VirD4 T4SS (Cascales and Christie, 2004b). VirB<sub>2At</sub>–DNA crosslinking also was observed with variant channels harboring Tra“, Pil“ ‘uncoupling’ mutations, but not among T pili isolated from the cell surface by shearing (Cascales and Christie, 2004b; Jakubowski et al., 2005). Very recently, structural studies of the F pilus revealed the striking finding that the inner lumen is composed of phospholipids derived from the inner membrane and in stoichiometric association with the Tra<sub>Af</sub> pilin subunit (Costa et al., 2016). In line with early models describing the dynamics of F pilus assembly and retraction (Manchak et al., 2002), these findings suggest that Tra<sub>Af</sub> pilin–phospholipid complexes comprise the building blocks for polymerization of the F pilus from an inner membrane platform (Costa et al., 2016). Some evidence also has been presented for the capacity of extended F pili to mediate substrate transfer in the apparent absence of direct donor–recipient cell contacts (Harrington and Rogerson, 1990; Babic et al., 2008). Such transfer events are rare, in line with extensive biochemical and some ultrastructural evidence that efficient conjugal transfer requires direct cell-to-cell contact (Samuels et al., 2000; Lawley et al., 2002; Arutyunov and Frost, 2013). Nevertheless, the capacity of extended pili to mediate substrate transfer is consistent with a model in which a pilus polymer extending across the donor cell envelope, and potentially beyond, functions as a conduit for substrate passage.

Our findings that the OMCC caps of the Tra<sub>pKM101</sub> and VirB/VirD<sub>4</sub> T4SSs are essential for pilus production (Fig. 2 and Supporting Information Fig. S2) confirms and extends results of an earlier study showing that an <i>A. tumefaciens</i> VirB10ΔAP mutation confers low levels of surface-exposed VirB2 pilin and defects in pilus polymerization (Jakubowski et al., 2009). In that study, however, the AP boundaries (residues 308–337) were assigned on the basis of a crystal structure of VirB10-like ComB10 associated with a <i>H. pylori</i> competence system (Terradot et al., 2005). With the availability of the X-ray structure for the Tra<sub>pKM101</sub>O-layer (Chandran et al., 2009), we reassigned the AP boundaries of both TraF and VirB10 so that the latter spans residues 282–335. The VirB10 AP mutant analyzed here (VirB10Δ282–335) completely blocked detection of
surface-exposed VirB2 pilin (Supporting Information Fig. S2), while the corresponding TraF mutant (TraFΔ307–355) similarly blocked pilus production as monitored by IKe uptake and transfer in liquid (Fig. 2). Further reinforcing the notion that the distal end of the OMCC regulates pilus biogenesis, we previously showed that an Arg substitution for a highly conserved Gly residue in VirB10At that maps in the OMCC’s interior chamber and near the OM-spanning cap also blocks T pilus production without affecting substrate transfer to plant cells (Banta et al., 2011).

We incorporate our findings into a working model for conjugation systems in Gram-negative bacteria in which the default pathway is pilus production (Fig. 7). These pili function in a ‘mate-seeking’ mode either through dynamic rounds of extension and retraction as shown for F pili (Clarke et al., 2008) or via a mechanism(s) in which adhesive pili accumulate abundantly in the milieu to promote formation of mating aggregates (Samuels et al., 2000). Upon establishment of donor–recipient cell contacts, the T4SS ceases pilus production and transitions to the ‘mating’ mode. Various signals regulate the
pilus-to-channel morphogenetic switch, including those propagated by the recipient cell (Frost and Koraimann, 2010; Arutyunov and Frost, 2013) and those from within the donor cell associated with substrate docking with VirD4-like receptors, engagement of the VirD4 receptor with the IMC, and ATP hydrolysis by the T4SS ATPases (Cascales and Christie, 2004a; De la Cruz et al., 2010; Lang et al., 2011; Lang and Zechner, 2012; Cascales et al., 2013). Our model is reminiscent of earlier models invoking a late-stage bifurcation in the T4SS assembly pathway for production either of the pilus or translocation channel (Christie et al., 2005; Trokter et al., 2014), but highlights the importance of the pilus tip protein and the distal end of the OMCC specifically for the pilus extension mode (Fig. 7). Accordingly, we propose that the pilus tip protein is recruited to and forms specific contacts with the OMCC as a prerequisite for pilus extension from the cell surface. Recruitment may occur at the extracellular surface, in view of evidence that VirB5-like subunits are exported across the OM independently of the T4SS (Schmidt-Eisenlohr et al., 1999). However, pilus tip proteins also have been reported to interact with IMC components (Yuan et al., 2005; Villamil Giraldo et al., 2012), raising the alternative possibility of their engagement with the T4SS in the periplasm. Regardless of the entry point, a central feature of our model is that the TraC/VirB5-like subunit engages—probably dynamically—with the distal end of the OMCC to drive extension of pilus from the cell surface. Extracellular and intracellular ‘matting’ signals thus might regulate the pilus-to-channel switch by blocking formation of productive contacts between the pilus tip protein and the OMCC cap or the major pilin subunit.

Our studies also supplied important new insights into the OMCC domain requirements for T4SS function. For example, TraF’s CT domain (residues 355–386) is important for protein stability, but the conserved RDLDF motif within this domain is critical for TraF function (Fig. 2B). On the basis of the X-ray structure for the Tra\textsubscript{pKM101} O-layer (Chandran et al., 2009), we had envisioned that CT domain–lever arm contacts among adjacent TraF subunits (see Supporting Information Fig. S2C) might be important for OMCC assembly or stability. Indeed, the CT and lever arm deletions did impact TraF stability (Fig. 2 and Supporting Information Fig. S4), yet the Δlever mutant retained near WT function with respect to substrate transfer and pilus production. These findings establish that the lateral intersubunit contacts formed by the lever arm in the lateral shelf are not essential for assembly of a functional OMCC scaffold. Analyses of the TraF β-barrel domain swaps supplied further evidence for conformational flexibility of the OMCC. The functionality of the TraF/βB-CT\textsubscript{VirB10} chimera (Fig. 3), and of equivalent chimeras composed of the β-barrel domains from the TrwE\textsubscript{R388} and PtlG\textsubscript{ap} homologs (Fig. 5 and data not shown), was particularly surprising in view of the low overall sequence relatedness of the VirB10 homologs (Supporting Information Fig. S3). Furthermore, TraF’s β-barrel extensively interacts with VirB9-like TraO in the Tra\textsubscript{pKM101} O-layer crystal structure (Supporting Information Fig. S3C) (Chandran et al., 2009), and only a few of the residues comprising the TraF–TraO subunit interfaces are conserved among the β-barrel domains of the TraF homologs (Supporting Information Fig. S3C). These findings suggest that TraF’s network of intra- and intersubunit contacts do not structurally lock the Tra\textsubscript{pKM101} OMCC, which is in line with evidence that VirB10\textsubscript{Ap} undergoes a conformational change in response to substrate docking and ATP energy signals, as well as unspecified extracellular signals, to regulate substrate passage.

