**INTRODUCTION**

Type IV secretion systems (T4SSs) are used by bacteria for different functions, the two most common being conjugation and effector protein translocation. These processes play important roles in, respectively, the spread of antibiotic resistance and infection by bacterial pathogens. During conjugation single-stranded DNA is translocated into recipient cells by a contact-dependent process. *Neisseria gonorrhoeae* is the only bacterium known to use a T4SS to secrete single-stranded DNA into the extracellular milieu (Dillard and Seifert, 2001; Hamilton *et al*., 2005; Salgado-Pabón *et al*., 2007).

The most studied T4SS is the P-type system from *Agrobacterium tumefaciens*, consisting of 12 core proteins named VirB1-VirB11 and VirD4 (Alvarez-Martinez and Christie, 2009). The F-plasmid...
encodes protein homologs to most of the Vir core proteins (VirB2-VirB10 and VirD4) found in P-type T4SSs, but the F-plasmid additionally encodes several proteins that are conserved only in F-type T4SSs (Lawley et al., 2003). F-type T4SSs have been found on many conjugative plasmids and in genetic islands on the bacterial chromosome (Lawley et al., 2003). The genes encoding the <i>N. gonorrhoeae</i> T4SS proteins are located on a 59-kb genetic island (the Gonococcal Genetic Island, GGI) (Callaghan et al., 2017). 21 genes organized in 4 operons are important for secretion of ssDNA by the <i>N. gonorrhoeae</i> T4SS (Pachulec et al., 2014). The structural T4SS proteins encoded by 17 genes can be divided into three groups: (1) Proteins showing homology to proteins found in most type IV secretion systems, (2) proteins showing homology to proteins conserved only in F-type T4SSs, and (3) proteins specific to the <i>N. gonorrhoeae</i> T4SS or only found in GGI-like T4SSs (Hamilton et al., 2005; Pachulec et al., 2014). (For an overview, see Table 1 and Figure 1).

The energy-providing ATPases TraC (a VirB4 homolog) is found in F-type T4SSs while F-type T4SSs are missing a homolog to the ATPase VirB11 found in P-type T4SSs (Alvarez-Martines and Christie, 2009). <i>N. gonorrhoeae</i> encodes a TraC homolog required for DNA secretion (Hamilton et al., 2005; Pachulec et al., 2014).

Structural studies of the outer membrane core complex (OMCC) of two conjugative P-type T4SSs have been published (Chandran et al., 2009; Fronzes et al., 2009; Low et al., 2014). The OMCC consists of three proteins that can form a double membrane-spanning complex. The hub protein VirB10 inserts into both the outer and the inner membrane, spans the periplasm and has a short N-terminal end in the cytoplasm (Chandran Darbari and Waksman, 2015). The two other proteins in the OMCC, VirB7 and VirB9 are associated with the outer membrane (Chandran et al., 2009; Low et al., 2014). Structural (Hu et al., 2019) and two-hybrid (Harris et al., 2001) data suggest that the F-plasmid T4SS has a similar OMCC consisting of the VirB10 homolog TraBp, the VirB7 homolog TraVp, and the VirB9 homolog TraKp, TraLp, TraEp, and TraGp from the F-plasmid are proteins associated with the inner membrane with some homology to proteins from the P-type T4SSs (Lawley et al., 2003) (Table 1 and Figure 1b). The <i>N. gonorrhoeae</i> GGI encodes homologs of TraBp, TraVp, TraKp, as well as TraGp, TraLp, and TraEp homologs (Table 1, Figure 1a). Mutational analyses have shown that all of these proteins are important for DNA secretion by the <i>N. gonorrhoeae</i> T4SS (Hamilton et al., 2001; 2005; Pachulec et al., 2014).

In addition to the proteins found in most other type IV secretion systems, F-type T4SSs have a group of periplasmic or peripheral membrane proteins (TraWp, TraUp, TraHp, TraFp, TraFp, and TraNp) that has been linked to assembly and extension of the conjugation pilus (Arutyunov and Frost, 2013). Although <i>N. gonorrhoeae</i> does not have pilus-dependent DNA secretion, the TraWp, TraUp, TraHp, TraFp, TrbCp, and TraNp homologs encoded by the <i>N. gonorrhoeae</i> GGI are essential for DNA secretion (Hamilton et al., 2001; 2005; Pachulec et al., 2014).

F-type T4SSs generally encode periplasmic thiol-oxidoreductases that promote disulfide bond formation in the periplasm (Hemmis and Schildbach, 2013; Pachulec et al., 2014). Some plasmids with F-type T4SSs such as the F-plasmid encode the protein TrbB which has a redox-active site and a TraF protein without a redox-active site, while others such as the <i>N. gonorrhoeae</i> GGI and the plasmid R27 encode a periplasmic DsbC (disulfide bond) homolog often in combination with a TraF-like protein, both proteins having redox-active sites (Elton et al., 2005; Hemmis and Schildbach, 2013). DsbCp and TraFp are both essential for DNA secretion by the <i>N. gonorrhoeae</i> T4SS (Hamilton et al., 2005).

Lytic transglycosylases capable of peptidoglycan degradation are believed to play a role in the assembly of transport complexes in the cell envelope (Koraimann, 2003). Three of the proteins that are important for DNA secretion by <i>N. gonorrhoeae</i> AtIA<sub>N</sub>, Yag<sub>N</sub>, and LtgX<sub>N</sub> are thought to be associated with the peptidoglycan layer (Dillard and Seifert, 2001; Kohler et al., 2007; Pachulec et al., 2014). AtIA<sub>N</sub> and LtgX<sub>N</sub> are both lytic transglycosylases (Kohler et al., 2007). LtgX<sub>N</sub> shows homology to Orf169, a lytic transglycosylase from the F-plasmid T4SS, while AtIA<sub>N</sub> is specific to the <i>N. gonorrhoeae</i> T4SS (Kohler et al., 2007).

