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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[1,3], Elena V. Orlova[2], Gabriel Waksman[2], and Peter J. Christie[1*]

Running Head: Chimeric T4SSs Reveal Compositional Flexibility of Outer Membrane Subassemblies

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

Present Address: School of Health Professions, The University of Texas Medical Branch (UTMB) at Galveston, Texas 77555.

*Correspondence to: Peter J. Christie

Department of Microbiology and Molecular Genetics, McGovern Medical School, Houston, TX 77005.

Phone: 713-500-5440, Fax: 713-500-5499

E-mail: Peter.J.Christie@uth.tmc.edu

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 functionally characterized the OMCC, 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 IMC_{pKM101} 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. A structure of the *A. tumefaciens* VirB/VirD4 OMCC, solved by transmission electron microscopy, adopts a cage structure similar to that of the pKM101 OMCC. Our findings indicate that the OMCCs are highly structurally and functionally conserved - but also intrinsically conformationally flexible - scaffolds for translocation channels. Importantly, the distal end of the OMCC functions as a morphogenetic checkpoint controlling extension of the conjugative pilus.

INTRODUCTION

The bacterial type IV secretion systems (T4SSs) are a versatile superfamily of macromolecular transporters (Costa *et al.*, 2015, Christie, 2016). An interesting feature of this superfamily is that members sharing a common ancestry and, likely, overall architecture have evolved the capacity to carry out a wide range of biological functions. An early review highlighted the functional diversity of the T4SSs by describing striking similarities in T4SS gene organization and subunit composition among the *Escherichia coli* pKM101 Tra, *Agrobacterium tumefaciens* VirB/VirD4, and *Bordetella pertussis* Ptl T4SSs (Winans *et al.*, 1996). Now grouped as members of the type IVa or P-type subfamily, these systems respectively promote conjugative transfer in *E. coli*, deliver oncogenic T-DNA and effector proteins to plant cells, and export the multisubunit pertussis toxin (PT) across the *B. pertussis* outer membrane to the milieu (Christie & Vogel, 2000, Christie, 2016). Each of these systems is assembled from a set of 9 - 11 conserved subunits, all 'signatures' of this T4SS subfamily (Christie *et al.*, 2005, Bhatty *et al.*, 2013, Chandran Darbari & Waksman, 2015). These observations raise the intriguing and unsolved question of how such systems diversified over evolutionary time to specify contact-dependent substrate transfer to bacterial or eukaryotic target cells or contact-independent export.

Recent structural studies have provided an architectural blueprint for the type IVa subfamily. The *E. coli* pKM101 Tra (designated Tra_{pKM101}) and closely related R388-encoded Trw (Trw_{R388}) conjugation machines are composed of a large (~185 Å in width and height) cage-shaped outer membrane (OM) core complex (the OMCC) of 1 MegaDalton (MDa) in size (Fronzes *et al.*, 2009, Chandran *et al.*, 2009, Low *et al.*, 2014). The OMCC is composed of 14 copies of three proteins, VirB7, VirB9, and VirB10 (we use here the *A. tumefaciens* VirB/VirD4 system, $VirB/VirD4_{At}$, as a unifying nomenclature). The OMCC is made of two layers, the O- and I-layers. The O-layer is assembled from 14 copies each of the VirB7- and VirB9-like subunits together with the C-terminal halves of VirB10 homologs. The VirB10 C-terminal region folds as a β-barrel connected to a helix-loop-helix extension termed the antennae projection (AP). In the assembled OMCC, APs from the 14 TraF monomers form a cap of ~100 Å in diameter with a central channel of ~10 - 30 Å in diameter (Fronzes *et al.*, 2009, Chandran *et al.*, 2009). The cap spans the outer membrane and is postulated to participate in various surface phenomena, including biogenesis of conjugative pili, conveyance of secretion substrates across the OM, and establishment of target cell contacts. The proximal end of the Trw_{R388} OM core complex is connected by a narrow stalk to an even larger inner membrane complex (IMC) of 2.5 MDa in size, which is composed of the N-terminal halves of VirB10-like subunits, 24 copies of VirB6 homologs, and 12 copies each of VirB3, VirB4 ATPase, VirB5 and VirB8 homologs (Low *et al.*, 2014). Missing from the solved Trw_{R388} subassembly, designated as the $T4SS_{3\text{-}10}$ structure, are the VirB2-like pilins, the conjugative pilus, and the VirD4- and VirB11-like ATPases. Recently, a structure of a conjugative pilus showed the pilus to be composed of a 5-start helical assembly consisting of a stoichiometric complex of an α-helical hairpin bound to a phospholipid (Costa et al. (2016) Cell reference to be added). However, how this pilus is mounted onto the T4SS IMC and OMCC is unknown.

In this study, we characterized the contributions of the OMCCs associated with the type IVa systems to formation of cell-cell contacts, substrate transfer, and pilus biogenesis. Results of mutational and domain swapping studies of the Tra_{pKM101} and $VirB/VirD4_{At}$ model systems established the requirement for the OMCC cap for assembly of the conjugative pilus, but not for establishment of productive mating junctions or even for contact-dependent activation of a type VI secretion system (T6SS). We also show that chimeric machines consisting of the IMC_{pKM101} coupled to heterologous OMCCs from the Trw_{R388}, $VirB/VirD4_{At}$, and Ptl_{Bp} 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 structural 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 first constructed a set of pKM101 derivatives lacking individual *tra* genes to define the genetic requirements for T4SS machine assembly (Fig. S1). Eight of the 11 *tra* genes were successfully 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 *tra* gene cluster and upstream regulatory sequences cloned either into pBAD plasmids or joined to pKM101's *oriV* replication region along with a selectable Kan^r gene. These mini-pKM101 plasmids encode fully functional Tra T4SSs (see below) and served as templates for construction of complete collections of *tra* gene deletion mutations by inverse PCR.

E. coli donors conjugatively transfer pKM101 at frequencies approaching 1 transconjugant per donor (Tc/D) in 2 h solid-surface matings (Winans & Walker, 1985). In contrast to previous reports that IncN plasmids typically transfer poorly in liquid matings (Bradley *et al.*, 1980, Jorgensen & Stenderup, 1982), MG1655(pKM101) cells delivered pKM101 fairly efficiently at $\sim 10^{-3}$ Tc's/D in 2 h and at $\sim 10^{-1}$ Tc's/D in overnight liquid matings subjected to constant agitation (Fig. S1). Another donor strain carrying the mini-pKM101 plasmid pRP100 mobilized the transfer of plasmid pJG142, which carries the entire pKM101 *mob* region (Paterson *et al.*, 1999), at frequencies slightly higher than observed for pKM101 in both solid surface and liquid matings (Fig. 1).