The functionality of the chimeric T4SSs (Tra::VirB, Tra::Trw, Tra::Ptl) confirmed the modular nature of the IMC and OMCC subassemblies and supplied further evidence that these machines are intrinsically adaptive and flexible. While prior studies have shown that certain subunits of the OMCC (Llosa et al., 2003; De Paz et al., 2005) or to a limited degree the VirB8 subunit of the IMC (Paschos et al., 2006; Bourg et al., 2009) are exchangeable between closely related T4SSs, this is the first demonstration of the functionality of chimeric systems built from phylogenetically diverse IMC and OMCC subassemblies. At one level, the observed architectural similarities of the OMCCs solved previously (Chandran et al., 2009; Fronzes et al., 2009; Low et al., 2014) and here for the VirB OMCC (Fig. 6) provide a structural basis for understanding how heterologous OMCCs might substitute for the Tra\textsubscript{pKM101} subassembly. Moreover, given the wide-spread phylogenetic distribution of the VirB7, VirB9, and VirB10 homologs among the type IVa systems, and the recent identification of ring-shaped OMCCs associated with type IVb systems (represented by the Legionella pneumophila Dot/Icm T4SS) (Kubori et al., 2014; Kubori and Nagai, 2015), it is reasonable to predict that the OMCC structures solved to date are paradigmatic for T4SSs associated with Gram-negative species. However, the OMCC also must physically and functionally interact with the IMC to build the translocation channel and the pilus, and to regulate their dynamic activities. A complex network of contacts involving the VirB9- and VirB10-like OMCC subunits and the VirB6- and VirB8-like IMC subunits are required for elaboration of these structures (Hafelnmeier et al., 2000; Das and Xie, 2000; Krall et al., 2002; Jakubowski et al., 2003; Jakubowski et al., 2004; Baron, 2006). Contacts between the VirB10-like subunits and VirD4-like subset receptors also are implica ted in transduction of the aforementioned intracellular (substrate docking/ATP energy) and extracellular (target cell binding)
signals necessary for transitioning to the ‘mating’ mode (Llosa et al., 2003; Atmakuri et al., 2004; Cascales and Christie, 2004a; De Paz et al., 2005; Mihajlovic et al., 2009; Lang et al., 2011; Arutyunov and Frost, 2013; Cascales et al., 2013). While our use of chimeric TraF proteins facilitated productive coupling of pKM101’s IMC with the heterologous OMCCs, the functionality of the chimeric T4SSs almost certainly requires other IMC–OMCC contacts as well as signal-activated conformational changes. Further studies of these and other chimeric T4SSs should reveal additional structure-function relationships between these two subassemblies.

Finally, our studies employing the P. aeruginosa T6SS killing assay confirmed that neither the Tra<sub>pKM101</sub> OMCC cap nor the extended pilus was required for establishment of donor–target cell contacts. Studies with the pKM101<sub>A</sub>tra mutants supplied evidence that an intact Tra<sub>pKM101</sub> T4SS is essential for T6SS activation (Supporting Information Fig. S5), which agrees with earlier findings for the RP4 conjugation system (Ho et al., 2013). Strikingly, however, we also found that the chimeric T4SSs (Fig. 4), as well as Tra<sub>pKM101</sub> cap deletion and other Pil<sup>+</sup> and Tra<sup>-</sup> mutant strains (Fig. 5), triggered this killing system. That the Tra:<Pta>Ptr</Pta> chimera efficiently activated P. aeruginosa T6SS killing was particularly surprising since the native Pil system supports neither pilus biogenesis nor binding of B. pertussis to eukaryotic target cells (Burns, 2003; Locht et al., 2011). These findings establish that in absence of the OMCC cap or the extended pilus, another pKM101-encoded surface feature(s) must be capable of contacting and transmitting a potentiating signal to the P. aeruginosa cell envelope. Such a feature might correspond to (i) a motif of the OMCC that becomes surface-exposed only upon target cell sensing (ii) a short, surface-exposed pilus structure that was not detectable by our available assays or (iii) another surface-exposed protein that is not encoded by the pKM101 <i>tra</i> cluster but physically or functionally interacts with the OMCC. Future studies utilizing the T6SS killing assay should continue to refine our understanding of the T4SS surface features required for initiation of donor–recipient cell contacts and mating junction formation.

**Experimental procedures**

**Strains and growth conditions**

<i>E. coli</i> DH5<sup>a</sup> (GIBCO-BRL) was used for plasmid constructions and the type VI secretion system (T6SS) killing assay. <i>E. coli</i> MG1655 (<i>E. coli</i> Genetic Stock Center) served as donors in the conjugation assays and for the phage infection assays. <i>E. coli</i> CAG18477 served as recipients in the conjugation assays (Singer et al., 1989). <i>E. coli</i> HME45 (Thomason et al., 2014) was used for construction of <i>tra</i> gene deletions from native pKM101. <i>Pseudomonas aeruginosa</i> PAO1 (Holloway, 1955) containing an ISPhoA insertion in the retS locus was used for the T6SS killing assay (<i>Pseudomonas</i> Transposon Mutant Collection, University of Washington Genome Sciences). <i>E. coli</i> strains were grown in Luria Broth (LB) at 37°C with shaking. <i>E. coli</i> strains were cultured in the following antibiotics: carbenicillin (50 μg ml<sup>-1</sup>), kanamycin (50 μg ml<sup>-1</sup>), spectinomycin (100 μg ml<sup>-1</sup>), chloramphenicol (20 μg ml<sup>-1</sup>), tetracycline (20 μg ml<sup>-1</sup>) and rifampicin (50 μg ml<sup>-1</sup>).<i>P. aeruginosa</i> PAO1 was grown in LB without antibiotic selection.

**Plasmid constructions**

Plasmids and oligonucleotides used in these studies are listed in Supporting Information Tables S1 and S2 respectively.

**Vectors**

pBAD24Spc was created by isolation of the <i>spc</i> gene as a Smal fragment from pHP45 and inserting it into the Scal site within the <i>crb</i> gene on pBAD24.

**pKM101 tra mutant plasmids.** Eight of the 11 <i>tra</i> genes were deleted from pKM101 by recombineering (Thomason et al., 2014). Briefly, pKM101 or pKM101Spc<sup>−</sup> were transferred by conjugation into <i>E. coli</i> strain HME45, which contains the bacteriophage λ red system under the control of the cl857 repressor. For construction of each <i>tra</i> gene deletion, the <i>kan</i> cassette from plasmid pUC4K was PCR amplified so that it carried flanking NcoI sites and 35 basepairs (bps) of 5′ and 3′ sequences that were complementary to regions immediately upstream and downstream of a <i>tra</i> gene of interest. HME45(pKM101Crb<sup>+</sup>) or HME45(pKM101Spc<sup>−</sup>) cells were temperature-induced for expression of the red-gam genes, and the <i>kan</i> amplicons were introduced by electroporation with <i>Kan</i> selection for transformants. Because pKM101 is a multicopy plasmid, we eliminated plasmids lacking the integrated <i>kan</i> cassette by subculturing the <i>Kan</i> transformants for 4 days in LB broth containing kanamycin (200 μg ml<sup>-1</sup>). Isolated plasmids were digested with Ncol and religated to delete the <i>kan</i> cassette, and ligation mixes were introduced into DH5α with selection for Crb<sup>+</sup> or Spc<sup>−</sup>. Transformants were screened for <i>Kan</i> sensitivity, and <i>tra</i> deletion mutations were confirmed by sequencing across the deletion junction.