The T4SS from <i>N. gonorrhoeae</i> shows some amino acid sequence similarity to the T4SS from the F-plasmid but the sequence identity is generally low, typically around 25% (see Table 2) (Hamilton et al., 2005; Ramsey et al., 2014), and while other F-type T4SSs are involved in contact-dependent DNA secretion, the T4SS from <i>N. gonorrhoeae</i> carries out contact-independent DNA secretion. Comparing the two systems could potentially be used to define generic features of

### Table 1: Predicted localization of 17 T4SS proteins essential for DNA secretion by <i>N. gonorrhoeae</i> and the corresponding homologous proteins from the F-plasmid T4SS and P-type T4SSs

| <i>N. gonorrhoeae</i> T4SS | F-plasmid T4SS | P-type T4SSs |
|-------------------------|----------------|--------------|
| TraC (CP)               | TraC (CP)      | VirB4 (CP/IM/PP) |
| TraE (CP/IM/PP)         | TraE (CP/IM/PP) | VirB8 (CP/IM/PP) |
| TraG (CP/IM/PP)         | TraG (CP/IM/PP) | VirB6 (CP/IM/PP) |
| TraL (CP/IM)            | TraL (CP/IM)   | VirB3 (CP/IM)  |
| TraF (PP)               | TraF(PP)       |               |
| TraH (PP)               | TraH (PP)      |               |
| TraU (PP)               | TraU (PP)      |               |
| TraW (PP)               | TraW (PP)      |               |
| TrbC (PP)               | TrbC (PP)      |               |
| DsbC (PP)               |                |               |
| AtlA (PP/PG)            | Orf169 (PP/PG) | VirB1 (PP/PG)  |
| LtgX (PP/PG)            |                |               |
| Yag (PP/PG)             |                |               |
| TraB (CP/IM/PP/OM)      | TraB (CP/IM/PP/OM) | VirB10 (CP/IM/PP/OM) |
| TraK (PP/OM)            | TraK (PP/OM)   | VirB9 (PP/OM)  |
| TraV (PP/OM)            | TraV (PP/OM)   | VirB7 (PP/OM)  |
| TraN (PP/OM)            | TraN (PP/OM)   |               |
|                        |                | VirB11 (CP)    |

Abbreviations: CP, cytoplasm; IM, inner membrane; PP, periplasm; PG associated with peptidoglycan; OM, outer membrane.
F-type T4SSs as well as giving information about the specific features associated with the unique function of the *N. gonorrhoeae* T4SS.

We have compiled sequence-based and published localization information for the *N. gonorrhoeae* T4SS, and experimentally determined outer membrane localization of TraHN. Only a limited number of studies explore the Tra protein interaction network; we, therefore, systematically tested 17 proteins important for DNA secretion by *N. gonorrhoeae* for protein–protein interactions using bacterial two-hybrid systems. To determine if the identified interactions are likely to be specific to the *N. gonorrhoeae* T4SS or general for F-type T4SSs, we tested the corresponding proteins from the F-plasmid for interactions. Interactions of particular interest were confirmed by co-purification. We have shown cross-system interchangeability of homologous T4SS proteins from the two systems in several cases using both two-hybrid and co-purification approaches, and present interaction models for both systems.

2 | RESULTS

2.1 | TraHN localizes to the outer membrane dependent on TraGN

To use bacterial two-hybrid systems correctly, it is important to know the cellular localization of the proteins.

TraHF has been implicated in F-pilus extension (Arutyunov and Frost, 2013); however, without a pilus, the Neisseria TraHN likely plays a distinct role. Since the localization of TraHN was unknown, we epitope-tagged TraHN with a triple FLAG tag at the C-terminus and examined its subcellular localization in *N. gonorrhoeae*. When traHN-FLAG3 was expressed from the native site, no TraHN-FLAG3 was detected by western blot. Therefore, traHN-FLAG3 was expressed using an inducible promoter from a distant site on the gonococcal chromosome. TraHN-FLAG3 was detectable in that strain upon induction. Cell fractions containing outer membrane, total membrane, or soluble protein were examined, and TraHN-FLAG3 was found in the outer membrane and total membrane fractions only (Figure 2a). This localization pattern matched that of known outer membrane protein LtgA and was distinct from that of known inner membrane protein SecY and known soluble protein CAT (Figure 2a).

The traHN, traGN, and atlAN genes are in an operon separate from the one encoding most of the structural proteins of the gonococcal T4SS (Pachulec et al., 2014). The transcript is found at significantly higher levels than that encoding the other T4SS structural proteins, and the translation of TraHN and TraGN, and possibly also AtlAN, is controlled by an RNA switch (Ramsey et al., 2015). The coregulation of these proteins suggested that they might work together for assembly of part of the T4SS. We hypothesized that perhaps TraHN requires the lytic transglycosylase AtlAN to make an opening in the cell wall for TraHN to pass through and that perhaps AtlAN might need TraGN in the inner membrane to access the periplasm.

We used an atlAN deletion mutant to test the necessity of atlA for TraH localization. Outer membrane preparations were examined for TraHN-FLAG3 by western blot. Contrary to our hypothesis, the deletion of atlAN did not significantly reduce TraHN-FLAG3 in the outer membrane (Figure 2b).