Most of the Δ*tra* gene mutations abolished Tra T4SS function, in agreement with previous findings for the *A. tumefaciens* VirB/VirD4 T4SS and the *E. coli* Trw_{R388} conjugation system (Figs. 1 & S1) (Berger & Christie, 1994, Larrea *et al.*, 2013). Most strains harboring the pRP100Δ*tra* and pKM101Δ*tra* variant plasmids were fully complemented by *trans*-expression of the corresponding *tra* genes from the P_{BAD} promoter. Strains carrying the pKM101Δ*traA*, Δ*traB*, or Δ*traD* mutant plasmids were only partially complemented, as evidenced by transfer frequencies of 3- to 4-orders of magnitude lower than those of the pKM101-carrying donor (Fig. S1). Regardless of possible negative effects accompanying nonstoichiometric production of certain Tra proteins on machine assembly or slight polar effects on downstream gene expression, results of the complementation studies established that 9 of the 11 Tra proteins are essential for elaboration of a functional Tra_{pKM101} T4SS.

virB1-like *traL* 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 for other T4SSs documenting attenuating effects of hydrolase gene deletions on substrate transfer (Berger & Christie, 1994, Bayer *et al.*, 1995, Zahrl *et al.*, 2005). *traL* expression from the strong P_{BAD} promoter in Δ*traL* mutant donors conferred an apparent 10- to 100-fold increase in transfer efficiencies (Fig. 1). These P_{BAD}::*traL* expressing cells, however, grew poorly and exhibited protrusions and enhanced vesicle production compared with cells expressing *traL* from the native promoter (data not shown), as observed previously for the P19 hydrolase associated with the plasmid R1-encoded conjugation systems (Bayer *et al.*, 2001). The apparent increase in mating frequency, recorded as the number of transconjugants per donor, is therefore likely due to a reduction in donor cell viability as a result of TraL overproduction.

The last *tra* gene, *virB5*-like *traC*, is postulated to encode a pilus tip protein on the basis of previous findings for VirB5 homologs associated with the $VirB/VirD4_{At}$ system and the *Helicobacter pylori* Cag T4SS (Aly & Baron, 2007, Shaffer *et al.*, 2011). Interestingly, Δ*traC* mutant donors retained the capacity to transfer DNA substrates at frequencies of $\sim 5x10^{-4}$ Tc's/D on solid surfaces, but were completely defective for plasmid transfer in liquid matings (Figs 1& S1). In the F plasmid transfer system, F pili dynamically extend and retract to initiate distant contacts and then formation of direct donor-recipient cell contacts in liquid matings, which is thought to account for the observed high-frequency transfer of F plasmids under these conditions (Clarke *et al.*, 2008). At this time, there is no evidence that the Tra_{pKM101} pilus dynamically extends and retracts, yet its production of the Tra_{pKM101} pilus might still promote donor-recipient contacts enabling pKM101 transfer in liquid. 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 Δ*tra* mutant strains, only the Δ*traL* mutant was dispensable for pilus production. The requirement of TraC for IKe infection and plasmid transfer in liquid, but not solid-surface, matings is consistent with a proposal that this putative pilus tip protein contributes specifically to pilus extension from the cell surface and not directly to elaboration of the translocation channel.

The outer membrane core complex (OMCC) cap also is dispensable for substrate transfer but required for pilus biogenesis. With a pKM101 molecular 'toolkit' in hand, we next sought to define the T4SS machine requirements for productive engagement with recipient cells. While conjugative pili clearly play a role in initiating donor-target cell interactions, mutations conferring a transfer-positive, pilus-minus (Tra^+, Pil^-) "uncoupling" phenotype, reminiscent of that observed for Δ*traC*, have been isolated in various subunits of the $VirB/VirD4_{At}$ (Sagulenko *et al.*, 2001, Jakubowski *et al.*, 2003, Jakubowski *et al.*, 2005) and *E. coli* RP4-encoded (Haase *et al.*, 1995) T4SSs. These observations suggest, first, that conjugative T4SSs can assemble alternatively as pilus-producing or substrate transfer machines and, second, these systems possess surface features other than extended pili that promote formation of productive mating junctions on solid surfaces.

To explore this latter proposal, we sought to define the functional importance of the OMCC cap, which to date is the only 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 Tra_{pKM101} 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 steady-state levels and conferred only a modest reduction in DNA transfer compared with donors producing native TraF (Figs. 2B,C). To evaluate the generality of these findings, we inserted duplicate (2X) or triplicate (3X) FLAG tags at several positions within the AP domain of *A. tumefaciens* VirB10 (Fig. S3A).

These insertions also 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 (Figs. S3B, C). The AP domains of TraF and VirB10 share only 26 % identity (Figs. 3A, S2), but interestingly reciprocal swaps of these domains yielded stable (Figs. 3C, S3C) 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 (Fig. S3B). We conclude that the OMCC caps of the Tra_{pKM101} and VirB/VirD4$_{At}$ T4SSs augment, but are not required for, substrate transfer to target cells.

Complete deletions of the APs from TraF and VirB10, however, abolished production of WT pili, as evidenced by lack of pKM101 transfer in liquid, resistance to IKe phage infection, and lack of surface display of the VirB2 pilin on *A. tumefaciens* cells (Figs. 2B & S3B) which serves as a convenient assay for T pilus production by the VirB/VirD4$_{At}$ T4SS (Sagulenko *et al.*, 2001, Kerr & Christie, 2010). Further mutational analyses indicated 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 FLAG insertion in this domain remained sensitive to infection by IKe (Fig. 3B). Similarly, *A. tumefaciens* strains producing VirB10 variants with FLAG insertions in the APL accumulated abundant amounts of VirB2 pilin on the cell surface indicative of T pilus production, whereas strains with FLAG insertions in VirB10's α2 or α3 helices had comparatively low levels of surface pilin (Fig. S3B). Also of note, reciprocal swaps of TraF's and VirB10's AP domains supported pilus production in *E. coli* and *A. tumefaciens* (Fig. 3B, S3B). Together, these findings suggest that elaboration of the extended pilus requires formation of an α-helical channel across the OM regardless of its sequence composition.

The C-terminal (CT) domain, but not the lever arm, is critical for TraF function. The extreme C termini of TraF and VirB10 are highly related (72 % identity) and both domains possess several residues, most notably an RDLDF motif, that are highly conserved among the VirB10 family members (Fig. 3A) (Jakubowski *et al.*, 2009). As shown by the Tra_{pKM101} O-layer structure, TraF's C-terminal (CT) domain forms a β-strand that extends along the β-barrel domain (see Figs. 2A, S2)(Chandran *et al.*, 2009). An AP-CT deletion mutant was undetectable and a ΔCT mutant accumulated at low levels suggesting that the CT domain contributes to TraF stability (Fig. 2C). However, within the CT domain, smaller deletions of a highly-conserved RDLDF motif or the C-terminal 9 residues (CT9) did not affect steady-state protein accumulation but completely eliminated function as monitored by substrate transfer and IKe phage infection (Fig. 2B, C). To further examine the functional importance of the CT domain, we exchanged VirB10$_{At}$'s AP and CT domains or just the CT domain for the corresponding domains of TraF. In *E. coli*, the respective TraF/AP-CT_{VirB10} and TraF/CT_{VirB10} chimeras supported substrate transfer on solid surfaces, but not transfer in liquid or IKe phage infection (Fig. 3B, data not shown). Interestingly, VirB10's CT domain extends 11 residues beyond that of TraF and when we deleted this extension, the resulting chimera, TraF/CTΔ11_{B10}, accumulated at abundant levels and supported substrate transfer and IKe phage infection (Figs. 3A, B). In *A. tumefaciens*, a VirB10 chimera bearing TraF's AP-CT (VirB10/AP-CT_{TraF}) (Fig. S3), or VirB10 deleted of its C-terminal 11 residues (data not shown), also supported WT levels of substrate transfer to plants but not T pilus production. Together, these findings suggest that the CT domains contribute to stabilization of the VirB10 proteins and also mediate intra- or intersubunit contacts necessary for channel formation and pilus production. VirB10's C-terminal 11 residues also are required for T pilus production by the VirB/VirD4$_{At}$ T4SS. This motif poisons pilus production by the Tra_{pKM101} system, although interestingly TraF bearing a FLAG tag at its C terminus exhibited WT function (Fig. 2B, C).