**Mini-pKM101 plasmids.** We constructed 2 mini-pKM101 plasmids with a goal of simplifying genetic manipulations of the <i>tra</i> gene cluster. pCGR108 was generated by introduction of the <i>tra</i> region from pKM101 into pBAD24. We amplified a ~10-kb region of pKM101 encompassing the upstream regulatory region and <i>tra</i> promoter through trAG. This fragment was amplified with primers pKM101_2700NcoI_F and pKM101_13500XbaI_R and the resulting PCR product was introduced into pBAD24 using Ncol and XbaI restriction sites. The second mini-pKM101 plasmid, pRP100, was constructed by joining three PCR products: (i) the <i>tra</i> gene cluster
extending from the 3’ end of kikA through the end of traG, (ii) the pKM101 oriV replication origin and (iii) an nptII gene encoding KanR. The ~9-kb tra gene cluster was amplified from pKM101 with primers pKM101_1921Nco1_F and pKM101_13500XbaR, a ~3-kb region encompassing the replication origin was amplified with primers RSP007 and RSP008, and the nptII gene was amplified with primers RSP005 and RSP006 using plasmid pUC4K as a template. The replication origin and nptII gene were joined together using overlapping PCR, digested with NcoI and XbaI, and the resulting fragment was ligated to the tra gene cluster. The resulting circularized product was transformed into E. coli DH5α with KanR as a selection for self-replicating plasmids. Transformants were screened for plasmids bearing the three PCR fragments followed by sequence analysis of the PCR fragment junctions. We also confirmed that each of the mini-pKM101 plasmids encodes a fully functional TraT4SS (see Results).

pRP100::tra and pCGR108::tra variants. We precisely deleted each of the tra genes from pRP100 by inverse PCR using the 5’ phosphorylated primers listed in Supporting Information Table S1 and pRP100 as a template. The resulting plasmids, designated pRP101-pRP111, sustain deletions of traL through traG, respectively. We also deleted traF and traN-traF from pCGR108 to create pJG125 and pJG143, respectively, using a similar inverse PCR protocol, except that SacI and XhoI restriction sites were incorporated at the 5’ and 3’ ends of the deletion junctions.

pKM101 mob plasmid. We constructed a mobilizable plasmid bearing the pKM101 origin-of-transfer (oriT) sequences and adjacent traK, traJ and traF genes. These genes code for the oriT processing proteins, relaxase TraL and accessory factor TraK, and the coupling protein TraJ. A PCR fragment spanning the oriT-traL region was generated with primers oriT_NcoI_F and TraL_HindIII_R and pKM101 as a template, and then introduced as a blunt-ended fragment into a blunt-ended HindIII site on the pSC101 derivative pGB2 to make pJG142.

Tra gene expression plasmids. Plasmids pMS1 through pMS11 express the pKM101 tra gene cluster tra genes, respectively, from the PBAD promoter. Each tra gene was PCR amplified using primers listed in Supporting Information Table S2, and pKM101 as a template. The resulting PCR fragments were digested with NcoI and KpnI for introduction into NcoI/KpnI-digested pBAD24Kan. Plasmids pJG59 and pJG62 express native and his6-tagged traF, respectively, from the PBAD promoter on pBAD24Sp. They were constructed by PCR amplification of traF using primers TraF_FWD_NcoI or TraF_NT_His_FWD_NcoI and TraF_RVS_XhoI with pKM101 as a template, digestion of the PCR fragments with NcoI and XhoI, and introduction of the resulting fragments into NcoI/Sall-digested pBAD24Spc. Plasmid pJG103 expresses PBAD::traF-CT_FLAG, producing C-terminally FLAG-tagged TraF. It was constructed by amplifying traF using primers TraF_FWD_NcoI and TraF_386-FLAG_CT_RVS_XhoI with pKM101 as a template. The resulting PCR fragment was digested with NcoI and XhoI and introduced into a NcoI/Sall-digested pBAD24Spc.

traF mutant plasmids. The following plasmids expressing traF mutant alleles from the PBAD promoter were constructed by PCR amplification of gene fragments of interest using primers listed in Supporting Information Table S2 and pKM101 as a template, digestion of the final products with NcoI and XhoI, and introduction of the digested fragments into NcoI/Sall-digested pBAD24Spc. Plasmids: pJG95 produces TraF::AP-CT from PBAD::traF1–301 (numbers correspond to traF codons); pJG96 produces TraF::ACT from PBAD::traF1–353; pJG76 produces TraF::AP from PBAD::traF307–354 (traF1–307 and traF355–386 were amplified and joined by overlapping PCR, and cloned as above); pJG61 produces TraF::APL-5xGly from PBAD::traF::APL-5xGly (traF1–322 and traF346–386 were amplified to carry a 5xGly residues at their 3’ and 5’ ends, respectively, and then joined by overlapping PCR); pJG64 produces His6::TraF::FLAG330 from PBAD::his6::traF::FLAG330 (traF1–330 and 331–386 were amplified to carry a FLAG tag at their 3’ and 5’ ends, respectively, and then joined by overlapping PCR); pJG101 produces TraF::ARLDLF from PBAD::traF373–377; pJG97 produces TraF::ACT9; pJG92 produces TraF::lever from PBAD::traF::lever (traF1–170 and traF200–386 were amplified, and joined by overlapping PCR).

diversity at the 3’ end of kikA through the end of traG, (ii) the pKM101 oriV replication origin and (iii) an nptII gene encoding KanR. The ~9-kb tra gene cluster was amplified from pKM101 with primers pKM101_1921Nco1_F and pKM101_13500XbaR, a ~3-kb region encompassing the replication origin was amplified with primers RSP007 and RSP008, and the nptII gene was amplified with primers RSP005 and RSP006 using plasmid pUC4K as a template. The replication origin and nptII gene were joined together using overlapping PCR, digested with NcoI and XbaI, and the resulting fragment was ligated to the tra gene cluster. The resulting circularized product was transformed into E. coli DH5α with KanR as a selection for self-replicating plasmids. Transformants were screened for plasmids bearing the three PCR fragments followed by sequence analysis of the PCR fragment junctions. We also confirmed that each of the mini-pKM101 plasmids encodes a fully functional TraT4SS (see Results).

pRP100::tra and pCGR108::tra variants. We precisely deleted each of the tra genes from pRP100 by inverse PCR using the 5’ phosphorylated primers listed in Supporting Information Table S1 and pRP100 as a template. The resulting plasmids, designated pRP101-pRP111, sustain deletions of traL through traG, respectively. We also deleted traF and traN-traF from pCGR108 to create pJG125 and pJG143, respectively, using a similar inverse PCR protocol, except that SacI and XhoI restriction sites were incorporated at the 5’ and 3’ ends of the deletion junctions.

pKM101 mob plasmid. We constructed a mobilizable plasmid bearing the pKM101 origin-of-transfer (oriT) sequences and adjacent traK, traJ and traF genes. These genes code for the oriT processing proteins, relaxase TraL and accessory factor TraK, and the coupling protein TraJ. A PCR fragment spanning the oriT-traL region was generated with primers oriT_NcoI_F and TraL_HindIII_R and pKM101 as a template, and then introduced as a blunt-ended fragment into a blunt-ended HindIII site on the pSC101 derivative pGB2 to make pJG142.