Next, we tested the requirement for co-transcribed TraGN, as well as the structural protein TraKN, for TraH localization to the outer membrane. A traGN deletion strain showed significantly reduced TraHN-FLAG3 in the outer membrane, whereas the deletion

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**FIGURE 1**  Schematic drawing of T4SSs showing known and/or predicted localization of (a) 17 T4SS proteins important for DNA secretion by *N. gonorrhoeae*, (b) homologous proteins from the F-plasmid T4SSs (c) homologous proteins from the P-type T4SSs. Previously identified interactions are shown by lines: (a) The TraV/TraK interaction described in Ramsey et al. (2014) (b) Interactions described in Harris et al. (2001) and Harris and Silverman (2004) (c) Interactions from Das et al. (1997) and Das and Xie (2000). OM, outer membrane, IM, inner membrane, PG, peptidoglycan. Proteins shared between the three systems are shown in red/violet colors while F-type-specific proteins are shown in green. Proteins specific to *N. gonorrhoeae* are shown in yellow while VirB11 found only in the P-type system is shown in blue.
|                       | Signal peptide | Topology prediction | Amino acid identity<sup>b</sup> | Experimental evidence for localization (N. gonorrhoeae protein) | Experimental evidence for localization (F-plasmid protein) |
|-----------------------|----------------|---------------------|-------------------------------|---------------------------------------------------------------|-----------------------------------------------------------|
| **Core complex proteins** |                |                     |                               |                                                               |                                                           |
| TraB<sub>N</sub>      | No             | No                  | 1 TMN-In, C-out               | 24%                                                           | Ramsey et al. (2014)                                      | Hu et al. (2019)                                          |
| TraK<sub>N</sub>      | Yes            | Yes                 | No TM                         | 21%                                                           | Ramsey et al. (2014)                                      | Hu et al. (2019)                                          |
| Tra<sub>V</sub>N      | Yes            | Yes                 | No TM                         | 18%                                                           | Ramsey et al. (2014)                                      | Hu et al. (2019)                                          |
| **Other proteins with homology to both F and Vir proteins** |                |                     |                               |                                                               |                                                           |
| TraL<sub>N</sub>      | No             | No                  | 2 TMN-In, C-In<sup>a</sup>    |                                                               |                                                            | Lawley et al. (2003)                                      |
| TraE<sub>N</sub>      | No             | No                  | 1 TMN-In, C-out               | 21%                                                           |                                                            | Arutyunov et al. (2010)                                   |
| TraG<sub>N</sub>      | No             | No                  | 8 TMN-Out, C-Out              |                                                               |                                                            | Firth and Skurray (1992)                                 |
| TraC<sub>N</sub>      | No             | No                  | No TM                         | 24%                                                           |                                                            | Hu et al. (2019)                                          |
| **Proteins with homology to proteins from F-like T4SSs (Hamilton et al., 2005)** |                |                     |                               |                                                               |                                                           |
| Tra<sub>W</sub>N      | Yes            | Yes                 | No TM                         | 26%                                                           | Ramsey et al. (2012)                                      | Arutyunov et al. (2010)                                   |
| TraU<sub>N</sub>      | Yes            | Yes                 | No TM                         | 34%                                                           |                                                            | Arutyunov et al. (2010)                                   |
| Trb<sub>C</sub>N      | No             | Yes                 | No TM                         | 25%                                                           |                                                            | Lawley et al. (2003)                                      |
| TraN<sub>N</sub>      | Yes            | Yes                 | 1 TM C-In                     |                                                               |                                                            | Klimke et al. (2005)                                      |
| Tra<sub>F</sub>N      | Yes            | Yes                 | No TM                         | 26%                                                           |                                                            | Arutyunov et al. (2010)                                   |
| Tra<sub>H</sub>N      | Yes            | Yes                 | No TM                         | 23%                                                           |                                                            | Arutyunov et al. (2010)                                   |
| LtgX<sub>N</sub>      | No             | Yes                 | No TM                         |                                                               |                                                            | Kohler et al. (2007)                                      |
| Dsb<sub>B</sub>N      | Yes            | Yes                 | No TM                         |                                                               |                                                            | Orf169 has a signal sequence                             |
| **Proteins specific for GGI-like T4SS or for the N. gonorrhoeae T4SS (Pachulec et al., 2014)** |                |                     |                               |                                                               |                                                           |
| AtIA<sub>N</sub>      | No             | No                  | No TM                         |                                                               |                                                            | Kohler et al. (2007)                                      |
| Yag<sub>B</sub>N      | No             | No                  | No TM                         |                                                               |                                                            | Proposed peptidoglycan association (Pachulec et al., 2014) |

Abbreviations: N/C-In, N/C terminal end of the protein in the cytoplasm; N/C-Out, N/C terminal end in the periplasm; TM, transmembrane helix.

<sup>a</sup>For TraL<sub>N</sub>, the 6 different predictions shown by the TOPCONS webserver differed concerning the localization of the N-terminal end while all predictions indicated a cytoplasmic localization of the C-terminus.

<sup>b</sup>Amino acid identity between the protein from <i>N. gonorrhoeae</i>, and the corresponding protein from the F-plasmid. The amino acid identity is only calculated for the F-plasmid proteins used in this study.
of traKN had no effect (Figure 2c). Thus TraGN, independent of AtIA\textsubscript{N} and TraKN, is needed for TraHN to be present at significant levels in the gonococcal outer membrane.

To obtain information about the localization of other \textit{N. gonorrhoeae} T4SS proteins, we performed bioinformatic analyses as described in experimental procedures and compiled data from the literature. The outcome is summarized in Table 2.

2.2 | The BACTH and BACTH-TM systems

In the bacterial adenylate cyclase two-hybrid system (BACTH) (Karimova et al., 1998), the proteins of interest fused with the two fragments (T18 and T25) from the catalytic domain of \textit{Bordetella} pertussis adenylate cyclase and interaction between the proteins result in functional complementation between T18 and T25 leading to cAMP synthesis and transcriptional activation of the lactose operon. The BACTH-TM system (Ouellette et al., 2014) inserts a transmembrane helix between the proteins of interest and the T18 and T25 fragments of the adenylate cyclase. While the BACTH system requires the proteins of interest to be located in the cytoplasm or the inner membrane (Karimova et al., 1998; 2005), the BACTH-TM system enables the study of protein interactions in the periplasm (Ouellette et al., 2014). A combination of the BACTH and the BACTH-TM systems can be used to study interactions between inner membrane proteins with a cytoplasmic domain and a periplasmic protein.

