Structural Analysis and Inhibition of TraE from the pKM101 Type IV Secretion System*  

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Gram-negative bacteria use type IV secretion systems (T4SSs) for a variety of macromolecular transport processes that include the exchange of genetic material. The pKM101 plasmid encodes a T4SS similar to the well-studied model systems from Agrobacterium tumefaciens and Brucella suis. Here, we studied the structure and function of TraE, a homolog of VirB8 that is an essential component of all T4SSs. Analysis by X-ray crystallography revealed a structure that is similar to other VirB8 homologs but displayed an altered dimerization interface. The dimerization interface observed in the X-ray structure was corroborated using the bacterial two-hybrid assay, biochemical characterization of the purified protein, and in vivo complementation, demonstrating that there are different modes of dimerization among VirB8 homologs. Analysis of interactions using the bacterial two-hybrid and cross-linking assays showed that TraE and its homologs from Agrobacterium, Brucella, and Helicobacter pylori form heterodimers. They also interact with heterologous VirB10 proteins, indicating a significant degree of plasticity in the protein-protein interactions of VirB8-like proteins. To further assess common features of VirB8-like proteins, we tested a series of small molecules derived from inhibitors of TraE and its homologs from Agrobacterium tumefaciens; 11 of these proteins (TraL to TraG) are encoded in an operon similar to the VirB1–11 operon in Agrobacterium. Similar to other secretion systems of this class, pKM101 encodes homologs of putative ATPases (VirB4, VirB11, and VirD4), of core secretion system components (VirB1, VirB3, VirB6, VirB7, VirB8, VirB9, and VirB10), and of pilus components (VirB2 and VirB5). Whereas the biochemistry and the functions of individual T4SS components of the Agrobacterium and Brucella systems have been studied more thoroughly, structure biological approaches using co-expression of a subset of pKM101 core components (TraN, TraE, TraF, and TraO, homologs of VirB7 to VirB10) led to the first high resolution structure of the T4SS core complex using cryo-electron microscopy and X-ray crystallography (9–11). Surprisingly, co-expression of the VirB8 homolog TraE was not necessary for the formation of the TraN-Traf-TraO complex under these conditions, but VirB8 homologs are essential for the function of T4SSs, and they are thought to be assembly factors (12).

VirB8 homologs are small periplasmic proteins of about 25 kDa comprising a short N-terminal cytoplasmic region, one transmembrane helix, and a periplasmic region of 18 kDa. They are encoded in an operon similar to the VirB1–11 operon in Agrobacterium. Similar to other secretion systems of this class, pKM101 encodes homologs of putative ATPases (VirB4, VirB11, and VirD4), of core secretion system components (VirB1, VirB3, VirB6, VirB7, VirB8, VirB9, and VirB10), and of pilus components (VirB2 and VirB5). Whereas the biochemistry and the functions of individual T4SS components of the Agrobacterium and Brucella systems have been studied more thoroughly, structure biological approaches using co-expression of a subset of pKM101 core components (TraN, TraE, TraF, and TraO, homologs of VirB7 to VirB10) led to the first high resolution structure of the T4SS core complex using cryo-electron microscopy and X-ray crystallography (9–11). Surprisingly, co-expression of the VirB8 homolog TraE was not necessary for the formation of the TraN-Traf-TraO complex under these conditions, but VirB8 homologs are essential for the function of T4SSs, and they are thought to be assembly factors (12).

VirB8 homologs are small periplasmic proteins of about 25 kDa comprising a short N-terminal cytoplasmic region, one transmembrane helix, and a periplasmic region of 18 kDa. They are essential for all T4SSs in which they have been studied, and VirB8 was shown to be present in a helical arrangement around the cell in A. tumefaciens (13, 14). The results of extensive
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geneic and biochemical analyses suggest that VirB8-like proteins are assembly factors that undergo a series of mostly transient interactions with other T4SS components (15–21). The X-ray structures of the periplasmic domains of VirB8 homologs from *A. tumefaciens* (VirB8a) and from *Brucella suis* (VirB8b) were solved, and both proteins were predicted to form dimers of similar geometry via an α-helical region (22, 23). Interestingly, analysis of the TraM protein from the plasmid pIP501 conjugation system from Gram-positive Enterococci and of the TcpC protein from *Clostridium perfringens* showed that despite the absence of apparent sequence similarity, these proteins had a very similar fold (24, 25). However, these proteins form trimers, suggesting that VirB8-like proteins may be able to interact via different interfaces of their core structure. This notion is consistent with biochemical analysis suggesting that, in line with its predicted role as an assembly factor, VirB8 undergoes relatively weak protein-protein interactions with other T4SS components (19, 21, 26).

Comparative analysis of different VirB8 homologs from *Bartonella* species showed homodimerization and a limited degree of heterodimer formation, suggesting a mechanistic solution preventing non-functional interactions of homologs that are simultaneously expressed in one organism (27). VirB8b was shown to interact with the close homolog TraJ from the pSB102 conjugation system, adding further evidence to the notion that VirB8 interactions are probably transient and may even be promiscuous (28, 29).