Tra gene expression plasmids. Plasmids pMS1 through pMS11 express the pKM101 tra gene cluster tra genes, respectively, from the PBAD promoter. Each tra gene was PCR amplified using primers listed in Supporting Information Table S2, and pKM101 as a template. The resulting PCR fragments were digested with NcoI and KpnI for introduction into NcoI/KpnI-digested pBAD24Kan. Plasmids pJG59 and pJG62 express native and his6-tagged traF, respectively, from the PBAD promoter on pBAD24Sp. They were constructed by PCR amplification of traF using primers TraF_FWD_NcoI or TraF_NT_His_FWD_NcoI and TraF_RVS_XhoI with pKM101 as a template, digestion of the PCR fragments with NcoI and XhoI, and introduction of the resulting fragments into NcoI/Sall-digested pBAD24Spc. Plasmid pJG103 expresses PBAD::traF-CT_FLAG, producing C-terminally FLAG-tagged TraF. It was constructed by amplifying traF using primers TraF_FWD_NcoI and TraF_386-FLAG_CT_RVS_XhoI with pKM101 as a template. The resulting PCR fragment was digested with NcoI and XhoI and introduced into a NcoI/Sall-digested pBAD24Spc.

-traF mutant plasmids. The following plasmids expressing traF mutant alleles from the PBAD promoter were constructed by PCR amplification of gene fragments of interest using primers listed in Supporting Information Table S2 and pKM101 as a template, digestion of the final products with NcoI and XhoI, and introduction of the digested fragments into NcoI/Sall-digested pBAD24Spc. Plasmids: pJG95 produces TraF::AP-CT from PBAD::traF1–301 (numbers correspond to traF codons); pJG96 produces TraF::ACT from PBAD::traF1–353; pJG76 produces TraF::AP from PBAD::traF307–354 (traF1–307 and traF355–386 were amplified and joined by overlapping PCR, and cloned as above); pJG61 produces TraF::APL-5xGly from PBAD::traF::APL-5xGly (traF1–322 and traF346–386 were amplified to carry a 5xGly residues at their 3’ and 5’ ends, respectively, and then joined by overlapping PCR); pJG101 produces TraF::ARLDLF from PBAD::traF373–377; pJG97 produces TraF::ACT9; pJG92 produces TraF::lever from PBAD::traF::lever (traF1–170 and traF200–386 were amplified, and joined by overlapping PCR).

traF virB10 chimera plasmids. The following plasmids expressing traF virB10 chimeric genes were constructed by PCR amplification of gene fragments of interest using primers listed in Supporting Information Table S2 and pKM101 or traF fragments or pKVD10 for virB10 fragments. The amplification products (listed in parentheses) were joined by overlapping PCR, and the resulting fragments were cloned as above; pJG64 produces His6::TraF::FLAG330 from PBAD::his6::traF::FLAG330 (traF1–330 and 331–386 were amplified to carry a FLAG tag at their 3’ and 5’ ends, respectively, and then joined by overlapping PCR); pJG101 produces TraF::ARLDLF from PBAD::traF373–377; pJG97 produces TraF::ACT9; pJG92 produces TraF::lever from PBAD::traF::lever (traF1–170 and traF200–386 were amplified, and joined by overlapping PCR).

Chimeric T4SSs reveal compositional flexibility of outer membrane subassemblies
pSU1443 and pKM101 as templates, and the resulting amplicon was digested with SacI and XhoI for introduction into pJG143. Plasmid pJG143 contains pKM101 traL-traD-SacI/XhoI-traG; it was derived from pCGR108 by inverse PCR. Plasmid pJG144 expresses the chimeric gene cluster tra::virB. A DNA fragment encoding virB7-traE-virB9-traFl

#Beta-virB10 was designed with codon-optimization for expression in E. coli, and synthesized by Genewiz Inc. The DNA fragment was isolated from pJG202 by digestion with SacI and XhoI and introduced into similarly-digested pJG143. Plasmid pJG151 expresses the chimeric gene cluster tra::ptl. A DNA fragment encoding ptl-traE-ptaF-

#traF1–172/jB-CTvirB10 was designed with codon-optimization for expression in E. coli, synthesized by Genewiz Inc. The DNA fragment was isolated from pJG202 and introduced into pJG143 as described above.

**Mini-pKM101 plasmids with traF variants.** Plasmid pJG125 carries the pKM101 tra genes except that XhoI and SacI sites were substituted for traF. It was constructed by inverse PCR using 5’ phosphorylated primers listed in Supporting Information Table S2 and pCGR125 as a template. pJG125 derivatives expressing different tra alleles were constructed by introduction of PCR fragments generated with primers listed in Supporting Information Table S2 into SacI/XhoI-digested pJG125. Plasmids pJG152 produces N-terminally FLAG-tagged TraF; pJG158 produces FLAG-TraF::AP (deleted of codons 307–354); pJG154 produces FLAG_TraF::ADLD (deleted of codons 373–377); pJG153 produces FLAG_TraF::CT9 (deleted of 9 codons at the 3’ end); pJG155 produces FLAG_TraF/AP-

#CTFvirB10 (traF1–307, virB10.286–377); pJG157 produces FLAG_TraF/AP-CT

#TraE (traF1–307/trwE303–395); pJG156 produces FLAG_TraF/AP-CT

#TraE2 (traF1–307/trg294–374).

A. tumefaciens virB10 expression plasmids. We incorporated a Strept (St) sequence at the 3’ end of virB10 on plasmid pTiA6NC of strain A348 (Garfinkel et al., 1981). virB10-St was amplified with 500 bp of 5’ and 3’ flanking sequences using overlapping PCR and primers listed in Supporting Information Table S2. We then cloned this fragment into pBB50 for introduction into the ΔvirB10 derivative PC1010 by marker-exchange eviction mutagenesis, as previously described (Berger and Christie, 1994). The resulting strain, A348virB10-St, carrying the incorporated virB10-St gene was used for purification and structural characterization of the A. tumefaciens VirB/VirD4 OMCC.