In the Tra_{pKM101} 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, resulting 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 β_n1 in the lever arm of an adjacent TraF monomer (Fig. 2, S2) (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 protein migrated in SDS-polyacrylamide gels as multiple, presumptive degradation products, suggestive of a contribution of the lever arm to TraF stability (Fig. S3). Strikingly, however, TraFΔlever-producing donors delivered DNA substrates to recipient cells nearly at WT levels and also exhibited IKe phage sensitivity (Fig. S3). Despite an apparent stabilizing effect by the lever arm on TraF, the lever arm shelf and its associated network of lateral contacts in the assembled OMCC do not impact the OMCC's contribution as a scaffold for assembly of the translocation channel and extended pilus.

TraF chimeras with substituted OM core complexes 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). TraF chimeras composed of VirB10's N-proximal cytoplasmic (Cyto) or transmembrane (TM) domains joined to the remaining portions of TraF accumulated at abundant levels but were nonfunctional (Figs. 3B, C). Chimeras bearing VirB10's PRR domain (TraF/PRR_{B10}) or the entire portion of VirB10 extending from the TM domain through the C terminus (TraF/TM-CT_{B10}) accumulated at low levels or moderate levels, respectively, but failed to support plasmid transfer or IKe phage uptake. These findings are in agreement with previous studies showing that the N-terminal regions of VirB10-like proteins form extensive interactions with cognate IMC components and VirD4 coupling proteins (see Discussion) (Das & Xie, 2000, Atmakuri *et al.*, 2004, Llosa *et al.*, 2003, Rivera-Calzada *et al.*, 2013).

Strikingly, however, chimeras consisting of TraF's N-terminal half joined to VirB10's β-barrel domain accumulated at low but detectable levels and also supported substrate transfer at frequencies of 10^{-5} - 10^{-6} Tc's/D (Fig. 3B). The functionality of these chimeras was particularly striking given that the TraF and VirB10 β-barrel domains share low sequence relatedness (19 % identity, Fig. S2) and results from the Tra_{pKM101} O-layer crystallography studies establishing that TraF's β-barrel forms extensive contacts with its OMCC partner subunit TraO (Fig. S2) (Chandran *et al.*, 2009).

We next tested whether the TraF/βB-CT_{VirB10} chimera would function more efficiently if the VirB7 and VirB9 subunits of the VirB/$VirD4_{At}$ OMCC also were substituted for their Tra_{pKM101} counterparts, essentially creating an IMC_{Tra}::$OMCC_{VirB}$ chimera (designated Tra::VirB). To ensure temporal and stoichiometric synthesis of the IMC and OMCC subassemblies, we substituted codon-optimized *virB7*, *virB9* and the *traF/βB-CT_{B10}* chimeric gene for *traN*, *traO*, and *traF* within the pKM101 *tra* region expressed from the mini-pKM101 plasmid pJG144. Plasmid pJG144 phenocopies WT pKM101 in conferring highly efficient transfer of the mobilizable plasmid pJG142 and pilus production, as monitored by substrate transfer in solid-surface and liquid matings and IKe phage susceptibility (Fig. 4A, B). The Tra::VirB chimera also supported transfer of the mobilizable plasmid, although at a frequency comparable to that observed by donors in which TraF/βB-CT_{VirB10} was substituted for native TraF (compare Figs. 3B & 4B). The Tra::VirB chimera did not support formation of the WT pilus, however, as evidenced by IKe phage resistance and lack of substrate transfer in liquid media.

To further explore the compositional flexibility of the OM core complex, we engineered two more chimeric T4SSs bearing heterologous OMCCs. The first was derived from the *E. coli* Trw_{R388} conjugation machine, a system closely related phylogenetically and functionally to that of Tra_{pKM101} (Llosa *et al.*, 2003). The second was from the Ptl_{Bp} T4SS, which has diverged considerably from ancestral conjugation systems both in primary sequence (Fig. S2) and through its adapted function as a PT export system (Locht *et al.*, 2011). Strikingly, both systems supported conjugative transfer of the pKM101 *oriT* plasmid in solid surface matings. Donors producing the Tra::Trw chimera transferred the pKM101 substrate at 10^{-3} Tc's/D, whereas donors producing the Tra::Ptl chimera transferred the plasmid at low frequencies of ~10^{-7} Tc's/D. Despite the low level of transfer of the Tra::Ptl chimera, our findings clearly establish the modular and interchangeable nature of OMCCs from diverse type IVa systems in supporting conjugative DNA transfer.

T4SS requirements for triggering of Type VI-mediated killing. We sought to define the contributions of surface-exposed structures of the T4SS to formation of donor-target cell contacts even in the absence of detectable substrate transfer or pilus production, and to this end we employed a T6SS killing assay. Previously, it was shown 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 conveying 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 DH5α cells lacking pKM101 exhibit comparable growth in the presence or absence of *P. aeruginosa* (Fig. 4C), as also shown previously (Ho *et al.*, 2013). However, DH5α pKM101) cells producing the Tra system exhibited a ~2-log reduction in colony forming units (CFUs) when incubated in the presence vs the absence of *P. aeruginosa* PAO-1 (Fig. 4C) or the presence of a $T6SS^-$ (*vipA*) mutant (data not shown). Strains competent for production only of the pKM101 Tra IMC or OMCC, respectively, were not killed in the presence of *P. aeruginosa*, confirming that both subassemblies of the Tra_{pKM101} T4SS are required for activation of the T6SS. Interestingly, *E. coli* strains engineered to produce the chimeric T4SSs (Tra::Trw, Tra::VirB, Tra::Ptl) also triggered T6SS killing at levels comparable to the native Tra T4SS (Fig. 4C). These chimeric systems thus phenocopy the intact Tra_{pKM101} T4SS in transducing an activating signal to *P. aeruginosa* target cells, despite exhibiting various degrees of attenuation in substrate transfer and deficiencies in detectable pilus production (Fig. 4B).

As expected from the above findings (Fig. 4) and our earlier phenotypic analyses (Fig. 1), the Δ*traL* mutant retained the capacity to activate the *P. aeruginosa* T6SS, whereas strains individually deleted of the remaining *tra* genes failed to trigger killing (Fig. S4). All of the complemented Δ*tra* mutants triggered T6SS killing (Fig. S4), confirming the importance of an intact T4SS for signal transmission to *P. aeruginosa*. To further evaluate the requirements for intercellular signaling, *E. coli* strains harboring TraF mutations in the AP or CT domains were analyzed for T6SS activation. Intriguingly, strains deleted of the AP (confers a Tra^+,Pil^- phenotype) and with CT mutations (Tra^-,Pil^-) triggered a T6SS killing response (Fig. 5A). These findings establish that the Tra_{pKM101} T4SS retains the capacity to transduce an intercellular potentiating signal even in the absence of DNA transfer, elaboration of the OMCC cap, or formation of the WT pilus.