Here, we have extended the analysis of interactions between VirB8 homologs. We show that even distant homologs from different species interact and that this promiscuity extends to interactions with VirB10 homologs. Structural and biochemical analysis of TraE reveals a divergence as to the mode of dimerization compared with previously characterized homologs, underlining the cognate plasticity of this protein. Based on structural information and on previous work showing that VirB8b is a target for small molecule inhibitors (30, 31), we analyzed small molecules that bind to TraE and inhibit the conjugation of pKM101. We conclude that despite their divergent sequences and the transient nature of their interactions, VirB8-like proteins have common features that can be exploited for structure-based design of T4SS inhibitors.

**Results**

**The Bacterial Two-hybrid Assay Shows Heterologous Interactions between Distantly Related VirB8 Homologs**—To assess the capacity of VirB8 proteins to interact with homologs from other T4SSs, we expressed full-length VirB8a, VirB8b, TraE, and the VirB8 homolog CagV from *Helicobacter pylori* as fusions to the T25 and the T18 domains of *Bordetella* CyaA cytoplotoxin. The interactions were analyzed in the bacterial two-hybrid (BTH) assay using *Escherichia coli* cyaA deletion strain BTH101 and β-galactosidase activity as a readout fused to the cytoplasmic N terminus of the full-length proteins. The results show that all of these proteins homodimerize and that TraE also forms heterodimers with VirB8a, VirB8b, and CagV (Fig. 1A). Whereas the relative strength of the heterologous interactions was somewhat lower than that of TraE homodimerization, these results were nevertheless surprising because the sequence identities between the different homologs are low (14–35% as compared with TraE), the CagV protein from *H. pylori* being the most distantly related. *In vitro* cross-linking between the purified periplasmic domains of TraE, VirB8b, and CagV provided further evidence that these proteins have the capacity to form homomultimers as well as heteromultimers (Fig. 2). To test whether VirB8 proteins also interact in a heterologous fashion with other essential T4SS components from other organisms, we tested binding to VirB10 (19). Using this assay, TraE was shown to bind to VirB10 homologs from *B. suis* and from *A. tumefaciens*, further underlining its promiscuous binding capacity (Fig. 1B).

**Surface Plasmon Resonance Analysis of Interactions between CagV, TraE, and VirB8b**—As an alternative approach to measure interactions between homologs CagV, TraE, and VirB8b, ligands were amine-coupled to individual flow cells of SPR chips, and then each was flowed overtop in solution (analyte). Single-cycle kinetic analyses (*i.e.*, titration of low to high protein concentration without regeneration until the end of the series) showed that there was no nonspecific binding of bovine serum albumin (baseline control) to any of the reference or protein-immobilized surfaces (Fig. 3). When tested against CagV-coupled surfaces, TraE bound in a significant, dose-dependent manner with rapid association and dissociation kinetics (Fig. 3A). Similarly, the responses for
CagV and TraE binding to TraE-coupled surfaces were significant (Fig. 3B); lower yet increasing signal responses with VirB8b were also detected. When tested in the reverse orientation, TraE bound to VirB8b-coupled surfaces in a dose-dependent manner, and VirB8b also interacted with itself (Fig. 3C). When analyzed according to a “steady-state affinity” model, the orthogonal SPR data provides direct evidence for CagV-TraE, TraE-TraE, and TraE-VirB8b interactions in the same low micromolar affinity range (Fig. 3D).

**Structural Analysis of TraE Reveals an Altered Mode of Dimerization**—To gain high resolution insights into the molecular basis of the heterologous interactions, the X-ray structure of the periplasmic domain of TraE was determined by X-ray crystallography. Crystals of purified TraE diffracted up to 2.4 Å resolution (Table 1), and the structure was solved by molecular replacement using the homolog VirB8a that shares 25% amino acid identity as a search model. Structural comparison of TraE with its closest homologs VirB8a and VirB8b showed that the
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TABLE 1
Data collection and refinement statistics for TraE
All values in parentheses are given for the highest resolution shell.

| Parameters                  | Values                                    |
|-----------------------------|-------------------------------------------|
| Wavelength (Å)              | 1.1                                       |
| Resolution range (Å)        | 38.6–2.441 (2.529–2.441)                 |
| Space group                 | C2 2 2,                                    |
| Unit cell parameters: a (Å), b (Å), c (Å) | 112.049, 123.362, 109.8                   |
| α, β, γ (°)                 | 90°                                       |
| Total reflections           | 184,794                                   |
| Unique reflections          | 28,596 (2.826)                            |
| Multiplicity                | 6.0                                       |
| Completeness (%)            | 99.99 (99.93)                             |
| Mean I/σ(I)                 | 32.7 (2.3)                                |
| Wilson B-factor             | 46.46                                     |
| Rwork                       | 0.121                                     |
| Rfree                       | 0.132                                     |
| Reflections used for Rfree  | 2,000                                     |
| Rmerge                      | 0.2472 (0.3429)                           |
| Rmerge                       | 0.2801 (0.3475)                           |
| No. of non-hydrogen atoms   | 4,636                                     |
| Macromolecules              | 4,569                                     |
| Water                       | 117                                       |
| Protein residues            | 558                                       |