We introduced the following plasmids expressing virB10 alleles into strain PC1010. Plasmid pKV10 produces native VirB10 from P

#P_lac::virB10 and fully complements the ΔvirB10 mutation (Jakubowski et al., 2009). Plasmids pSJ510, pSJ511 and pSJ512 were constructed by inserting an SphI restriction site at codons 298, 329 and 332, respectively, by inverse PCR using primers listed in Supporting Information Table S2 and pKV10 as a template. We then PCR amplified 3xFLAG tag sequences, each with flanking SphI sites, using primers listed in Supporting Information Table S2 and pSJ503 as a template. Plasmid pSJ503 contains a 3xFLAG tag and was constructed by annealing oligonucleotides listed in Supporting Information Table S2, digesting the product with Ncol and BamHI, and inserting the digested fragment into similarly-digested pBSISK+/Ndel. We then digested the amplified 3xFLAG tag sequences with SphI for insertion at codons 298, 329 and 332, creating plasmids pJG40, pJG42 and pJG44 respectively. Plasmid pJS52 producing VirB10 with a 2xFLAG tag inserted at codon 310 was constructed by two-step overlapping PCR using primers listed in Supporting Information Table S2 and pKV10 as a template. Plasmid pSJ500 producing VirB10 with a C-terminal FLAG tag was constructed by amplification of virB10 with a 3’ terminal FLAG sequence using oligonucleotides listed in Supporting Information Table S2 and pKV10 as a template.

Plasmid pJS504 producing VirB10/AAP (deleted of residues 288–337) was constructed by inverse PCR using primers listed in Supporting Information Table S2 and pKV10 as a template. Plasmids pJS501 and pJS502 producing VirB10/AAP and VirB10/AAP-CT, respectively, were constructed by overlapping PCR using the primers listed in Supporting Information Table S2 and pKV10 or pKM101 as templates. Following amplification, the virB10/AAP and virB10/AAP-CT products were digested with Ndel-XhoI for insertion into pBSISK+/Ndel.

All ColE1 plasmids expressing the virB10 alleles were ligated to broad-host-range plasmids pXZ151 or pBBR-Kan for introduction into A. tumefaciens (Berger and Christie, 1994).

**Conjugation assays**

E. coli conjugation assays on solid surfaces were carried out essentially as previously described (Whitaker et al., 2016). Briefly, overnight cultures of donors and recipients were diluted 1:100 in antibiotic-free media and incubated for 1 h with shaking at 37°C. For induction from the P

#P_BAD promoter, arabinose was added (0.2% final concentration) followed by incubation for 1 h with shaking at 37°C. Donors and recipients (2.5 µl) were mixed on a nitrocellulose filter on LB media containing 0.2% arabinose and the mating mix was incubated for 2 h at 37°C. For broth matings, induced donors were mixed with recipients at a 1:1 volumetric ratio and incubated at 37°C for 2 h. Filter and broth mating mixtures were serially diluted and plated on media selective for transconjugants and donors. Frequency of transfer was calculated as the number of transconjugants per donor (Tcs/D). Experiments were performed at least three times in duplicate or triplicate, and results are reported as the mean frequency of transfer.

**Phage infection assays**

IKe bacteriophage was propagated as described previously for R17 (Lang et al., 2011). Strains carrying plasmids of interest were grown and assayed for susceptibility to IKe infection as previously described with slight modifications (Cellini et al., 1997). Briefly, cells were induced with arabinose as described above for the conjugation assays. Fifty microliters of cells at a concentration of 10^8 ml^{-1} were spread on an LB plate containing appropriate antibiotics and arabinose, and allowed to dry. Five microliters of IKe...
(10⁶ pfu, final concentration) was spotted onto the lawns of cells, and plates were incubated overnight at 37°C.

**Type VI killing assay**

T4SS-mediated killing of *E. coli* by the *Pseudomonas aeruginosa* type VI secretion system (T6SS) was carried out as previously described (Ho et al., 2013). Briefly, 2 ml of *E. coli* DH5α donors and a *P. aeruginosa* PAO1retS were incubated overnight with shaking at 37°C, then resuspended in 2 ml of antibiotic-free LB followed by a 1:100 dilution in 5 ml of antibiotic-free LB. Cells were then incubated with shaking at 37°C for 2 h, pelleted and resuspended in 100 μl LB. *P. aeruginosa* (17 μl) were mixed with *E. coli* (3 μl) on filters placed on LB plates and incubated for 3 h at 37°C. Cells were resuspended in 1 ml of LB and serial dilutions were spotted onto plates containing spectinomycin (300 μg/ml) and rifampicin (100 μg/ml) to select for growth of *E. coli*. T6SS killing of *E. coli* is presented as *E. coli* cell viability in CFU per ml.

**Protein detection**

*E. coli* strains were grown and induced for expression of His₆ or FLAG-tagged TraF variants of interest in LB media, harvested, and normalized to equivalent optical densities (OD₆₀₀). Total protein extracts were subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes, and blots were developed with FLAG primary antibodies and HRP-conjugated secondary antibodies for detection of the TraF proteins by chemiluminescence (Whitaker et al., 2016). For VirB10 detection in *A. tumefaciens*, cells were grown and induced for expression of the *vir* genes (see below), and total cell extracts were analyzed by SDS-PAGE and immunostaining of western blots with α-VirB10 antibodies (Jakubowski et al., 2009).

**Extracellular VirB2 blot assay**

Surface-exposed VirB2 was detected by colony immunoblotting using α-VirB2 antibodies as described previously (Kerr and Christie, 2010).

**A. tumefaciens outer-membrane complex expression and purification**

*A. tumefaciens* strain A348virB10-St was inoculated in 100ml of MG/L media supplemented with 100μg/ml of Kanamycin. After overnight incubation at 26°C, 10 ml of culture pellet was inoculated into 200 ml of fresh MG/L media and incubated with shaking to an OD₆₀₀ of 0.5–0.8. The culture was further harvested by centrifugation and resuspended in 6 L of ABIM media (supplemented with 100 mM of acetosyringone for *vir* genes expression) to an OD₆₀₀ of 0.1–0.2. After 12–14 h of incubation at 23°C, the cultures were harvested by centrifugation and resuspended in cooled 50 mM Tris-HCl pH 8.0, treated with DNase I, lysosome and EDTA-free protease inhibitor tablets, and sonicated on ice. After cell disruption, 1 mM EDTA was added and the lysate was clarified by centrifugation at 38,000g for 20 min. The membrane fraction was then collected by centrifugation at 98,000g for 45 min and membrane pellets were mechanically homogenized and solubilized in 50 mM of Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% w/v Digitonin (Sigma), 0.75% w/v DMNPG (Anatrace), 0.5% w/v DDM (Anatrace) and 1 mM DTT for 1 h at 4°C. The suspension was clarified by centrifugation at 98,000g for 20 min and the supernatant was loaded onto a 5 ml Strep Trap HP (GE Healthcare) column and washed with 50 mM of Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1% w/v Digitonin, 0.05% w/v DMNPG and 1 mM DTT at 4°C. The outer-membrane complex was eluted with the equivalent wash buffer supplemented with 2.5 mM of desethylbixin. The single peak fractions were pooled and loaded onto a Superose 6 10/300 column (GE Healthcare) equilibrated with the same buffer without desethylbixin. The sample eluting as a peak after the column void was used immediately for the preparation of negative stain EM grids.