Structural resolution of the *A. tumefaciens* VirB/VirD4 OM core complex and comparisons with the pKM101 OMC. 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 by negative-stain electron microscopy (see Fig. S5). This structure has dimensions of 180 Å in diameter and 155 Å in height, closely resembling the pKM101- and R388-encoded OMCCs (Fronzes *et al.*, 2009, Chandran *et al.*, 2009, Low *et al.*, 2014). The VirB OMCC also has 14-fold symmetry with openings at its proximal and distal ends of dimensions 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. Regardless, 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 functional interchangeability of the heterologous OMCCs for that of 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 & Walker, 1985, Paterson *et al.*, 1999), and here we capitalized on the construction of a pKM101 'molecular toolkit' to address a central question in the type IV secretion field of how these machines establish productive contacts with target cells. Among the systems functioning in Gram-negative species, conjugative pili initiate donor - recipient cell contacts either through dynamic extension - retraction or pilus sloughing or release mechanisms (Samuels *et al.*, 2000, Lawley *et al.*, 2003, Christie, 2004, Clarke *et al.*, 2008). Once pilus-mediated cell-cell contacts are established, stable mating junctions form thereby enabling efficient DNA transfer. Extended pili are not obligatory for conjugative DNA transfer, however, since Gram-positive bacterial conjugation machines function efficiently in their absence (Bhatty *et al.*, 2013) and various pilus-blocking mutations in Gram-negative systems have been shown to permit efficient substrate transfer on solid surfaces (Eisenbrandt *et al.*, 2000, Sagulenko *et al.*, 2001, Jakubowski *et al.*, 2003, Jakubowski *et al.*, 2005). These observations suggest that surface features of conjugation systems other than pili are capable of mediating formation of productive donor-recipient cell mating junctions. Here, guided in part by solved structures of T4SS subassemblies, we evaluated the contributions of the pKM101 Tra subunits and OMCC domains to pilus production, donor-target cell contacts, and intercellular substrate transfer. Intriguingly, we discovered that T4SSs lacking prominent surface features implicated in target cell binding, including the OM-spanning cap domain and the TraC pilus tip protein, display the Tra^+, Pil^- "uncoupling" phenotype. We also showed that heterologous OMCC subassemblies could be substituted for that of the Tra_{pKM101} system. Mechanistically, functionality of the chimeric T4SSs is remarkable, first, because swapped OMCCs were derived from T4SSs adapted over evolutionary time for distinct purposes of conveying substrates interbacterially or to eukaryotic cells through contact-dependent or -independent processes. Second, T4SSs are known to activate substrate transfer in response to transduction of intracellular and extracellular signals across the cell envelope, implying that the Tra_{pKM101} IMC not only physically interacts with the heterologous OMCCs, but also must transduce signals in the form of conformational changes required for channel activation.

We defined the functional importance of TraC and the other Tra subunits through a systematic *tra* deletion/complementation analyses using native pKM101 as well as mini-pKM101 plasmids that were more amenable to genetic manipulation (Figs. 1 & S1). These studies confirmed an early report of the dispensability of TraC for pKM101 transfer on solid surfaces (Winans & Walker, 1985) but further established the importance of TraC for transfer in liquid. Previous work has shown that conjugative transfer on solid surfaces does not require production of extended pili due to close packing of donor and recipients (Lessl *et al.*, 1993, Haase *et al.*, 1995, Samuels *et al.*, 2000), whereas by contrast pilus production is important for efficient transfer in liquid (Lawley *et al.*, 2003, Clarke *et al.*, 2008, Arutyunov & Frost, 2013). Coupled with the demonstrated importance of TraC for infection by IKe (Figs. 1 and S1)(Yeo *et al.*, 2003), which binds the tip of the pKM101 pilus (Bradley, 1979), our findings strongly indicate that TraC contributes specifically to pilus nucleation. Of further interest, Winans and Walker (1985) also supplied genetic evidence for surface exposure and even intercellular transfer of TraC. In a phenomenon termed extracellular complementation, a TraC-producing (but non-conjugative) 'helper' strain is capable of restoring mating proficiency of a Δ*traC* mutant when the two strains are mixed with a third, plasmid-free recipient strain. These findings prompted a proposal that the 'helper' strain delivers surface-localized TraC to the Δ*traC* mutant where it then associates with the Tra_{pKM101} T4SS to promote pilus assembly, mating pair formation, and substrate transfer. Studies have since confirmed that TraC is surface-localized even on cells lacking the Tra_{pKM101} T4SS (Schmidt-Eisenlohr *et al.*, 1999), although there is still no direct evidence for cell-to-cell transmission.

The selective importance of VirB5/TraC subunits for pilus production also is supported by the observation that the Ptl_{Bp} system lacks a VirB5 homolog and does not produce detectable pili, yet efficiently exports the multisubunit PT across the OM (Farizo *et al.,* 2000, Locht *et al.,* 2011). In some characterized systems, however, mutations of *virB5/traC*-like genes completely abolish substrate transfer (Berger & Christie, 1994, Fischer *et al.*, 2001, de Paz *et al.*, 2005, Larrea *et al.*, 2013). This could be explained by findings that VirB5-like subunits have functionally diversified during evolution to carry out additional activities of importance for intercellular transfer. In several species, for example, the VirB5-like subunits are implicated in specifying contacts with target cells. In *H. pylori*, VirB5-like CagL carries an RGD motif as well as other motifs that mediate binding to β integrin receptors on human host cells (Kwok *et al.*, 2007, Barden & 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 *H. pylori* strains (Olbermann *et al.*, 2010, Gorrell *et al.*, 2016). In *A. tumefaciens*, VirB5 also carries an RGD motif (Backert *et al.*, 2008), which similarly might contribute to establishment of productive contacts with susceptible plant cells in view of findings $VirB5_{At}$ overproduction or exogenous addition of purified VirB5 enhances *A. tumefaciens*-mediated T-DNA transfer efficiencies. Finally, in *Bartonella henselae*, variant forms of surface-located VirB5 subunits are thought to determine host-specificity of erythrocyte parasitism (Dehio, 2008). Evidence for localization of $CagL_{Hp}$ and $VirB5_{At}$ at the tips of pili produced by the respective T4SSs also is consistent with a dual role for the VirB5 subunits in pilus nucleation and target cell binding in some systems (Aly & Baron, 2007, Kwok *et al.*, 2007, Lacroix & Citovsky, 2011, Barden & Niemann, 2015).

The OM-spanning caps of the Tra_{pKM101} and $VirB/VirD4_{At}$ T4SSs were surprisingly permissive to mutation with respect to substrate transfer (Figs. 2, S3), 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 (see Fig. 7). In our earlier crosslinking studies, we showed that the $VirB2_{At}$ pilin forms formaldehyde (FA)-crosslinkable contacts with DNA substrates during their transit through the *A. tumefaciens* VirB/VirD4 T4SS (Cascales & Christie, 2004b). $VirB2_{At}$ - 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 (Jakubowski *et al.*, 2005, Cascales & Christie, 2004b). 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, in stoichiometric association with the $TraA_F$ 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 $TraA_F$ pilin - phospholipid complexes comprise the building blocks for polymerization of the F pilus from an inner membrane platform (Costa *et al.*, 2016). Finally, some evidence has been presented for the capacity of extended F pili to mediate substrate transfer in the apparent absence of direct donor-recipient cell contacts (Babic *et al.*, 2008, Harrington & Rogerson, 1990). Such transfer events are rare, as suggested by a wealth of data for this and other systems that efficient conjugative transfer requires formation of direct cell-cell contacts and stable mating junctions (Samuels *et al.*, 2000, Lawley *et al.*, 2002, Arutyunov & 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.