2.3 | Interactions: \textit{N. gonorrhoeae} genes cloned into the BACTH and the BACTH-TM system vectors

All proteins were fused with both T18 and T25 fragments. The BACTH vectors pUT18C and pKT25 put the T18 and the T25 fragments in the N-terminal end of the protein while pUT18 (Karimova et al., 2001) and p25N (Claessen et al., 2008) put the T18 and the T25 fragment in the C-terminal end of the protein. The BACTH-TM vectors pUTM18C (Ouellette et al., 2014) and pKTM25 (this study) add a transmembrane domain, and the T18 or T25 fragment in the N-terminal end of the proteins.

According to, respectively, previous work (Ramsey et al., 2014) and our bioinformatics analysis (Table 2), transmembrane proteins Tra\textsubscript{BN} and Tra\textsubscript{EN} are likely to have their N-terminals in the cytoplasm, thus we cloned them in the BACTH vectors pUT18C and pKT25. For the transmembrane proteins Tra\textsubscript{GN} and Tra\textsubscript{LN}, respectively, experimental evidence (Kohler et al., 2013) and bioinformatics analyses indicate that the C-terminal ends of the proteins are likely to be in the cytoplasm while the N-terminal ends are likely to be in the periplasm (Table 2). The genes encoding Tra\textsubscript{GN} and Tra\textsubscript{LN} were therefore cloned in pUT18 and p25N.

Because the BACTH-TM system (Ouellette et al., 2014) had not previously been used for studying periplasmic proteins, we cloned Tra\textsubscript{HN}, Tra\textsubscript{UN}, Tra\textsubscript{NN}, Tra\textsubscript{KN}, and AtIA\textsubscript{N} in both the BACTH vectors pUT18C and pKT25 and the BACTH-TM system vectors pUTM18C and pKTM25 to be able to compare the results obtained with the two systems. Ltg\textsubscript{XN}, Yag\textsubscript{N}, Dsb\textsubscript{CN}, Tra\textsubscript{WN}, Tra\textsubscript{FN}, and Trb\textsubscript{CN} were cloned only in the BACTH-TM system vectors (Table 3). For Tra\textsubscript{HN}, Tra\textsubscript{UN}, Tra\textsubscript{NN}, Tra\textsubscript{KN}, and AtIA\textsubscript{N}, the sequences encoding the signal peptide were detected with SignalP, or in the case of Trb\textsubscript{CN} and Ltg\textsubscript{XN} with TOPCONS, and removed from the sequences before cloning. For Tra\textsubscript{WN} the first 19 amino acids including the assumed lipobox (Ramsey et al., 2014) were removed before cloning. For AtIA\textsubscript{N} and Tra\textsubscript{CN}, we chose to put the T18 and T25 fragments at both ends of the protein, thus these proteins were cloned in all four BACTH vectors pUT18C, pUT18, pKT25, or p25N (Table 3).
| Transmembrane/cytoplasmic proteins | Periplasmic/outer membrane proteins cloned in both the BACHT and the BACHT-TM system | Periplasmic proteins cloned only in the BACHT-TM system |
|-----------------------------------|--------------------------------------------------------------------------|-----------------------------------------------------|
| T18TraCN | TraCNT18 | T18TraBN | TT18 | T18TraEN | TraGNT18 | TraLNT18 | T18AtLAN | TM18AtlA | N | TM18DN | T18TraFN |
| T25TraCN | – | + | – | – | – | – | – | – | – | – | – |
| T25TraBN | – | – | + | + | – | – | – | – | – | – | – |
| T25TraEN | – | – | – | – | – | – | – | – | – | – | – |
| T25AtLAN | – | – | – | – | – | – | – | – | – | – | – |
| T25TraWN | + | – | – | – | – | – | – | – | – | – | – |

Note: +, –, and w indicate, respectively, interaction, no interaction, and weak interaction. Space indicates that the interaction was not tested. The placement of T18 and T25 relative to the protein name indicates N or C-terminal fusion. T18 or T25 indicate that the gene encoding the protein was cloned into the BACTH vectors. TM18 or TM25 indicates that the gene encoding the protein was cloned into the BACTH-TM vectors.
2.4 | Combinations of *N. gonorrhoeae* proteins tested

TraB<sub>N</sub>, TraG<sub>N</sub>, TraE<sub>N</sub>, and TraL<sub>N</sub> are believed to be transmembrane proteins with potential interaction partners in the cytoplasm, the inner membrane, the periplasm and in the case of TraB<sub>N</sub> also the outer membrane. Due to the possible interactions of these proteins with proteins in several cellular compartments, we tested all possible combinations of these proteins cloned in the BACTH system with all other proteins cloned in the BACTH or the BACTH-TM system by co-transformation into *E. coli* BTH10. Functional complementation was assayed as described in the method section (for an overview of the tested combinations of plasmids, see Table 3).

For the proteins cloned only in the BACTH-TM system (LtgX<sub>N</sub>, YagN, DsbC<sub>N</sub>, TraW<sub>N</sub>, TraF<sub>N</sub>, and TrbC<sub>N</sub>), we tested for interactions between proteins cloned in BACTH-TM vectors and for interactions between transmembrane proteins cloned in BACTH-vectors and periplasmic proteins cloned in BACTH-TM vectors (Table 3). A similar test was done for the six proteins cloned in both the BACTH and the BACTH-TM system, that is, TraK<sub>N</sub>, TraV<sub>N</sub>, TraU<sub>N</sub>, TraH<sub>N</sub>, TraN<sub>N</sub>, and Atia<sub>N</sub> (Table 3).