**Electron microscopy and image processing**

Sample preparation for EM. Four microliters of the OMCC diluted to 0.01 mg/ml in the gel filtration buffer (see above) was applied on glow-discharged carbon-coated copper grids (400 mesh grid copper, Agar Scientific). After incubation for 2 min, the sample was washed twice with 10 μl water and then stained for 1 min with 10 μl 2% uranyl acetate. Then the grids were blotted to remove excess stain, dried, and kept for the microscope sessions. The data were collected on a F20 microscope (FEI) operating at 200 kV at a magnification of 45,500× using a low dose mode (~ 30 e Å²) and a defocus range of –0.7 to –2.0 μm. Images were recorded on a Gatan UltraScan 4000 CCD camera (Gatan) with a calibrated pixel size of 3.3 Å. 60 micrographs were collected. Quality was assessed visually and through CTF estimation. Images with distorted Thon rings and drift were not considered for the further processing.

Preprocessing. The contrast transfer function (CTF) of the microscope was estimated using CTFFIND3 (Mindell and Grigorieff, 2003) and the CTF correction of entire images was done by phase flipping using Bsoft (Heymann and Bellenap, 2007). Particle images were picked from the CTF corrected micrographs. A total of 1746 particles were manually selected and extracted with a box size of 240 × 240 pixels using EMAN/BOXER (Ludtke, 2010). The following processing was done using IMAGIC software (Van Heel et al., 1996). Images of particles were normalized, band pass filtered, centered, subjected to reference-free multistatistical analysis (MSA), and then the alignment and classification were refined using iteratively multireference-alignment (MRA) and MSA where the best classes were used as new references. Quality of classes was assessed by variations between images that constitute classes: the lower variations → the better class (Van Heel et al., 2000). This refinement procedure was considered complete when changes in
Acknowledgements

The authors thank members of the Christie laboratory for helpful discussions. Studies in the Christie laboratory were supported by National Institutes of Health Grants R01GM48476 to PJC and F32 AI114182 to C. G.-R., and in the Waksman laboratory by Wellcome Trust grant 098302 to GW. They also thank National Institute of Allergy and Infectious Diseases grant F32 AI114182 and National Institute of General Medical Sciences grant R01GM48476. The authors declare no conflict of interest.

References

Aly, K.A., and Baron, C. (2007) The VirB5 protein localizes to the T-pilus tips in Agrobacterium tumefaciens. Microbiology 153: 3766–3775.

Ames, B.N., McCann, J., and Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res 31: 347–364.

Arutyunov, D., and Frost, L.S. (2013) F conjugation: back to the beginning. Plasmid 70: 18–32.

Atmakuri, K., Cascales, E., and Christie, P.J. (2004) Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. Mol Microbiol 54: 1199–1211.

Bacic, A., Lindner, A.B., Vulic, M., Stewart, E.J., and Radman, M. (2008) Direct visualization of horizontal gene transfer. Science 319: 1533–1536.

Backert, S., Fronzes, R., and Waksman, G. (2008) VirB2 and VirB5 proteins: specialized adhesins in bacterial type-IV secretion systems? Trends Microbiol 16: 409–413.

Banta, L.M., Kerr, J.E., Cascales, E., Giuliano, M.E., Bailey, M.E., McKay, C., et al. (2011) An Agrobacterium VirB10 mutation conferring a type IV secretion system gating defect. J Bacteriol 193: 2566–2574.

Barden, S., and Niemann, H.H. (2015) Adhesion of several cell lines to Helicobacter pylori CagL is mediated by integrin alphaVbeta6 via an RGDLXXL motif. J Mol Biol 427: 1304–1315.

Baron, C. (2006) VirB8: a conserved type IV secretion system assembly factor and drug target. Biochem Cell Biol 84: 890–899.

Bayer, M., Eferl, R., Zellwig, G., Tefeler, K., Dijkstra, A., Koraimann, G., and Hogenauer, G. (1995) Gene 19 of plasmid R1 is required for both efficient conjugative DNA transfer and bacteriophage R17 infection. J. Bacteriol 177: 4279–4288.

Bayer, M., Iberer, R., Bischof, K., Rassi, E., Stabenheimer, E., Zellwig, G., and Koraimann, G. (2001) Functional and mutational analysis of p19, a DNA transfer protein with muramidase activity. J Bacteriol 183: 3176–3183.

Berger, B.R., and Christie, P.J. (1994) Genetic complementation analysis of the Agrobacterium tumefaciens virB operon: virB2 through virB11 are essential virulence genes. J Bacteriol 176: 3646–3660.

Bour, G., Sube, R., O’Callaghan, D., and Patey, G. (2009) Interactions between Brucella suis VirB8 and its homolog TraJ from the plasmid pSB102 underline the dynamic nature of type IV secretion systems. J Bacteriol 191: 2985–2992.

Bradley, D.E. (1979) Morphology of pili determined by the N incompatibility group plasmid N3 and interaction with bacteriophages PR4 and IKe. Plasmid 2: 632–636.

Bradley, D.E., Taylor, D.E., and Cohen, D.R. (1980) Specification of surface mating systems among conjugative drug resistance plasmids in Escherichia coli K-12. J Bacteriol 143: 1466–1470.

Burns, D.L. (2003) Type IV transporters of pathogenic bacteria. Curr Opin Microbiol 6: 29–34.

Cascales, E., and Christie, P.J. (2004a) Agrobacterium VirB10, an ATP energy sensor required for type IV secretion. Proc Natl Acad Sci USA 101: 17228–17233.

Cascales, E., and Christie, P.J. (2004b) Definition of a bacterial type IV secretion pathway for a DNA substrate. Science 304: 1170–1173.

Cascales, E., Atmakuri, K., Sarkar, M.K., and Christie, P.J. (2013) DNA substrate-induced activation of the Agrobacterium VirB/VirD4 type IV secretion system. J Bacteriol 195: 2691–2704.

Cellini, C., Kalogeraki, V.S., and Winans, S.C. (1997) The hydrophobic TraM protein of pKM101 is required for conjugal transfer and sensitivity to donor-specific bacteriophage. Plasmid 37: 181–188.

Chandran Darbari, V., and Waksman, G. (2015) Structural biology of bacterial type IV secretion systems. Annu Rev Biochem 84: 603–629.

Chandran, V., Fronzes, R., Duquerroy, S., Cronin, N., Navaza, J., and Waksman, G. (2009) Structure of the outer membrane complex of a type IV secretion system. Nature 462: 1011–1015.

Christie, P.J. (2004) Type IV secretion: the Agrobacterium VirB/VirD4 and related conjugation systems. Biochim Biophys Acta 1694: 219–234.

Christie, P.J. (2016) The mosaic type IV secretion systems. EcoSal Plus 7: 1128.

Christie, P.J., and Vogel, J.P. (2000) Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. Trends Microbiol 8: 354–360.

Christie, P.J., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S., and Cascales, E. (2005) Biogenesis, architecture, and
function of bacterial type IV secretion systems. *Annu Rev Microbiol* **59**: 451–485.

Clarke, M., Madder, L., Harris, R.L., and Silverman, P.M. (2008) F-pili dynamics by live-cell imaging. *Proc Natl Acad Sci USA* **105**: 17978–17981.