The OMCC cap was essential for detection of surface pili in both the Tra_{pKM101} and $VirB/VirD4_{At}$ systems (Figs. 2, S3). In *A. tumefaciens*, we previously determined that a VirB10ΔAP mutation confers low levels of surface-exposed VirB2 pilin and striking defects in pilus polymerization. In that study, the AP boundaries (residues 308-337) were assigned on the basis of a crystal structure of VirB10-like ComB10 associated with a *H. pylori* competence system (Terradot *et al.*, 2005, Jakubowski *et al.*, 2009). With the availability of the entire O-layer of the Tra_{pKM101} OMCC X-ray structure, AP boundaries of both TraF and VirB10 were reassigned so that the latter spans residues 288-339 (Chandran *et al.*, 2009). Our present mutational analyses confirmed that the VirB10Δ288-339 variant, and the corresponding $TraF_{pKM101}$ mutant, conferred strong blocks in delivery of pilin subunits or extension of the pilus to the cell surface. We also have shown that VirB10 homologs possess an invariant Gly residue that in the Tra_{pKM101} O-layer crystal structure is positioned in the interior chamber of the OMCC near the OM-spanning cap. Interestingly, substitution of Arg for this residue (Gly272) in $VirB10_{At}$ completely blocks production of T pili without affecting substrate delivery to plant cells, reinforcing the notion that the distal end of the OMCC plays a critical role in regulating extension of pili from the cell surface (Banta *et al.*, 2011).

To reconcile our findings, we propose a working model that is consistent with previous models (Christie *et al.*, 2005, Trokter *et al.*, 2014), in which the default pathway for conjugation systems in the absence of any target cell interactions is the production of extended pili (see Fig. 7). These pili function in a 'mate-seeking' mode 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 direct donor - recipient cell contacts, the T4SS ceases production of extended pili and transitions to the 'mating' mode presumably through translocation of substrates through a short, envelope-spanning pilus structure. Various signals regulate this morphogenetic switch, including recipient contact-propagated signals of an undefined nature (Frost & Koraimann, 2010, Arutyunov & Frost, 2013) and activating signals within the donor cell including i) productive binding of VirD4-like substrate receptors with the IMC (de la Cruz *et al.*, 2010, Lang *et al.*, 2011), ii) docking of DNA substrates with the channel ATPases VirD4 and VirB11 (Cascales *et al.*, 2013, Lang & Zechner, 2012), and iii) ATP hydrolysis activities of the VirD4 and VirB11 ATPases (Cascales & Christie, 2004a). In the framework of this model, our present findings point to the importance of the TraC/VirB5-like pilus tip protein and the distal end of the OMCC, specifically for the 'mate-seeking' mode of pilus extension. A central prediction of our findings, currently under investigation, is that the exported form of TraC is recruited to the OMCC cap where it interacts both with the cap and the tip of the pilus to nucleate extension from the cell surface. Contact-mediated extracellular and intracellular signals induce pilus retraction or sloughing and block further rounds of TraC-driven pilus extension, thereby transitioning the T4SS to the 'mating' mode. It is important to note that TraC/VirB5-like subunits have been shown to interact with IMC components (Yuan *et al.*, 2005, Villamil Giraldo *et al.*, 2012), raising the possibility that the pilus tip proteins are recruited to the T4SS from a periplasmic location. Nevertheless, a central feature of our model is that that VirB5-like subunits engage with the distal end of the OMCC to drive pilus extension from the cell surface.

Our studies also supplied important insights into the OMCC domain requirements for T4SS function. For example, TraF's CT domain (residues 355-386) appears to contribute to protein stability, whereas the conserved RDLDF motif within this domain is critical for function (Fig. 2B). We had envisioned that the CT domain contacts with the lever arm of the adjacent TraF monomer that were identified in the Tra_{pKM101} O-layer structure (Fig. S2C) (Chandran *et al.*, 2009) contributed to OMCC assembly or stability. Although the TraFΔlever mutant protein exhibited some degradation, it nevertheless supported efficient substrate transfer and pilus production (Fig. S4). Thus, while the lever arm platform and its CT domain contacts might stabilize the OMCC scaffold, mutant scaffolds devoid of these stabilizing contacts still assemble with sufficient structural integrity to support elaboration of the extended pilus and a fully functional translocation channel.

We also gained evidence for conformational flexibility of the OMCC through TraF β-barrel domain swaps. The functionality of the $TraF/\beta B\text{-}CT_{VirB10}$ chimera (Fig. 3), and of equivalent chimeras composed of the β-barrel domains from the $TrwE_{R388}$ and $PtlG_{Bp}$ homologs (Fig. 5 & data not shown), was particularly surprising in view of the low overall sequence relatedness of the VirB10 homologs (Fig. S2) coupled with findings that TraF's β-barrel forms a large number of contacts with VirB9-like TraO in the Tra_{pKM101} O-layer crystal structure (Fig. S2C) (Chandran *et al.*, 2009). Only a few of the residues that form the TraF-TraO subunit interfaces are conserved among the β-barrel domains of the TraF homologs (Fig. S2C), minimally suggesting that TraF's network of intra- and intersubunit contacts do not structurally lock the Tra_{pKM101} OMCC. The notion that the OMCC is intrinsically flexible also agrees with previous work showing that $VirB10_{At}$ undergoes a conformational change in response to sensing of substrate docking and ATP hydrolysis signals to regulate substrate passage through the distal portion of the VirB/VirD4 channel (Cascales & Christie, 2004a, Cascales *et al.*, 2013).

The functional interchangeability of the entire Tra_{pKM101} OMCC with corresponding subassemblies from the $VirB/VirD4_{At}$, Trw_{R388} and Ptl_{Bp} systems (Fig. 4) further underscores the intrinsic flexibility of the entire T4SS in supporting assembly of the translocation channel. At one level, the observed architectural similarities of the OMCCs solved to date provide a structural basis for understanding how heterologous OMCCs were able to substitute for the Tra_{pKM101} subassembly (Fig. 6). Moreover, given the widespread 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 & 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 both to build the translocation channel and to regulate its dynamic activity. 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 the channel (Hapfelmeier *et al.*, 2000, Das & 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 implicated in transduction of the aforementioned intracellular (substrate docking/ATP energy) and extracellular (target cell binding) signals necessary for transitioning the T4SS to the 'mating' mode (Llosa *et al.*, 2003, Atmakuri *et al.*, 2004, de Paz *et al.*, 2005, Cascales & Christie, 2004a, Mihajlovic *et al.*, 2009, Lang *et al.*, 2011, Cascales *et al.*, 2013, Arutyunov & Frost, 2013). Although our use of TraF chimeras clearly facilitated productive coupling between pKM101's IMC and the heterologous OMCCs, it is reasonable to predict that functionality of the chimeric systems also required establishment of other IMC-OMCC contacts as well as signal-activated conformational changes. Further studies of these or other heterologous T4SSs should reveal underlying mechanistic and structural relationships between these two subassemblies.

Finally, although the OMCC cap or the extended pilus potentially mediate productive binding of donors with target cells, the Tra^+ phenotypes of the chimeric T4SSs (Fig. 4) and of various Tra_{pKM101} mutant T4SSs, including Δcap and Pil^- variants (Figs. 2 & 3), argues against a requirement for either structure for donor – recipient contacts. We further evaluated the contribution of the OMCC to formation of donor-target cell interactions, even in the absence of DNA transfer, by capitalizing on the discovery that *E. coli* cells harboring conjugation machines propagate a signal to the *P. aeruginosa* cell envelope that activates a T6SS killing response (Ho *et al.*, 2013). Strikingly, each of the chimeric T4SSs efficiently triggered *P. aeruoginosa* T6SS killing, which was most surprising for the Tra::Ptl system whose OMCC in the native Ptl system neither supports pilus biogenesis nor direct binding of *B. pertussis* with eukaryotic target cells (Fig. 5) (Burns, 2003, Locht *et al.*, 2011). Our further analyses of the killing potential of mutant strains sustaining Δ*tra* mutations further confirmed that substrate transfer is not necessary for T6SS activation, as was also previously shown for the RP4 conjugation system (Ho *et al.*, 2013). Furthermore, a cap deletion mutant (Tra^+, Pil^- phenotype) or CT domain mutations (Tra^-,Pil^-) confirmed that neither the extended pilus nor the OMCC cap was essential for triggering the killing response. Taken together, these findings suggest that features other than the OMCC cap or pilus can mediate formation of intercellular mating junctions. We envision that such surface features might include i) surface-exposed motif of the OMCC that forms only transiently in response to target cell sensing ii) another surface-exposed protein that is not encoded by the *tra* operon but physically or functionally interacts with the OMCC, or iii) short, surface-exposed pilus structures that were not detectable by our available assays. Our future work will continue to employ *P. aeruginosa* T6SS killing as a surrogate assay to further define T4SS surface features required for the initiation of donor-recipient cell contacts and formation of productive mating junctions.