As controls, we tested for interactions between the periplasmic proteins cloned in both the BACTH and the BACTH-TM systems (Table 3). Cloning the periplasmic proteins in the BACTH and the BACTH-TM vectors should result in protein expression in different cellular compartments, therefore no interactions should be observed between periplasmic proteins cloned in the two different vector systems. As expected, we did not observe any interactions between a periplasmic protein cloned in a BACTH vector and a periplasmic protein cloned in a BACTH-TM vector (Table 3).

For TraC<sub>N</sub>, we tested for both a TraC<sub>N</sub>/TraCN interaction and interactions with transmembrane proteins (Table 3).

2.5 | The detected interactions

To examine if the interactions observed for the *N. gonorrhoeae* T4SS proteins were specific for the *N. gonorrhoeae* T4SS proteins or could potentially be general for F-type T4SS proteins, we analyzed interactions among the corresponding F-plasmid proteins using the protein-adenylate fusions summarized in Table 4.

The observed interactions could be classified into three groups. Group 1 consists of interactions observed both with the *N. gonorrhoeae* T4SS proteins and with the corresponding proteins from the F-plasmid (Figure 3a). The OMCC protein homologs TraB and TraV participate in the majority (8/10) of these interactions. The following interaction partners were observed: TraB (TraB, TraW, TraK, TraE, and TrbC) and TraV (TraK, TraV, and TraW). The two additionally shared interactions were TraC/TraC and TraW/TrbC. The TM-TrbCN/TraB<sub>N</sub>, TM-TraV<sub>N</sub>/TM-TraV<sub>N</sub>, and TM-TraV<sub>N</sub>/TM-TraW<sub>N</sub> are seen as weak interactions in Figure 3a. β-Galactosidase measurements showed that the signal was approximately 4, 5, and 8 times.
FIGURE 3  Bacterial 2-hybrid interactions. Left side: schematic drawings showing the observed interactions and the supposed cellular localization of the proteins. Right side: Colonies of *E. coli* BTH101 transformants carrying plasmids encoding the proteins indicated in the order T18/T25. Protein names in green indicate that the gene encoding the protein was cloned in a BACTH-TM system vector, black names indicate that the gene was cloned in the BACTH system vector. C1, C2, C3, and C4 are vector controls; respectively, pUT18C/pKT25, pUTM18C/pKTM25, pUTM18C/pKT25, and pUT18C/pKTM25. (a) Interactions observed both between proteins from the *N. gonorrhoeae* T4SS and the between the corresponding proteins from the F-plasmid. (b) Interactions observed only between proteins from the *N. gonorrhoeae* T4SS. (c) Interactions observed only between proteins from the F-plasmid.
above the background level for the 3 interactions, respectively (data not shown).

Group 2 comprises 10 interactions observed only with periplasmic or membrane-spanning proteins from *N. gonorrhoeae*, not with the F-plasmid homologs (Figure 3b). TraV₉ or TraH₉ are involved in 9 out of the 10 interactions. The following interaction partners were observed for TraV₉ and TraH₉, respectively: TraV₉ (TraH₉, TraF₉, TraB₉, TraB₉, and DsbC₉), TraH₉ (TraB₉, TraU₉, TraF₉, and TraW₉). The last Neisseria-specific interaction observed was between DsbC₉ and TraB₉. Interactions involving DsbC₉ were only tested with proteins from *N. gonorrhoeae* since no relevant homolog is encoded by *N. gonorrhoeae* F-plasmid.

Group 3 comprises 11 interactions observed only with proteins from the F-plasmid (Figure 3c). The main proteins involved in the F-plasmid specific are TrbCF, TraF F, TraU F, and TraK F. TraF F has a redox-active site that is missing in TraF₂, indicating different roles for the two proteins. The interaction observed for TrbC F, TraF₂ and TraU₂ are, respectively, TrbC F (TraV₂, TraF₂, TraU₂, TraK₂, and TrbC₂), TraF₂ (TraB₂, TraW₂, and TraK₂), and TraU₂ (TraW₂, and TrbC₂). The last F-plasmid-specific interaction is between TraC F and TraB₂.

Some of the studied proteins were able to interact with a relatively large number of other proteins, that is, TraB₉ and TraB₂ are involved in 8 and 7 interactions, respectively, TraV₉ is involved in 8 interactions, TrbC₂ is involved in 7 interactions, and TraK₂ is involved in 5 interactions (Figure 3). Some proteins with intrinsic tendency to interact with any protein, the so-called “sticky proteins,” can give rise to false positives in two-hybrid screens (Battesti and Bouveret, 2012); however, all the proteins tested in this study showed selectivity with regard to interaction partners. Whether these interactions would be formed if several, possibly competing interaction partners were present at the same time is, however, a question that cannot be addressed by two-hybrid studies.

In some cases, for instance, for the TraB₉/TraE₂ interaction, an interaction was only observed with one of the two possible combinations of T18 and T25. This has been observed in previous two-hybrid analyses, one possible reason being the different copy numbers of the T18 and T25 plasmids (Battesti and Bouveret, 2012).

No interactions were detected for AtlA₉, LtgX₉, Ya₉, TraG₉, TraN₉, TraN₉, TraL₉, and TraH₉.