| Root mean square deviation  |                                            |
| Bonds (Å)                   | 0.004                                     |
| Angles (degrees)            | 0.93                                      |
| Ramachandran favored (%)    | 99                                        |
| Ramachandran allowed (%)    | 1                                         |
| Ramachandran outliers (%)   | 0                                         |
| Clashscore                  | 4.21                                      |
| Average B-factor (Å²)       | 38.20                                     |
| PDB number                  | 507                                       |

a $R_{merge} = \frac{\sum_{hkl} |I_hkl| - |\langle I_{hkl} \rangle|}{\sum_{hkl} |I_{hkl}|}$, where $T$ is a test data set randomly selected from the observed reflections before refinement. The test data set was not used throughout refinement and contained 7% of total unique reflections.

b $R_{free} = \frac{\sum_{hkl} |I_hkl| - |\langle I_{hkl} \rangle|}{\sum_{hkl} |I_{hkl}|}$. 

c Analyzed by Molprobity.

overall fold, comprising five α-helices on one side of the protein and four β-sheets on the opposite side, is similar. However, the orientations of the α-helices 1, 4, and 5 of TraE are somewhat different from VirB8a and VirB8b; the root mean square deviation is 0.66 Å compared with VirB8a and 1.67 Å compared with VirB8b (Fig. 4, A–C). The most interesting difference is that the dimer interface is clearly altered from that of VirB8a and VirB8b regarding the participating residues (Fig. 4D) and in its overall geometry (Fig. 5). The dimer interface buries a total of 844 Å² for VirB8a and 925 Å² for VirB8b, whereas the area of the TraE dimer interface is only 577 Å². The second and third contact regions described in VirB8a and VirB8b structures are not present in the TraE dimer, and the two monomers are tilted in comparison with the more symmetrical arrangement of its homologs (Fig. 5). Despite these differences, in silico docking suggests that heterodimer formation of TraE with VirB8a and VirB8b is possible (Fig. 6), which is consistent with the results from the BTH assay (Fig. 1).

The Predicted Dimerization Site Is Important for VirB8 Interactions in vitro and for Its Functionality in Vivo—To assess whether the atypical dimerization site predicted in the X-ray structure is biologically significant, variants of residues Glu97, Gln105, Lys168, and Tyr214, which form hydrogen bonds across the dimer interface were constructed. Single amino acid changes, such as Y214A or K168A, had little or no effect on dimerization using the BTH assay. In contrast, dimerization of the variants E97A and Q105A and of those carrying double changes E97A/Q105A and K168A/Y214A was strongly reduced (Fig. 7A). Similarly, when variants of the periplasmic domain of TraE carrying single and double amino acid changes were analyzed by gel filtration, we found that the concentration-dependent dimerization observed in the wild type protein was significantly diminished if not abolished in both single and double variants (Fig. 7B). Finally, to assess functionality in the natural biological context, we tested the ability of TraE and its variants to complement conjugation of a non-polar pKM101:traE transposon insertion mutant. This analysis revealed that conjugation was markedly reduced or not detectable in the case of all single and double variants (Fig. 7C), despite the fact that the proteins were stably expressed. Taken together, these results support the biological relevance of the dimerization site predicted by X-ray crystallography. We next analyzed whether the structural information can be exploited for the design of inhibitors of TraE-dependent plasmid conjugation.

Docking of VirB8b Inhibitors and of Novel Derivates to TraE—
A common feature of VirB8 homologs is the presence of a surface groove located between the α-helical and the β-sheet faces of the protein, and it was hypothesized that this groove has a functional role in mediating protein interactions by VirB8 homologs (22, 26). We therefore explored whether putative binding by small molecules to this site in turn inhibits bacterial conjugation. Previous docking results showed that the most potent compounds that bound to this site in VirB8b were the inhibitors B8I-1, B8I-2, and B8I-3 (31). These and other small molecules also tested (31) were shown to bind the equivalent surface groove of TraE (Fig. 8). To further probe this binding site, we also synthesized seven novel analogues of compound B8I-2 (Fig. 9), which from our previous work inhibited Brucella virulence (31). Analogues of B8I-2 were designed to probe for enhanced interaction with the binding site, and in silico docking predicted that all of these compounds would bind to TraE (Fig. 8C). We therefore tested their impact on TraE interactions in vitro and in vivo.