EXPERIMENTAL PROCEDURES

Strains and growth conditions. *E. coli* DH5α (GIBCO-BRL) was used for plasmid constructions and the type VI secretion system (T6SS) killing assay. *E. coli* MG1655 (*E. coli* Genetic Stock Center) was used for conjugation and phage infection assays. *E. coli* HME45 (Thomason *et al.*, 2014) was used for construction of *tra* gene deletions from native pKM101. *Pseudomonas aeruginosa* PAO1(Holloway, 1955) containing an IS*phoA* insertion in the *retS* locus was used for the T6SS killing assay (*Pseudomonas* Transposon Mutant Collection, University of Washington Genome Sciences). *E. coli* strains were grown in Luria Broth (LB) at 37°C with shaking. *E. coli* strains were cultured in the following antibiotics: carbenicillin (50μg ml^{-1}), kanamycin (50μg ml^{-1}), spectinomycin (100μg ml^{-1}), chloramphenicol (20μg ml^{-1}), tetracycline (20μg ml^{-1}), and rifampicin (50μg ml^{-1}). *P. aeruginosa* PAO1 was grown in LB without antibiotic selection.

Plasmid constructions. Plasmids and oligonucleotides used in these studies are listed in Tables S1 and S2, respectively.
Vectors. pBAD24Spc was created by isolation of the *spc*^{r} gene as a SmaI fragment from pHP45Ω and inserting it into the ScaI site within the *crb*^{r} gene on pBAD24. pKM101Spc^r was constructed by introduction of the same *spc*^{r} gene as an EcoRI fragment into the unique EcoRI site within the *crb*^{r} gene of pKM101.

pKM101Δ*tra* mutant plasmids. Eight of the 11 *tra* genes were deleted from pKM101 by recombineering (Thomason *et al.*, 2014). Briefly, pKM101 or pKM101Spc^r were transferred by conjugation into *E. coli* strain HME45, which contains the bacteriophage λ *red* system under the control of the cI857 repressor. For construction of each *tra* gene deletion, the *kan*^{r} *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 *tra* gene of interest. HME45(pKM101Crb^r) or HME45(pKM101Spc^r) cells were temperature-induced for expression of the *red-gam* genes, and the *kan*^{r} amplicons were introduced by electroporation with Kan^r selection for transformants. Because pKM101 is a multicopy plasmid, we eliminated plasmids lacking the integrated *kan*^{r} cassette by subculturing the Kan^r transformants for 4 days in LB broth containing kanamycin (200 μg ml^{-1}). Isolated plasmids were digested with NcoI and religated to delete the *kan*^{r} cassette, and ligation mixes were introduced into DH5α with selection for Crb^r or Spc^r. Transformants were screened for Kan sensitivity, and *tra* 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 *tra* gene cluster. pCGR108 was generated by introduction of the *tra* region from pKM101 into pBAD24. We amplified a ~10-kilobase region of pKM101 encompassing the upstream regulatory region and *tra* 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 NcoI and XbaI restriction sites. The second mini-pKM101 plasmid, pRP100, was constructed by joining three PCR products: i) the *tra* 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 Kan^r. The ~10-kb *tra* gene cluster was amplified from pKM101 with primers pKM101_1921Nco1_F and pKM101_13500Xba_R, 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 Kan^r as a selection for self-replicating pRP100. 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 Tra T4SS (see Results).

pRP100Δ*tra* and pCGR108Δ*tra* variants. We precisely deleted each of the *tra* genes from pRP100 by inverse PCR using the 5'phosphorylated primers listed in 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 *traI* genes. These genes code for the *oriT* processing proteins, relaxase TraI and accessory factor TraK, and the coupling protein TraJ. A PCR fragment spanning the *oriT-traI* region was generated with primers oriT_NcoI_F and TraI_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 *traL* through *traG* genes, respectively, from the P_{BAD} promoter. Each *tra* gene was PCR amplified using primers listed in 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 *his*$_6$-tagged *traF*, respectively, from the P_{BAD} promoter on pBAD24Spc. 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/SalI-digested pBAD24Spc. Plasmid pJG103 expresses P_{BAD}::*traF-CT_FLAG*, producing C-terminally FLAG-tagged TraF. It was constructed by amplifying *traF* using primers TraF_FWD_NcoI and TraF_FLAG_CT_RVS_XhoI with pKM101 as a template. The resulting PCR fragment was digested with NcoI and XhoI and introduced into a NcoI/SalI digested pBAD24Spc

traF mutant plasmids. The following plasmids expressing *traF* mutant alleles from the P_{BAD} promoter were constructed by PCR amplification of gene fragments of interest using primers listed in Table S2 and pKM101 as a template, digestion of the final products

with NcoI and XhoI, and introduction of the digested fragments into NcoI/SalI-digested pBAD24Spc. Plasmids: pJG95 produces TraFΔAP-CT from P_{BAD}::*traF1-301* (numbers correspond to *traF* codons); pJG96 produces TraFΔCT from P_{BAD}::*traF1-353*; pJG76 produces TraFΔAP from P_{BAD}::*traFΔ307-354* (*traF1-307* and *traF355-386* were amplified and joined by overlapping PCR, and cloned as above); pJG61 produces TraFAPL-5xGly from P_{BAD}::*traFAPL-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 His_6-TraF-FLAG330 from P_{BAD}::*his$_6$-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); JG101 produces TraFΔRDLDF from P_{BAD}::*traFΔ373-377*; pJG97 produces TraFΔCT9 from P_{BAD}::*traFΔCT9*. pJG92 produces TraFΔlever from pBAD::*traFΔlever* (*traF1-170* and *traF200-386* were amplified, and joined by overlapping PCR).