### 2.6 Confirmation of selected interactions by co-purification

Since two-hybrid systems can give both false-negative and false-positive results, we aimed to further validate our findings with other methods. For many of the interactions observed in this study, some evidence for the interaction between the studied proteins or homologs from other T4SSs can be found in the literature (Das et al., 1997; Das and Xie, 2000; Gilmour et al., 2001; Harris et al., 2001; Ding et al., 2002; Harris and Silverman, 2004; Chandran et al., 2009; Fronzes et al., 2009; Sivanesan et al., 2010; Low et al., 2014; Ramsey et al., 2014; Casu et al., 2016; Oliveira et al., 2016; Shala-Lawrence et al., 2018; Hu et al., 2019). However, only limited evidence exists for interactions between OMCC proteins homologs (TraB, TraV, and TraK) and F-type-specific periplasmic proteins (Arutyunov et al., 2010), and the function of the F-type-specific periplasmic proteins is poorly understood. Therefore, we chose to concentrate on the confirmation of interactions between the OMCC protein homolog TraV and the periplasmic proteins TraW and TrbC. For TraV₂ and TraV₉ expression, the N-terminal lipobox was omitted. Instead, the proteins were equipped with an N-terminal pelB sequence for periplasmic expression and a C-terminal His-tag. For TraW₉, the whole reading frame was expressed without a His-tag. TraW₉ was retained on Ni-NTA beads in the presence but not in the absence of TraV₉-His, confirming that TraW₉ interacts with TraV₉ (Figure 4 and for further details see Figure S4). We were, however, unable to pull down TraW₂ with TraV₂-His. TrbC₂ was expressed without a His-tag to confirm the F-plasmid-specific interaction between TrbC₂ and TraV₂ by co-purification. Only a construct without the signal sequence gave a

**Figure 4** Co-purification of TraW₉ and TrbC₂ with TraV₉-His and TraV₂-His on Ni-NTA beads. (a) The predicted outcome of the co-purifications if the proteins interact. (b) The samples are separated by SDS-PAGE and visualized with Coomassie blue. Samples eluted from Ni-NTA beads with 250 mM imidazole are shown. For the TrbC₂ the results shown are for TrbC₂ expressed in E. coli Origami2(DE3), all other proteins were expressed in E. coli BL21(DE3) as described under experimental procedures. The calculated molecular weights are 19.5, 17.2, 28.2, and 21.4 kDa for TraV₉, TraV₂, TraW₉, and TrbC₂, respectively.
### Table 5  Mixed interactions *N. gonorrhoeae* T4SS/ F-plasmid T4SS (periplasmic and membrane-spanning proteins)

| Proteins from the F-plasmid T4SS | Proteins of the *N. gonorrhoeae* T4SS |
|----------------------------------|--------------------------------------|
| **T25TraBF**                     | **T25TraCN**                         |
| **T25TraCF**                     | **T25TraEN**                         |
| **T25TraEF**                     | **T25DsbC**                          |
| **TM25TraF**                     | **TM25TraBF**                        |
| **TM25TraHF**                    | **TM25TraCF**                        |
| **TM25TraKF**                    | **TM25DsbC**                         |
| **TM25TraUF**                    | **TM25TraEF**                        |
| **TM25TraVF**                    | **TM25DsbC**                         |
| **TM25TrbCF**                   | **TM25TraEF**                        |
| **TM25TraBN**                    | **TM25TraCN**                        |
| **TM25TraCN**                    | **TM25TraEN**                        |
| **TM25TraEN**                    | **TM25TraKN**                        |
| **TM25TraF**                     | **TM25TraKN**                        |
| **TM25TraF**                     | **TM25TraKN**                        |
| **TM25TraF**                     | **TM25TraKN**                        |
| **TM25TrbCN**                   | **TM25TraKN**                        |

Note: + and – indicate, respectively, interaction and no interactions. Space indicates that the interaction was not tested. The placement of T18 and T25 relative to the protein name indicates N or C-terminal fusion. T18 or T25 indicate that the gene encoding the protein was cloned into the BACTH vectors. TM18 or TM25 indicates that the gene encoding the protein was cloned into the BACTH-TM vectors.
high level of expression and only this protein was used. For pull-down experiments with His-tagged TraVF. TrbC_F was found to co-purify with TraV_F (Figures 4 and S4). Two cysteine residues are found toward the C-terminal end of TrbC_F, giving potentially different folding and protein interactions for TrbC_F expressed in the cytoplasm compared to TrbC expressed in the periplasm. E. coli Origami-2(DE3) is a protein expression strain with mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes. These alterations enhance disulfide bond formation in the cytoplasm. Similar results were obtained for TrbC expressed in the E. coli BL21(DE3) and E. coli Origami-2(DE3) indicating that disulfide bond formation is not important for the pull-down result observed.

2.7 Interactions between proteins from the F-plasmid T4SS and proteins from the N. gonorrhoeae T4SS

For interactions observed with proteins from the F-plasmid T4SS or with proteins from the N. gonorrhoeae T4SS, we examined if one of the proteins could be replaced by the corresponding F or N. gonorrhoeae T4SS proteins giving mixed F/N. gonorrhoeae T4SS interactions (Table 5). For all the interactions shared between the N. gonorrhoeae T4SS and F-plasmid T4SS proteins (Figure 3a), either one or both proteins could be replaced by the corresponding protein from the other system (Table 5 and Figure S3). While the TraV_N/TraK_N and TraV_F/TraK_F interactions were only observed when the genes encoding the two proteins were cloned in the BACTH system, the two mixed interactions TM-TraV_N/TraK_N and TM-TraV_F/TraK_F were observed only for the BACTH-TM clones (Table 5).

Also, for groups 2 and 3 containing, respectively, N. gonorrhoeae-specific interactions and F-specific interactions, several mixed interactions were observed (Table 5, Figures S1 and S2). TraB_N, TraB_F, TraV_N, TraV_F, TrbC_P, and TraK_P, which were able to form several non-mixed interactions, were also proficient in forming mixed interactions. Co-purification confirmed mixed interactions between TraV_F and TraW_N and between TraV_N and TrbC_F (Figure 4).