Small Molecules Bind to TraE and Inhibit pKM101 Conjugation—To measure small molecule binding, we established an in vitro assay based on the quenching of tryptophan fluorescence following the addition of inhibitors to the purified periplasmic domain of TraE. Using this assay, we determined $K_D$ values from low to high micromolar range for the different molecules (Table 2). The lowest $K_D$ value of 2.7 μM was observed for the B8I-2 analogue BAR-072 (Fig. 10A). Using chemical cross-linking, we showed that the purified periplasmic domain of TraE forms dimers and higher molecular mass multimers, which is consistent with the results from the BTH assay (Fig. 10B). The addition of BAR-072 attenuated cross-linker-dependent formation of multimers, suggesting that binding by this analogue interferes with dimerization (Fig. 10, B and C). Finally, we assessed whether the TraE-binding small molecules have an effect on plasmid conjugation. Conjugation experiments were conducted between a pKM101-carrying donor (FM433) and a plasmid-free recipient (WL400) in the presence of the small molecules at 50 μM concentration. No effects on bacterial growth were observed at this concentration except for molecule B8I-3, which was not further pursued (data not shown).
Analysis of plasmid transfer frequency showed that none of the original VirB8 inhibitors had a significant impact but that four of the tested molecules significantly reduced pKM101 transfer (B8I-16, BAR-072, BAR-073, and UM-024) (Fig. 11A). The strongest effect (10-fold reduction) was observed for molecule BAR-072, correlating with the lowest $K_D$ observed by fluorescence quenching. None of the molecules that reduced pKM101 transfer impacted the conjugation of the unrelated control plasmid RP4, suggesting that their effects are specific for pKM101 and not due to a nonspecific effect on bacterial metabolism or viability (Fig. 11B).

Discussion

VirB8 homologs are essential components of all T4SSs in which they have been studied. They are believed to undergo a series of transient protein-protein interactions contributing to T4SS assembly (15–21). Although the sequence identities between VirB8 homologs are in the range of 20–40%, the over-
all protein fold is well conserved (22, 23, 27), and this was confirmed here by determination of the TraE structure. In some cases, due to high divergence of the primary sequence, VirB8 homologs were found only upon their structural analysis (24, 25, 32), suggesting strong evolutionary pressure to conserve protein overall fold, with functional specificity emerging at a later date. These data are consistent with the early hypothesis that VirB8 is an assembly factor that was based on its similarity to a nuclear transfer factor (NTF-2) (22). X-ray crystallographic approaches have shown that most studied VirB8 homologs form dimers. However, the more distantly related homologs from Gram-positive bacteria, such as Clostridium TcpC and Enterococcus TraM, crystallized as trimers, suggesting significant structural plasticity regarding VirB8-like protein multimerization. Whereas TraE crystallized as a dimer, the spatial arrangement of the two subunits was clearly distinct from that of its closest homologs, VirB8a and VirB8b. The biological significance of this dimerization site was verified by the analysis of variants at the interaction interface, suggesting that different spatial orientations are biologically significant even in the case of closely related VirB8 homologs (20, 26).

The fact that the VirB8 fold is so highly conserved underlines the importance of this protein as a T4SS assembly factor. Several studies have shown that VirB8 undergoes multiple interac-
tions (15, 18, 19, 21, 26, 33), but until now only limited data were available on the molecular basis of these interactions. Considering that the periplasmic domain is relatively small (18 kDa), it is unlikely that VirB8 interactions are more than transient, which is consistent with the measured strength of interactions in the high nanomolar to micromolar range (19, 21). Nevertheless, specificity is important for ordered T4SS assembly, and a recent study on VirB8 homologue proteins from *Bartonella* and *Rickettsia* species suggests that structural barriers as well as differential expression prevent non-productive interactions between multiple VirB8 paralogs in these organisms (27). Whereas non-productive interactions are not pertinent in the case of VirB8 homologs from different bacteria, the high degree of interactions between VirB8 homologs from *Agrobacterium*, *Brucella*, pKM101, and *H. pylori* observed in the BTH assay as well as in *vitro* using cross-linking as well as SPR analysis was unexpected. In addition to homodimer formation, we observed heterologous binding to VirB10 homologs, indicating a high degree of plasticity of VirB8 interactions arising from the conserved protein fold. It would be of interest to test interactions with VirB8 homologs from Gram-positive bacteria in the future (TcpC, TraM) to determine whether the plasticity of interactions extends to trimer-forming homologs. Binding between VirB8 homologs in the BTH assay does not necessarily imply functional complementation in the natural biological context in which other additional protein-protein interactions are likely to be important. For example, despite the fact that Traf from plasmid pSB201 shares 50% sequence identity and bound *Brucella* VirB8 in the BTH assay, complementation with the full-length protein was not possible (28, 29). Similarly, we found that *Brucella* VirB8 does not complement *virB8* deletions in *A. tumefaciens*. The periplasmic domain from Traf did complement to a limited extent when it was fused to the N-terminal domain of VirB8b, but expression of Traf or of a Traf-VirB8b fusion protein inhibited virulence. This is probably due to strong binding with VirB8b, suggesting that both the dimer and the free monomer forms are functionally important.