Costa, T.R., Felisberto-Rodrigues, C., Meir, A., Prevost, M.S., Redzej, A., Trokter, M., and Waksman, G. (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* **13**: 343–359.

Costa, T.R., Ilangovan, A., Ukleja, M., Redzej, A., Santini, J.M., Smith, T.K., Egelman, E.H., and Waksman, G. (2016) Structure of the bacterial sex F pilus reveals an assembly of a stoichiometric protein-phospholipid complex. *Cell* **166**: 1436–1444.

Das, A., and Xie, Y.-H. (2000) The *Agrobacterium* T-DNA transport pore proteins VirB8, VirB9, and VirB10 interact with one another. *J Bacteriol* **182**: 758–763.

De la Cruz, F., Frost, L.S., Meyer, R.J., and Zechner, E.L. (2010) Conjugative DNA metabolism in Gram-negative bacteria. *FEBS Microbiol Rev* **34**: 18–40.

De Paz, H.D., Sangari, F.J., Bolland, S., Garcia-Lobo, J.M., Dehio, C., de la Cruz, F., and Llosa, M. (2005) Functional interactions between type IV secretion systems involved in DNA transfer and virulence. *Microbiology* **151**: 3505–3516.

Dehio, C. (2008) Infection-associated type IV secretion systems of *Bartonella* and their diverse roles in host cell interaction. *Cell Microbiol* **10**: 1591–1598.

Farizzo, K.M., Huang, T., and Burns, D.L. (2000) Importance of holotoxin assembly in Pti-mediated secretion of pertussis toxin from *Bordetella pertussis*. *Infect Immun* **68**: 4049–4054.

Fischer, W., Puls, J., Buhrdorf, R., Gebert, B., Odenbreit, S., and Haas, R. (2001) Systematic mutagenesis of the *Helicobacter pylori* cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. *J Bacteriol* **183**: 4779–4791.

Fronzes, R., Schafer, E., Wang, L., Orlova, E.V., and Waksman, G. (2009) Structure of a type IV secretion system core complex. *Science* **323**: 266–268.

Frost, L.S., and Koraimann, G. (2010) Regulation of bacterial conjugation: balancing opportunity with adversity. *Future Microbiol* **5**: 1057–1071.

Garfinkel, D.J., Simpson, R.B., Ream, L.W., White, F.F., Gordon, M.P., and Nester, E.W. (1981) Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. *Cell* **27**: 143–153.

Gorrell, R.J., Zwickel, N., Reynolds, J., Bulach, D., and Kwok, T. (2016) *Helicobacter pylori* CagL hypervariable motif: a global analysis of geographical diversity and association with gastric cancer. *J Infect Dis* **213**: 1927–1931.

Haase, J., Lurz, R., Grafh, A.M., Bamford, D.H., and Lanka, E. (1995) Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific plague propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. *J Bacteriol* **177**: 4779–4791.

Hapfelmeier, S., Domke, N., Zambryski, P.C., and Baron, C. (2000) VirB6 is required for stabilization of VirB5 and VirB3 and formation of VirB7 homodimers in *Agrobacterium tumefaciens*. *J Bacteriol* **182**: 4505–4511.

Harrington, L.C., and Rogerson, A.C. (1990) The F pilus of *Escherichia coli* appears to support stable DNA transfer in the absence of wall-to-wall contact between cells. *J Bacteriol* **172**: 7263–7264.

Heymann, J.B., and Belnap, D.M. (2007) Bsoft: image processing and molecular modeling for electron microscopy. *J Struct Biol* **157**: 3–18.

Ho, B.T., Basler, M., and Melkanos, J.J. (2013) Type 6 secretion system-mediated immunity to type 4 secretion system-mediated gene transfer. *Science* **342**: 250–253.

Holloway, B.W. (1955) Genetic recombination in *Pseudomonas aeruginosa*. *J Gen Microbiol* **13**: 572–581.

Jakubowski, S.J., Krishnamoorthy, V., and Christie, P.J. (2003) *Agrobacterium tumefaciens* VirB6 protein participates in formation of VirB7 and VirB9 complexes required for type IV secretion. *J Bacteriol* **185**: 2867–2878.

Jakubowski, S.J., Krishnamoorthy, V., Cascales, E., and Christie, P.J. (2004) *Agrobacterium tumefaciens* VirB6 domains direct the ordered export of a DNA substrate through a type IV secretion system. *J Mol Biol* **341**: 961–977.

Jakubowski, S.J., Cascales, E., Krishnamoorthy, V., and Christie, P.J. (2005) *Agrobacterium tumefaciens* VirB9, an outer-membrane-associated component of a type IV secretion system, regulates substrate selection and T-pilus biogenesis. *J Bacteriol* **187**: 3486–3495.

Jakubowski, S.J., Kerr, J.E., Garza, I., Krishnamoorthy, V., Bayliss, R., Waksman, G., and Christie, P.J. (2009) *Agrobacterium* VirB10 domain requirements for type IV secretion and T-pilus biogenesis. *Mol Microbiol* **71**: 779–794.

Jorgensen, N.H., and Stenderup, A. (1982) An InC plasmid exhibiting a high frequency of transfer in broth media. *Acta Pathol Microbiol Immunol Scand B* **90**: 323–324.

Kerr, J.E., and Christie, P.J. (2010) Evidence for VirB4-mediated dislocation of membrane-integrated VirB2 pilin during biogenesis of the *Agrobacterium* VirB/VirD4 type IV secretion system. *J Bacteriol* **192**: 4923–4934.

Krarl, L., Wiedemann, U., Unsinn, G., Weiss, S., Domke, N., and Baron, C. (2002) Detergent extraction identifies different VirB protein subassemblies of the type IV secretion machinery in the membranes of *Agrobacterium tumefaciens*. *Proc Natl Acad Sci USA* **99**: 11405–11410.

Kubori, T., and Nagai, H. (2015) The Type IVB secretion system: an enigmatic chimera. *Curr Opin Microbiol* **29**: 22–29.

Kubori, T., Koike, M., Bui, X.T., Higaki, S., Aizawa, S., and Kubori, T. (2016) Structural and molecular modeling for electron microscopy. *J Mol Biol* **291**: 250–253.

Lacroix, B., and Citovsky, V. (2011) Extracellular VirB5 enhances T-DNA transfer from *Agrobacterium* to the host plant. *PLoS One* **6**: e25578.

Lang, S., and Zechner, E.L. (2012) General requirements for protein secretion by the F-like conjugation system R1. *Plasmid* **67**: 128–138.

Lang, S., Kirchberger, P.C., Gruber, C.J., Redzej, A., Raff, S., Zeitlin, G., Zanger, K., and Zechner, E.L. (2011) An activation domain of plasmid R1 TraT protein delineates...
stages of gene transfer initiation. *Mol Microbiol* **82**: 1071–1085.

Larrea, D., de Paz, H.D., Arechaga, I., de la Cruz, F., and Llosa, M. (2013) Structural independence of conjugal coupling protein TrwB from its type IV secretion machinery. *Plasmid* **70**: 146–153.