3 DISCUSSION

3.1 Evaluation of the BACTH-TM system

Since the BACTH two-hybrid system (Karimova et al., 1998) requires the interaction between the T18 and the T25 fragments of the adenylate cyclase to occur in the cytoplasm, it is not optimal for studying periplasmic interactions. Ouellette et al. (2014) developed a modified BACTH system (the BACTH-TM system) where the E. coli OppB transmembrane domain is inserted between the protein of interest and the T18 or T25 fragment, exposing the protein of interest to the periplasmic environment but did not use the system to study interactions between periplasmic proteins. To evaluate the system, we cloned 13 proteins from the N. gonorrhoeae T4SS and 7 proteins from the F-plasmid T4SS system in the BACTH-TM system. For comparison, 6 proteins from the N. gonorrhoeae T4SS and 2 proteins from the F-plasmid T4SS were cloned into both the BACTH and the BACTH-TM systems. For TraH_F and TraU_N, we only observed interactions when the proteins were cloned in the BACTH-TM system (Table 3). Since TraH_N and TraU_N are proteins rich in cysteine residues (9 and 16, respectively), it seems likely that these proteins are unable to fold correctly in the cytoplasm. For periplasmic proteins cloned only in the BACTH-TM system, we observed interactions between TraW and TrbC from both the N. gonorrhoeae T4SS and the F-plasmid T4SS. In several F-type T4SSs, TrbC proteins are fused to the N-terminus of TraW, and TrbC and TraW were found to co-purify, indicating that the TraW/TrbC interaction is important for F-type T4SSs (Shala-Lawrence et al., 2018). These results indicate that BACTH-TM system can be used successfully to study periplasmic protein interactions.

For the peripheral membrane proteins, TraV_N and TraK_N, an interaction in the cytoplasm after removal of signal sequences have previously been demonstrated using a bacterial two-hybrid system (Ramsey et al., 2014). Since the only cysteine in TraK_N and TraK_F is in the predicted signal peptide, the TraV_TraK interaction is unlikely to involve a disulfide bridge (Ramsey et al., 2014). TraV_N, TraK_N, TraV_F, and TraK_F were cloned in both the BACTH and the BACTH-TM systems. The TraV_N/TraK_N and the TraV_F/TraK_F interactions were only observed for the proteins cloned in the BACTH system (Table 3). It is possible that linking the proteins to the inner membrane interferes with the TraV/TraK interaction. We did, however, observe other interactions for TM-TraV_N, TM-TraK_N, TM-TraV_F, and TM-TraK_F (Tables 3 and 4, Figure 3).

3.2 Interactions between F-type-specific T4SS periplasmic proteins

Two interaction groups for F-proteins have been defined by yeast two-hybrid screens (Harris et al., 2001; Harris and Silverman, 2004) - one consisting of the three proteins with homology to the outer membrane core proteins TraV_F, TraK_F, and TraB_F (Harris et al., 2001) and one consisting of F-specific proteins (TrbB_F, TrbL_F, TraW_F, TraU_F, TraH_F, and TraF_F) (Harris and Silverman, 2004). No interactions were observed between the two interaction groups. Our study using bacterial two-hybrid systems confirmed several of these previously observed interactions (Figure 3, Table 3). We have not included TrbB_F and TrbL_F in this study since the N. gonorrhoeae T4SS does not possess a TrbB homolog and the deletion of trbI has been found not to affect DNA secretion by N. gonorrhoeae (Pachulec et al., 2014). For TraW_F, TraH_F, TraU_F, and TraF_F, the study carried out by Harris and Silvermann (Harris and Silverman, 2004) demonstrated TraH_F/TraF_F, TraH_F/TraU_F, and TraW_F/TraU_F interactions. For TraH_F, we were unable to demonstrate any interaction (Table 3). It is possible that anchoring TraH_F to the inner membrane inhibits the formation of a correctly folded TraH_F protein. For TraH_N, we did observe TraH_N/TraF_N and TraH_N/TraU_N interactions similar to the interactions
observed by Harris and Silverman (Harris and Silverman, 2004) (Figure 3b). The TraW_N/TraU_F interaction observed by Harris and Silvermann (Harris and Silverman, 2004) was identified in this study as an F-specific interaction (Figure 3c). Besides, we observed the TrbC_F/TraW_F and TrbC_F/TraV_F interactions demonstrated by Shalawrence et al., 2018 (Figure 3a,c) as well as some new interactions between the F-type-specific proteins (Figure 3b,c).

We demonstrate that TraH_N is an outer membrane-associated protein in N. gonorrhoeae (Figure 2). Its F-plasmid homolog TraH_F also associates with the outer membrane in the presence of other T4SS proteins (Arutyunov et al., 2010). TrbF is required for correct TraH_N localization (Arutyunov et al., 2010). Gonococci do not require TrbI_N for DNA secretion (Pachulec et al., 2014). The transcriptomic study of Remmele, as well as the qRT-PCR results of Ramsey, indicated that the traH_N-traG_N-atlA_N transcript was found at much higher levels than the long transcript containing most other T4SS genes (Remmele et al., 2014; Ramsey et al., 2015). Thus, we sought to determine if TraH_N might work together with AtlA_N or TraG_N. TraG_N was found to affect the localization of TraH_N possibly by stabilizing TraH_N or by facilitating the transport of TraH_N to the gonococcal outer membrane.