The biological role of the prominent hydrophobic surface groove between the β-sheet face of the protein and the α-helices present at the dimerization site has been the subject of debate, and it was hypothesized that it may be a protein-protein interaction site (22). Currently, there is no evidence for this notion, but we subsequently discovered that this surface groove is the binding site for small molecule inhibitors of VirB8 dimerization (30, 31). At first, this result was counterintuitive because the dimer interface is on the side opposite to the binding site. However, substituting amino acids at the inhibitor binding site reduced the potency of the inhibitors as well as dimerization, suggesting that conformational changes of this surface groove may be linked to dimerization. NMR analysis of VirB8b showed significant conformational changes between the monomeric and the dimeric state that are consistent with this notion. Here, we probed Traf with small molecules that had been shown to inhibit VirB8b dimerization (31) as well as derivatives that were synthesized to improve the interaction potency to binding site residues. Using a fluorescence quenching assay, we showed that the VirB8 inhibitors and newly synthesized compounds bound to Traf with affinities in the micro-

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3 B. Casu, J. Smart, M. A. Hancock, M. Smith, J. Sygusch, and C. Baron, unpublished results.
4 M. Sharifahmadian, J. Omichinski, and C. Baron, unpublished results.
molar range despite apparent differences of the binding sites in the two proteins. Molecular docking suggested that almost all of the molecules bind to the surface groove between the β-sheet face and the α-helices of the protein that is equivalent to that in VirB8b. We determined a wide variety of binding affinities, and the molecule BAR-072 had the lowest $K_D$ value of 2.7 μM. Docking predicted that BAR-072 binds to four amino acids in the inhibitor-binding surface groove of TraE, whereas B8I-2 and other molecules only bind up to three amino acids, which could explain the difference in $K_D$ values (Fig. 8B). Interestingly, BAR-072 had a significant effect on T4SS function, leading to a 10-fold reduction of pKM101 transfer between bacteria. Furthermore, molecule UM-024 that was weakly active against VirB8b also significantly inhibited pKM101 conjugation, whereas the most potent VirB8b inhibitor, B8I-2, had no effect on pKM101 conjugation, suggesting that differences in the inhibitor binding sites can be exploited for the design of specific molecules.

The low degree of correlation between the $K_D$ values and the effects on pKM101 transfer (except for BAR-072) suggest the presence of an additional binding site or variations in the ability of the molecules to cross the cell envelope. Nevertheless, the observed attenuation of plasmid conjugation suggests for the first time that the inhibition of plasmid transfer is possible by targeting a specific component of a secretion/conjugation system. Our

| Name | Structure | Name | Structure | Name | Structure |
|------|-----------|------|-----------|------|-----------|
| B8I-1 | ![Structure](image1) | BAR-068 | ![Structure](image2) | UM-023 | ![Structure](image3) |
| B8I-2 | ![Structure](image4) | BAR-069 | ![Structure](image5) | UM-024 | ![Structure](image6) |
| B8I-3 | ![Structure](image7) | BAR-070 | ![Structure](image8) | UM-048 | ![Structure](image9) |
| B8I-4 | ![Structure](image10) | BAR-071 | ![Structure](image11) | UM-050 | ![Structure](image12) |
| B8I-5 | ![Structure](image13) | BAR-072 | ![Structure](image14) | | |
| B8I-8 | ![Structure](image15) | BAR-073 | ![Structure](image16) | | |
| B8I-16 | ![Structure](image17) | BAR-074 | ![Structure](image18) | | |

**FIGURE 9. Chemical structures of small molecules tested by in silico docking, in vitro binding, and in vivo conjugation assays.** Previously tested molecules (B8I-1 to B8I-16, UM-023 to UM-050) (31) and new B8I-2 analogues (BAR-068 to BAR-074) were used to probe the binding site.
results also suggest that the surface groove targeted by VirB8b inhibitors may be suitable as a target for TraE inhibitors, pointing to a new paradigm enabling discovery of inhibitors targeting VirB8-like proteins in a wide variety of pathogens.

**Experimental Procedures**

**Strains and Plasmids**—The strains and plasmids used are listed in Table 3. *E. coli* strains XL-1 Blue or DH5α were used as hosts for cloning and mutagenesis. Strain BL21star (ADE3) was used for overexpression of TraE, VirB8a, VirB8b, and CagV; strain BTH101 was used for BTH assays.

| Tested molecule | $K_D^{\mu M}$ | $\pm$ S.D. |
|-----------------|--------------|-----------|
| B8I-1           | 11.4 ± 0.6   |           |
| B8I-2           | 20.6 ± 1.4   |           |
| B8I-3           | 9.9 ± 0.5    |           |
| B8I-4           | 35.1 ± 3.1   |           |
| B8I-5           | 30.3 ± 2.8   |           |
| B8I-8           | 28.5 ± 2.5   |           |
| B8I-16          | 28.8 ± 2.2   |           |
| BAR-068         | 53.6 ± 3.9   |           |
| BAR-069         | 27.9 ± 2.4   |           |
| BAR-070         | 9.1 ± 0.3    |           |
| BAR-071         | 10.0 ± 0.4   |           |
| BAR-072         | 2.7 ± 0.1    |           |
| BAR-073         | 7.3 ± 0.6    |           |
| BAR-074         | 9.2 ± 0.4    |           |
| UM-0125823      | 9.6 ± 0.2    |           |
| UM-0125824      | 61.1 ± 5.5   |           |
| UM-0125848      | 14.4 ± 0.5   |           |
| UM-0125850      | 34.4 ± 1.6   |           |

* S.D. value derived from three replicas.