<u>*traF/virB10* chimera plasmids</u>. The following plasmids expressing *traF*/*virB10* chimeric genes were constructed by PCR amplification of gene fragments of interest using primers listed in Table S2 and pKM101 or *traF* fragments or pKVD10 for *virB10* fragments. The amplification products (listed in parantheses) were joined by overlapping PCR, and the resulting fragments were digested with NcoI and XhoI for introduction into NcoI/SalI digested pBAD24Spc. Plasmids: pJG68 produces TraF/NT_{VirB10} from P_{BAD}::*traF/NT$_{virB10}$* (*virB10.1-29* and *traF40-386*); pJG69 produces TraF/TMD_{VirB10} from P_{BAD}::*traF/TM$_{virB10}$* (*traF1-40, virB10.29-50, traF60-386*); pJG2005 produces TraF/TMD-CT_{VirB10} from P_{BAD}::*traF/TM-CT$_{virB10}$* (*traF1-40, virB10.29-377*); pJG70 produces TraF/PRR_{VirB10} from P_{BAD}::*traF/PRR$_{virB10}$* (*traF.1-60, virB10.51-172, traF194-386*); pJG150 produces TraF/βB_{VirB10} from P_{BAD}::*traF/βB$_{virB10}$* (*traF1-193, virB10.173-286, traF307-386*); pJG134 produces TraF/βB-AP_{VirB10} from P_{BAD}::*traF/βB-AP$_{virB10}$* (*traF1-193* and *virB10.173-335, traF356-386*); pJG57 produces TraF/AP-CT_{VirB10} from P_{BAD}::*traF/AP-CT$_{virB10}$* (*traF1-307, virB10.286-377*); pJG58 produces TraF/βB-AP-CT_{VirB10} from P_{BAD}::*traF/βB-CT$_{virB10}$* (*traF1-193, virB10.173-377*). pJG60 produces TraF/AP_{VirB10} from P_{BAD}::*traF/AP$_{virB10}$* (*traF1-307, virB10.286-335, traF356-386*); pJG65 produces TraF/APL_{VirB10} from P_{BAD}::*traF/APL$_{virB10}$* (*traF1-322, virB10.301-325, traF364-386*); pJG66 produces TraF/CT_{VirB10} from P_{BAD}::*traF/ CT$_{virB10}$* (*traF1-354, virB10.335-377*); pJG201 produces TraF/ CTΔ11_{VirB10} from P_{BAD}::*traF/* CTΔ11_{virB10} (*traF1-354, virB10.335-366.*

<u>Chimeric *tra* operons.</u> Plasmid pJG145 expresses the chimeric gene cluster *tra*::*trw*. A DNA fragment encoding *trwH-traE-trwF-traF/βB-CT$_{trwE}$* was generated by overlapping PCR (*trwH, traE, trwF, traF1-193, trwE197-395*) using primers listed in Table S2 and 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-traF/βB-CT$_{virB10}$* was designed with codon-optimization for expression in *E. coli*, and synthesized by Genewiz Inc. The DNA fragment was isolated from the pUC57-Amp vector by digestion with SacI and XhoI and introduced into similarly-digested pJG143. Plasmid pJG203 expresses the chimeric gene cluster *tra*::*ptl*. A DNA fragment encoding *ptlI-traE-ptlF-traF1-172/βB-CT$_{ptlG}$* (*ptlG160-374*) was designed with codon-optimization for expression in *E. coli* (Genewiz Inc) and introduced into pJG143 as described above.

<u>Mini-pKM101 plasmids with *traF* variants.</u> Plasmid pCGR125 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 Table S2 and pCGR125 as a template. pCGR125 derivatives expressing different *traF* alleles were constructed by introduction of PCR fragments generated with primers and templates listed in Table S2 into SacI/XhoI-digested pCG125. Plasmids: pJG152 produces N-terminally FLAG-tagged TraF; pJG1 produces FLAG-TraFΔAP (deleted of codons 307-354); pJG154 produces FLAG_TraFΔRDLDF (deleted of codons 373-377); pJG153 produces FLAG-TraFΔCT9 (deleted of 9 codons at the 3' end); pJG155 produces FLAG-TraF/AP-CT_{VirB10} (*traF1-307, virB10.286-377*); pJG157 produces FLAG-TraF/AP-CT_{TrwE} (*traF1-307/trwE303-395*); pJG156 produces FLAG-TraF/AP-CT_{PtlG} (*traF1-307/ptlG294-374*).

<u>*A. tumefaciens virB10* expression plasmids.</u> We incorporated a Strep-tag (St) sequence at the 3' end of *virB10* on plasmid pTiA6NC of strain A348(Garfinkel *et al.*, 1981). *virB10-St* was amplified with 500 basepairs (bps) of 5' and 3' flanking sequences using overlapping PCR and primers listed in Table S2. We then cloned this fragment into pBB50 for introduction into the Δ*virB10* derivative PC1010 by marker-exchange eviction mutagenesis, as previously described (Berger & Christie, 1994). The resulting strain, A348*virB10-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 pKVD10 produces native VirB10 from P_{lac}::*virB10* and fully complements the Δ*virB10* 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 Table S2 and pKVD10 as a template. We then PCR amplified 2xFLAG or 3xFLAG tag sequences, each with flanking SphI sites, using primers listed in Table S2 and pSJ503 as a template. Plasmid pSJ503 contains a 3xFLAG tag and was constructed by annealing oligonucleotides listed in Table S2, digesting the product with NcoI and BamHI, and inserting the digested fragment into similarly-digested pBSIISK$^+$.NdeI. We then digested the amplified 2x or 3x FLAG tag sequences with SphI for insertion at codons 298, 329, and 332, creating plasmids pJG40, pJG42, and pJG33, respectively. Plasmid pJG52 producing VirB10 with a 2xFLAG tag inserted at codon 310 was constructed by two-step overlapping PCR using primers listed in Table S2 and pKVD10 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 Table S2 and pKVD10 as a template.

Plasmid pSJ504 producing VirB10ΔAP (deleted of residues 288 - 337) was constructed by inverse PCR using primers listed in Table S2 and pKVD10 as a template. Plasmids pSJ501 and pSJ502 producing VirB10/AP_{TraF} and VirB10/AP-CT_{TraF}, respectively, were constructed by overlapping PCR using the primers listed in Table S2 and pKVD10 or pKM101 as templates. Following amplification, the *virB10/AP$_{traF}$* and *virB10/AP-CT$_{traF}$* products were digested with NdeI-XhoI for insertion into pBSIISK+.NdeI.

All ColE1 plasmids expressing the *virB10* alleles were ligated to broad-host-range plasmid pXZ151 for introduction into *A. tumefaciens* (Berger & 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_{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. PRD1 bacteriophage was propagated as described previously for R17 (Lang *et al.*, 2011). Strains carrying plasmids of interest were grown and assayed for susceptibility to PRD1 infection as previously described with slight modifications (Cellini *et al.*, 1997). Briefly, cells 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 the bacteriophages PRD1 (10^6 pfu, final concentration) were 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, 2ml of *E. coli* DH5α donors and a *P. aeruginosa* PAO1*retS* were incubated overnight with shaking at 37°C, then resuspended in 2ml of antibiotic-free LB followed by a 1:100 dilution in 5ml 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* (3µl) 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 (300µg/ml) and rifampicin (100µg/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 His_6- or FLAG-tagged TraF variants of interest in LB media, harvested, and normalized to equivalent optical densities (OD_{600}). Total protein extracts were subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes, and blots were developed with α-His or α-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 & Christie, 2010).