Lawley, T.D., Gordon, G.S., Wright, A., and Taylor, D.E. (2002) Bacterial conjugal transfer: visualization of successful mating pairs and plasmid establishment in live *Escherichia coli*. *Mol Microbiol* **44**: 947–956.

Llosa, M., Zunzunegui, S., and de la Cruz, F. (2003) Conjugal coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. *Proc Natl Acad USA* **100**: 10465–10470.

Locht, C., Coutte, L., and Mielcarek, N. (2011) The ins and outs of pertussis toxin. *FEBS Lett.* **468**: 4668–4682.

Low, H.H., Gubellini, F., Rivera-Calzada, A., Braun, N., Connelly, S., Dujeancourt, A., et al. (2014) Structure of a type IV secretion system. *Nature* **508**: 550–553.

Ludtke, S.J. (2010) 3D structures of macromolecules using single-particle analysis in EMAN. *Methods Mol Biol* **673**: 157–173.

Manchak, J., Anthony, K.G., and Frost, L.S. (2002) Mutational analysis of F-pilin reveals domains for pilus assembly, phage infection and DNA transfer. *Mol Microbiol* **43**: 195–205.

Mihajlovic, S., Lang, S., Sut, M.V., Strohmaier, H., Gruber, C.J., Koraimann, G., et al. (2009) Plasmid r1 conjugative DNA processing is regulated at the coupling protein interface. *J Bacteriol* **191**: 6877–6887.

Mindell, J.A., and Grigorieff, N. (2003) Accurate determination of local defocus and specimen tilt in electron microscopy. *J Struct Biol* **142**: 334–347.

Olbermann, P., Josenhans, C., Moodley, Y., Uhr, M., Stamer, C., Vauterin, M., et al. (2010) A global overview of the genetic and functional diversity in the *Helicobacter pylori cag* pathogenicity island. *PLoS Genet* **6**: e1001069.

Paschos, A., Patey, G., Sivanesan, D., Gao, C., Bayliss, R., Waksman, G., O’Callaghan, D., and Baron, C. (2006) Dimerization and interactions of *Brucella suis* VirB10 with VirB4 and VirB10 are required for its biological activity. *Proc Natl Acad USA* **103**: 7252–7257.

Paterson, E., More, M., Pillay, G., Cellini, C., Woodgate, R., Walker, G., Iyer, V., and Winans, S. (1999) Genetic analysis of the mobilization and leading regions of the IncN plasmids pKM101 and pCU1. *J Bacteriol* **181**: 2572–2583.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* **25**: 1605–1612.

Rivera-Calzada, A., Fronzes, R., Savva, C.G., Chandran, V., Lian, P.W., Laeremans, T., et al. (2013) Structure of a bacterial type IV secretion core complex at subnanometre resolution. *EMBO J* **32**: 1195–1204.

Sagulenko, E., Sagulenko, V., Chen, J., and Christie, P.J. (2001) Role of *Agrobacterium* VirB11 ATPase in T-pilus assembly and substrate selection. *J Bacteriol* **183**: 5813–5825.

Samuels, A.L., Lanka, E., and Davies, J.E. (2000) Conjugal junctions in RP4-mediated mating of *Escherichia coli*. *J Bacteriol* **182**: 2709–2715.

Schmidt-Eisenlohr, H., Domke, N., and Baron, C. (1999) TraC of IncN plasmid pKM101 associates with membranes and extracellular high-molecular-weight structures in *Escherichia coli*. *J Bacteriol* **181**: 5563–5571.

Shaffer, C.L., Gaddy, J.A., Loh, J.T., Johnson, E.M., Hill, S., Hennig, E.E., et al. (2011) *Helicobacter pylori* exploits a unique repertoire of type IV secretion system components for pilus assembly at the bacteria-host cell interface. *PLoS Pathol* **7**: e1002237.

Singer, M., Baker, T.A., Schnitzler, G., Deischel, S.M., Goel, M., Dove, W., et al. (1989) A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol Rev* **53**: 1–24.

Terradot, L., Bayliss, R., Oomen, C., Leonard, G.A., Baron, C., and Waksman, G. (2005) Structures of two core subunits of the bacterial type IV secretion system, VirB8 from *Brucella suis* and ComB10 from *Helicobacter pylori*. *Proc Natl Acad USA* **102**: 4956–4961.

Thomason, L.C., Sawitzke, J.A., Li, X., Costantino, N., and Court, D.L. (2014) Recombining: genetic engineering in bacteria using homologous recombination. *Curr Protoc Mol Biol* **106**: 11611–11639.

Trokter, M., Felisberto-Rodrigues, C., Christie, P.J., and Waksman, G. (2014) Recent advances in the structural and molecular biology of type IV secretion systems. *Curr Opin Struct Biol* **27**: 16–23.

Van Heel, M. (1987) Angular reconstitution: a posteriori assignment of projection directions for 3D reconstruction. *Ultramicroscopy* **21**: 111–123.

Van Heel, M., Harauz, G., Orlowa, E.V., Schmidt, R., and Schatz, M. (1996) A new generation of the IMAGIC image processing system. *J Struct Biol* **116**: 17–24.

Van Heel, M., Gowen, B., Matadeen, R., Orlowa, E.V., Finn, R., Pape, T., et al. (2000) Single-particle electron cryomicroscopy: towards atomic resolution. *Q Rev Biophys* **33**: 307–369.

Illamí Giraldo, A.M., Sivanesan, D., Carle, A., Paschos, A., Smith, M.A., Plesa, M., Coulton, J., and Baron, C. (2012) Type IV secretion system core component VirB8 from *Brucella* binds to the globular domain of VirB5 and to a periplasmic domain of VirB6. *Biochemistry* **51**: 3881–3890.

Whitaker, N., Berry, T.M., Rosenthal, N., Gordon, J.E., Winans, S.C., Burns, D.L., and Christie, P.J. (1996) Adapting a conjugation system for the export of pathogenic macromolecules. *Trends Microbiol* **4**: 64–68.

Winans, S.C., and Walker, G.C. (1985) Conjugal transfer system of the IncN plasmid pKM101. *J Bacteriol* **161**: 402–410.

Yeo, H.-J., Yuan, Q., Beck, M.R., Baron, C., and Waksman, G. (2003) Structural and functional characterization of the VirB5 protein from the type IV secretion system encoded
by the conjugative plasmid pKM101. *Proc Natl Acad Sci USA* **100**: 15947–15952.

Yuan, Q., Carle, A., Gao, C., Sivanesan, D., Aly, K.A., Hoppner, C., *et al.* (2005) Identification of the VirB4-VirB8-VirB5-VirB2 pilus assembly sequence of type IV secretion systems. *J Biol Chem* **280**: 26349–26359.

Zahrl, D., Wagner, M., Bischof, K., Bayer, M., Zavecz, B., Beranek, A., *et al.* (2005) Peptidoglycan degradation by specialized lytic transglycosylases associated with type III and type IV secretion systems. *Microbiology* **151**: 3455–3467.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.