**Quantitation of Conjugative DNA Transfer**—*E. coli* strains FM433 pKM101 (donor, ampicillin-resistant), FM433 pRP4 (donor, ampicillin-resistant), and WL400 (recipient, chloramphenicol-resistant) were grown in liquid LB medium at 37 °C (with 100 μg/ml for the pKM101- and RP4-carrying strains) to an $A_{600 nm}$ of 0.5–1, sedimented by centrifugation, and resuspended in an appropriate volume of LB medium without antibiotics. Equal amounts of donor and recipient strain (1 μl of each) were mixed on prewarmed LB agar and incubated for 2 h at 37 °C to enable conjugation, and the cells were then washed from the plate with 100 μl of liquid LB. To quantify conjugative transfer, dilutions of the conjugation mixture were plated on LB agar plates containing appropriate antibiotics for selection of pKM101-containing recipient strain WL400 (100 μg/ml ampicillin, 34 μg/ml chloramphenicol). To determine complementation with TraE variants, strain FM433 carrying a non-polar transposon insertion in the *traE* gene (pKM101 traE1228::Tn5 (3)) was transformed with complementation vector pTrc200 (negative control), pTrc200TraE (positive control), or pTrc200 expressing TraE variants; complementation experiments were carried out as above. For analysis of the inhibition of conjugation with small molecules, the cells were cultivated on agar and in liquid media in the presence of 50 μM concentration of the small molecules in the presence of 2.5% DMSO. The presence of DMSO and of the small molecules (with the exception of B8I-3) did not negatively impact bacterial growth.

**DNA Isolation and Manipulation**—Plasmid DNA was isolated using Qiagen Miniprep kits (Qiagen, Manchester, UK). Standard techniques were employed for the cloning, transformation, preparation, and restriction analysis of plasmid DNA from *E. coli* (34).

**FIGURE 10. Effect of BAR-072 on TraE.** A, *in vitro* binding assay based on tryptophan quenching upon the addition of BAR-072 to the purified periplasmic domain of TraE. B, cross-linking of TraE with DSS (0.4 mM) in the presence of BAR-072 at given concentrations (0–1.6 μM). DMSO final concentration of 2.5%; arrows indicate higher molecular weight complexes formed after cross-linking of TraE. C, quantification of the formation of DSS-dependent cross-linking products of TraE (indicated by arrows) in the absence and in the presence of BAR-072 (0–1.6 μM). Formation of the two cross-linking products was normalized by comparison with the control without BAR-072 (100% is the control); the data represent averages and S.E. (error bars) of the mean of three replicates.
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BTH Assay—Interactions between full-length TraE, VirB8a, VirB8b, CagV, VirB10a, VirB10b, and TraE variants were assessed in vivo using the BTH system as described (35). The genes encoding VirB8 homologs, TraE variants, and VirB10 homolog proteins were fused to the DNA sequences encoding the genes encoding VirB8 homologs, TraE variants, and VirB10 homologs in the presence of 50 µM TraE-binding small molecules. Conjugation assays between plasmid RP4-carrying donor strain FM433 and plasmid-free recipient WL400 were conducted in the presence of 50 µM TraE-binding small molecules. The numbers of colony-forming units compared with a control experiment in the absence of the small molecules are shown; data represent averages and S.E. (error bars) of three biological replicate cultures. Asterisks, statistically significant differences (p ≤ 0.0001, n = 3).

Crystallization of TraE and Data Collection—The TraE buffer was changed to 20 mM HEPES, 50 mM NaCl (pH 7.4), and the protein (15 mg/ml) was crystallized in 16% (w/v) PEG 10,000, 50 mM BisTris (pH 5.5), 100 mM ammonium acetate. The crystals were cryoprotected in 16% (w/v) PEG 10,000, 50 mM BisTris (pH 5.5), 100 mM ammonium acetate, and 20% ethylene glycol. The crystals were flash-frozen in liquid nitrogen, and the data were collected at beamline X25 of the National Synchrotron Light Source at Brookhaven National Laboratory. The intensity data were processed using the HKL2000 program.