***A. tumefaciens* outer-membrane complex expression and purification.** *A. tumefaciens* strain A348*virB10-St* was inoculated in 100ml of MG/L media supplemented with 100ug/ml of Kanamycin. After overnight incubation at 26°C, 10 ml of culture pellet was inoculated into 200ml of fresh MG/L media and incubated with shaking to an OD_{600} of 0.5-0.8. The culture was further harvested by centrifugation and re-suspended in 6L of ABIM media (supplemented with 100mM of acetosyringone for *vir* genes expression) to an OD_{600} of 0.1-0.2. After 12-14h of incubation at 23°C, the cultures were harvested by centrifugation and re-suspended in cooled 50mM Tris-HCl pH 8.0, treated with DNase I, lysosyme and EDTA-free protease inhibitor tablets, and sonicated on ice. After cell disruption, 1mM EDTA was added and the lysate was clarified by centrifugation at 38000*xg* for 20min. The membrane fraction was then collected by centrifugation at 98000*xg* for 45 min and membrane pellets were mechanically homogenized and solubilized in 50mM of Tris-HCl pH 8.0, 200mM NaCl, 1mM EDTA, 0.5% w/v Digitonin (Sigma), 0.75% w/v DM-NPG (Anatrace), 0.5% w/v DDM (Anatrace) and 1mM DTT for 1h at 4°C. The suspension was clarified by centrifugation at 98000*xg* for 20min and the supernatant was loaded onto a 5ml Strep Trap HP (GE Healthcare) column and washed with 50mM of Tris-HCl pH 8.0, 200mM NaCl, 1mM EDTA, 0.1% w/v Digitonin, 0.05% w/v DM-NPG and 1mM DTT at 4°C. The outer-membrane complex was eluted with the equivalent wash buffer supplemented with 2.5mM of desthiobiotin. The single peak fractions were pooled and loaded onto a Superose 6 10/300 column (GE Healthcare) equilibrated with the same buffer without desthiobiotin. 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.* 4 µl of the OM core complex diluted to 0.01mg/ml 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. The data were collected on a F20 microscope (FEI) operating at 200kV at a magnification of 45,500*xg* using a low dose mode (~ 30 e $Å^2$) 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.

Preprocessing. The contrast transfer function (CTF) of the microscope was estimated using CTFFIND3 (Mindell & Grigorieff, 2003) and the CTF correction of entire images was done by phase flipping using Bsoft (Heymann & Belnap, 2007). Particle images were picked from the CTF corrected micographs. A total of 1746 particles were manually selected and extracted with a box size of 240x240 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, centred, subjected to reference-free multi-statistical analysis (MSA), and then subjected to the iterative procedure that includes multi-reference-alignment (MRA), MSA and classification of particle images (van Heel *et al.*, 2000). After each round the best 10 classes representing characteristic views were further used as new references. This refinement procedure was considered complete when changes in image shifts were no longer observed. A subset of the best 96 representative class averages (from 300) were assigned Euler angles by angular reconstitution (Van Heel, 1987) using the structure of the pKM101 core complex (EMDB – 5031) as a starting model. An initial three-dimensional reconstruction of the OM core complex was then generated using the best classes with the lowest error evaluation for angular orientation; this initial map was further improved using anchor set refinement together with rounds of MRA and classification to produce the final three-dimensional map.

FIGURE LEGENDS

FIG. 1. *E. coli* pKM101 *tra* gene deletion and complementation analyses. The *E. coli* pKM101 *tra* and *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 *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*tra* mutations (histogram, upper bars) and results of complementation studies (histogram, lower bars) in which corresponding genes were *trans*-expressed from the P_{BAD} promoter (black arrow) on conjugative transfer. Matings (2 h) were carried 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). Transfer frequencies are presented as transconjugants/donor (Tc's/D). IKe phage sensitivity (S, sensitive, R, resistant) for Δ*tra* mutants and complemented strains is shown at the right.

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)** Steady-state 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.

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 IKe infection. **C)** Steady-state 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.

FIG. 4. Chimeric T4SSs support conjugative DNA transfer and activate T6SS killing. **A)** Sequences encoding the outer membrane core complex (OMCC) subunits TraN, TraO, and the C-terminal half (residues 194-386) of TraF were replaced with corresponding genes or gene fragments from the *A. tumefaciens* VirB, *E. coli* R388 Trw, or *B. pertussis* Ptl systems on mini-pKM101 plasmid pJG144. The chimeric T4SSs composed of the inner membrane complex (IMC) of pKM101 (yellow) joined to the OMCCs from the VirB, Trw, or Ptl systems (color-coded) are modeled on the R388 $T4SS_{3\text{-}10}$ structure (Low *et al.*, 2014). **B)** *E. coli* donors producing the IMC::OMCC chimeras transferred a pKM101 substrate (pJG142) at the frequencies shown in transconjugants per donor (Tc's/D) in solid-surface (histogram, solid bars) or liquid (stippled bars) matings, and were resistant to IKe phage infection (S, sensitive; R, resistant). **C)** *E. coli* survival when cultivated in the absence or presence of *P. aeruginosa* PAO1. *E. coli* DH5α cells lacked or produced intact or variant forms of the Tra_{pKM101} T4SS depicted. Statistical significance is shown based on a Student's *t* test corresponding to the values of plasmid-free DH5α or growth in the absence of *P. aeruginosa* (NS, not significant; $*P < 0.05$; $**P < 0.01$). For panels B and C, data presented are mean +/- SD, $n = 3$ independent replicates.

FIG. 5. Requirements for activation of T6SS killing by *P. aeruginosa* PAO1. *E. coli* DH5α lacking or producing the Tra_{pKM101} T4SS composed of the His_6-TraF variants shown; in each case, the *traF* allele was substituted for wild-type *traF* by incorporation into the pKM101 *tra* locus on plasmid pCGR108. Statistical significance is shown based on a Student's *t* test corresponding to the values of plasmid-free DH5α or growth in the absence of *P. aeruginosa* (NS, not significant; $*P < 0.05$; $**P < 0.01$). Data presented are mean +/- SD, $n = 3$ independent replicates. Lower panel: Steady-state levels of His-TraF variants in total cell extracts, as monitored by immunostaining with α-His antibodies. RNA polymerase β-subunit (α-RNAP) served as a loading control.

FIG. 6. Negative-stain EM structure of the *A. tumefaciens* outer-membrane core complex (OMCC) and comparison with the NS-EM structure of the OMCC (EMDB-5032) encoded by *E.coli* pKM101. **A)** *A. tumefaciens* OMCC side view (left) and cut-away side view (right). **B)** *E.coli* pKM101 OMCC side view (left) and cut-away side view (right). **C)** Representation of the cut-away side view of the overlay of *A. tumefaciens* and *E.coli* pKM101 OMCC's. **D)** Cross-section of overlaid *A. tumefaciens* and *E.coli* pKM101 OMCC complexes. Dashed line S in panel C indicates the level of the cross section shown in panel D.

FIG. 7. Working model for biogenesis of Type IVa secretion systems highlighting the importance of the postulated OMCC checkpoint in regulating pilus extension. Steps in the assembly pathway of the T4SS include **(A)** formation of the stable $T4SS_{3\text{-}10}$ substructure (Chandran *et al.*, 2009) and **(B)** elaboration of a short pilus that extends from an inner membrane platform to the cell surface by a mechanism requiring TraB/VirB4- and TraG/VirB11-type ATPases. Next, **(C)** the pilus extends from the cell surface in a mate-seeking mode by a mechanism activated by recruitment of surface-exposed TraC to the distal end of the OMCC (denoted by yellow lightning bolt). TraC alternatively might be recruited to the T4SS via a periplasmic location (red-dashed line, ?). Finally, **(D)** upon pilus-mediated

or direct contact with a recipient cell, a mating signal is transduced across the donor cell envelope resulting in recruitment of the TraJ/VirD4 substrate receptor, substrate docking and ATPase hydrolysis. These signals (denoted by lightning bolts) activate the morphogenetic switch to the T4SS 'mating' mode. The assembly intermediate depicted in **(B)** may bypass the pilus assembly (mate-seeking) mode **(C)** if presented with signals, e.g., recipient cell contact, required for activation of the substrate transfer (mating) mode **(D),** as could occur when donors and recipients grow in dense biofilm (solid-surface) communities. Abbreviations: OM, outer membrane; IM, inner membrane; P, peptidoglycan; OMCC, outer membrane complex; IMC, inner membrane complex; GSP, general secretory pathway. The pKM101 Tra proteins and their VirB counterparts required for each step of the assembly pathway are denoted.

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