Structure Determination of TraE—Structures were solved by molecular replacement using the structure of VirB8 from A. tumefaciens as a reference model (PDB code 2CC3) (23). Refinement was performed using Phenix software suite of program to the highest possible resolution (36) made possible by collecting diffraction data in 180° sweeps to take full advantage of the space group symmetry redundancy (C222₁) to maximize signal/noise ratio. Electron density maps were calculated to the resolution indicated in Table 1 to ensure at least 90% completeness in the highest resolution shell with an I/σ(I) > 2. Final model statistics, calculated with Phenix, molprobity, and PROCHECK, are shown in Table 1. The atomic coordinates and structure factors for TraE have been deposited at the Protein Data Bank (PDB code 5I97). The model of the final structure has an Rcryst (Rfree) value of 0.237 (0.275). All figures were prepared using the program PyMOL.

Large Zone Gel Filtration Chromatography—The molecular masses of the purified periplasmic domain of TraE and of its variants were determined by gel filtration using large zone elution (37). To this effect, 200-µl aliquots with protein concen-
| Strains     | Genotype/description | Source/reference |
|------------|----------------------|------------------|
| DH5a       | F−, F' lacZΔM15 (ΔlacZYA-argF) U169 recA1 endA1 hisD176 relA1 (F proAB lacIqZM15 Tn10 [TetR]) | Invitrogen       |
| XL-1 Blue  | recA1 endA1 gyrA96 thi-1 hasD176 supE44 recA1 lac (F proAB lacIqZM15 Tn10 [TetR]) | Agilent Technologies |
| BL21(DE3)star | F 80lacZ ΔlacZYA-argF 80lacZΔlacZYA-argF 80lacZΔlacZYA-argF | Invitrogen       |
| BTH101     | F' araD139, galE15, galK16, rpsL1 (strr), hsdR2, mcrA1, mcrB1, thi-1, gyrA96, relA1 | Ref. 40          |
| WL400      | F−, cya-99, araD139, galE15, galK16, rpsL1 (strr), hsdR2, mcrA1, mcrB1, thi-1, gyrA96, relA1 | W. Leinfelder, unpublished data |

| Plasmids   | Genotype/description | Source/reference |
|------------|----------------------|------------------|
| pHT        | kan' pET24d derivative | Ref. 31          |
| pHTVirB8bb | kan' pT7 promoter vector for the expression of His6-tagged periplasmic domain of B. suis VirB8 | Ref. 31 |
| pHTTraEp   | amp' pT7 promoter vector for the expression of His6-tagged periplasmic domain of H. pylori CagV | This work |
| pHTTraEp Y214A | pHTTraEp modified to encode TraE with amino acid change Y214A | This work |
| pHTTraEp E97A | pHTTraEp modified to encode TraE with amino acid change E97A | This work |
| pUT18      | pUC19 derivative including the T18 fragment (amino acids 225–399 of B. pertussis CyaA) N-terminal to the multiple cloning site | Ref. 40 |
| pKNT25     | pSU40 derivative including the T25 fragment (amino acids 1–224 of B. pertussis CyaA) N-terminal to the multiple cloning site | Ref. 40 |
| pUT18C     | pUC19 derivative including the T18 fragment (amino acids 225–399 of B. pertussis CyaA) C-terminal to the multiple cloning site | Ref. 40 |
| pK25       | pSU40 derivative including the T25 fragment (amino acids 1–224 of B. pertussis CyaA) C-terminal to the multiple cloning site | Ref. 40 |
| pUT18CvirB10b | amp', pUT18C harboring 1,176-bp XbaI/KpnI virB10 fragment from B. suis | Ref. 30 |
| pK25VirB10b | kan', pK25 harboring 1,176-bp XbaI/KpnI virB10 fragment from B. suis | Ref. 30 |
| pUT18CvirB8a | amp', pUT18C harboring 720-bp XbaI/KpnI virB8 fragment from A. tumefaciens | This work |
| pK25VirB8a | kan', pK25 harboring 720-bp XbaI/KpnI virB8 fragment from A. tumefaciens | This work |
| pUT18CCagV | amp', pUT18C harboring 765-bp XbaI/KpnI cagV fragment from H. pylori | This work |
| pK25CagV   | kan', pK25 harboring 765-bp XbaI/KpnI cagV fragment from H. pylori | This work |
| pUT18CTraE | amp', pUT18C harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |
| pK25TraE   | kan', pK25 harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |
| pUT18CTraE E97A | pUT18C modified to encode TraE with amino acid change E97A | This work |
| pK25TraE E97A | kan', pK25 harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |
| pUT18CTraE K168A | pUT18C modified to encode TraE with amino acid change K168A | This work |
| pK25TraE K168A | kan', pK25 harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |
| pUT18CTraE K168A | pUT18C modified to encode TraE with amino acid change K168A | This work |
| pK25TraE K168A | kan', pK25 harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |
| pUT18CTraE Q105A | pUT18C modified to encode TraE with amino acid change Q105A | This work |
| pK25TraE Q105A | kan', pK25 harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |
| pUT18CTraE Y214A | pUT18C modified to encode TraE with amino acid change Y214A | This work |
| pK25TraE Y214A | kan', pK25 harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |
| pUT18CTraE Q105A | pUT18C modified to encode TraE with amino acid change Q105A | This work |
| pK25TraE Q105A | kan', pK25 harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |
| pUT18CTraE Y214A | pUT18C modified to encode TraE with amino acid change Y214A | This work |
| pK25TraE Y214A | kan', pK25 harboring 699-bp XbaI/KpnI traE fragment from pKM101 | This work |

| Strains     | Genotype/description | Source/reference |
|------------|----------------------|------------------|
Dimerization and Inhibition of TraE

tractions ranging from 12 to 240 μM were applied to a Superdex 75 column (GE Healthcare) in 20 mM HEPES, 50 mM NaCl, pH 7.4, at 4 °C. The void volume of the column was determined with blue dextran 2000, and the calibration curve was determined by using the Low Molecular Weight Gel Filtration Calibration Kit (GE Healthcare).

Analysis of Protein-Protein Interactions by Cross-linking—
Chemical cross-linking with disuccinimidyl suberate (DSS; Pierce) was performed as described previously (18), and the formation of cross-linking products was quantified by SDS-PAGE, Western blotting, and using the ImageLab version 4.0 software (Bio-Rad).

Molecular Docking Analysis—In silico docking was performed using Autodock Vina (38) run through PyRx to manage the workflow and PyMOL to visualize the results. The chemical structures for each ligand were retrieved from the hit2lead.com website and converted to PDB format using openbabel software, followed by processing with Autodock Tools version 1.5.4 to assign Gasteiger charges, merging non-polar hydrogens, and to set torsional bonds. Docking runs were performed within a 47 × 52 × 33-Å rectangle search space surrounding the binding pocket, and output modes were ranked according to binding affinity. Autodock Vina identified molecular conformations with the best fit and strongest binding affinity (global minima) by a stochastic algorithm exploring surfaces/pockets of the rigid macromolecule, through an iterative series of local optimizations (changing shape, bond angles, and position of the ligand), evaluating both intramolecular (hydrophobic interactions, repulsions, hydrogen bonding, etc.) and intramolecular (torsion, rotational torque) factors.

Analysis of Small Molecule Binding by Fluorescence Spectroscopy—Changes in the intrinsic UV fluorescence emission of TraE upon binding of small molecules were recorded at 20 °C with a Cary Eclipse fluorometer (Varian) (λex, 295 nm; λem, 340 nm; 5-nm excitation and emission slit widths) in 20 mM HEPES, 50 mM NaCl, pH 7.4. The spectra were corrected for dilution effects, and the KD values were calculated from the ligand binding fluorescence data fitted to a single-site saturation curve with constant background using the Grafit version 6.0 software package.

Synthesis of Small Molecules—Small molecule derivatives of B81-2 were synthesized as described (31).

Surface Plasmon Resonance—Using label-free, real-time SPR, the interactions between CagV, TraE, and VirB8b were examined using a BIACORE 3000 system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; BIACore version 4.1 operating software). For quality control, each batch of purified protein was pretested on an Ultraflextreme MALDI-TOF/TOF system (Bruker Daltonics, Bremen, Germany) according to the manufacturer’s recommendations (intact mass analysis in linear-positive mode as well as top-down sequencing (in-source decay) to verify the N-/C-terminal amino acid sequences of the intact proteins).

SPR experiments were performed on research-grade CM5 sensor chips (Biacore) at 25 °C using filtered (0.2 μm) and degassed HBS-EP buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween 20). Immobilized protein surfaces (10 μg/ml in 10 mM sodium acetate, pH 5.0 (CagV, TraE) or pH 4.0 (VirB8b)) were prepared using the Biacore Amine Coupling Kit as recommended by the manufacturer (final immobilization levels = 300–900 RU for each protein); corresponding reference surfaces were prepared in the absence of protein.

To assess binding specificity and affinity, single-cycle kinetic analyses were performed in which increasing concentrations of BSA (negative control), CagV, TraE, and VirB8b were titrated in tandem (0–25 μM; 2-fold dilution series) over reference and protein-immobilized surfaces at 25 μl/min in KINJECT mode (1-min association + 30-s dissociation). Between titration series, the surfaces were regenerated at 50 μl/min using two 30-s pulses of solution I (HBS-EP containing 1.0 M NaCl and 0.05% (v/v) Empigen) followed by the EXTRACLEAN and RINSE procedures. Due to the rapid steady-state binding kinetics observed for each interaction pair, the binding responses were independent of mass transport limitations, and all double-referenced data (39) presented are representative of duplicate injections acquired from at least three independent trials. To predict overall equilibrium dissociation constants (KD) for each “ligand,” steady-state binding responses (Raq) were averaged near the end of each association phase, plotted as a function of “analyte” concentration (C), and then subjected to non-linear regression analysis (“Steady state affinity” model; BLAevaluation version 4.1 software). Complementary multicycle kinetic analyses (25 μl/min × 5-min association + 5-min dissociation) were performed in a similar manner and yielded consistent affinity constants.

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