3.3 | A possible biological implication of the interaction between TraV/TraK/TraB and F-type-specific proteins

In this study, we observed several interactions between the proposed OMCC protein TraB/TraV/TraK and F-type-specific proteins (Figures 3 and 4, Tables 3–5). The F-type-specific proteins have been assigned a function in pilus assembly/retraction and mating pair stabilization based on mutant studies (Arutyunov and Frost, 2013). Until recently the physical and functional relationship of the T4SS apparatus and the F-pilus has been undefined. However, a recent CryoET study (Hu et al., 2019) indicates that the F-pilus is connected to the T4SS outer membrane complex (OMC). Further, the study indicates that the F pilus nucleates assembly at the outer membrane in a process leading to a structural change in the OMC (Hu et al., 2019). It is tempting to speculate that the F-type-specific proteins are involved in this structural change. Although the N. gonorrhoeae T4SS lacks a pilus, a structural change of the OMC mediated by the F-type-specific proteins might still be needed to allow for substrate transfer.

3.4 | Interactions between TraV and F-type-specific proteins

TraV_F is an outer membrane lipoprotein (Doran et al., 1994). TraH_F, TraF_N, TraU_F, and TraW_F have been shown to localize to the outer membrane when in the context of the complete transfer apparatus, probably with TraF as the anchor protein (Arutyunov et al., 2010). We observed several interactions between TraV_N and TraV_F and F-type-specific periplasmic proteins using BACTH studies (Figure 3a–c). The TraW_N/TraV_N and the TrbC_F/TraV_F interactions were confirmed by co-purification (Figure 4). The results indicate that TraV can anchor F-type-specific periplasmic proteins to the outer membrane both for the N. gonorrhoeae T4SS and the F-plasmid T4SS. The TraV homolog VirB7 is a small lipoprotein that helps to stabilize the outer membrane complex at the outer membrane (Christie, 2016). VirB7 from the A. tumefaciens is only 55 amino acids; however, longer forms of VirB7 with additional functions have been described (Christie, 2016). TraV_F and TraV_N are, respectively, 193 and 171 amino acids with only the N-terminal part of the proteins showing weak homology to VirB7 (Ramsey et al., 2014). It is, therefore, possible that the C-terminal part of TraV could be involved in interactions with F-type-specific proteins.

3.5 | The TraB–TraE interaction

Due to the high divergence of the primary sequence, some VirB8 homologs have been identified only upon structural analysis (Goessweiner-Mohr et al., 2013). A bioinformatic study placed TraE_F in a universally present group of VirB8 homologs (Guglielmini et al., 2014) and secondary structure predictions also indicate that TraE_F and TraE_N are VirB8-like proteins (Goessweiner-Mohr et al., 2013). CryoEM of a P-type T4SS from the conjugative R388 plasmid shows that the inner membrane complex consists of the N-terminal part of VirB10 in connection with a set of other inner membrane-associated proteins including VirB8 (Low et al., 2014). Interactions between VirB8 homologs and VirB10 homologs have been demonstrated using the BACTH system (Casu et al., 2016) as well as other two-hybrid systems (Das and Xie, 2000; Ding et al., 2002). TraE_F has been shown to associate with the inner membrane (Arutyunov et al., 2010) and is essential for conjugation (Lawley et al., 2003), but the function of the protein is unknown. We observed the interaction between TraB and TraE for both proteins from the N. gonorrhoeae T4SS and proteins from the F-plasmid (Figure 3a) and a mixed TraE_F/TraB_F interaction (Table 5 and Figure S3). This result indicates that in addition to inner membrane localization, TraE shares with VirB8 the ability to interact with the VirB10 homolog TraB.

3.6 | Cross-system interchangeability of T4SS proteins

In this study, we observed several interactions between proteins from the F-plasmid T4SS and proteins from the N. gonorrhoeae T4SS system, indicating a high degree of cross-system interchangeability of homologous T4SS proteins despite low sequence homology (Table 5, Figures S1–S3). This phenomenon has been observed in several other studies (Gillespie et al., 2015; Casu et al., 2016; Carraro et al., 2017; Gordon et al., 2017). For P-type T4SS, there are indications for cross-system interchangeability between VirB8, VirB10, and VirB5 homologs (Schmidt-Eisenlohr et al., 1999; Gillespie et al.,...
2015; Casu et al., 2016; Gordon et al., 2017). In nature, this cross-system interchangeability might be important for bacteria carrying more than one T4SS (Gillespie et al., 2015). With regard to F-type T4SSs, it is not unusual for multidrug-resistant enterobacteria to carry both IncF and IncA/C plasmids (Rayamajhi et al., 2011; Silva et al., 2015). Like IncF plasmids, IncA/C plasmids encode F-type T4SSs (Harmer and Hall, 2015). An interesting example of crosstalk occurs between an IncA/C plasmid and *Salmonella* genomic island 1 (Carraro et al., 2017). While the IncA/C plasmid encodes an F-type T4SS, the *Salmonella* genomic island 1 only encodes homologs of TraN, TraH, and TraG with amino acid identity between 37% and 78% to the plasmid proteins (Carraro et al., 2017). The Tra subunits of the genomic island can complement their plasmid counterpart in mutant studies; however, the outcome of the conjugation is shifted toward the spread of the genomic island rather than the IncA/C plasmid (Carraro et al., 2017). The presence of an IncF plasmid increases the conjugation rate of co-residing IncA/C plasmids by an unknown mechanism (Gama et al., 2017). Our data support cross-system interchangeability between F-type T4SS proteins. This interchangeability might be a way different co-residing conjugative plasmids can interact in processes that could influence the spread of antibiotic resistance.

In conclusion, our results indicate that the T4SSs from the F-plasmid and *N. gonorrhoeae* share an overall architecture, especially with regard to conserved T4SS protein homologs (Figure 3a). However, interactions between F-type-specific proteins and between F-type-specific proteins and conserved T4SS proteins (TraV, TraK, and TraB) exhibit more variation between systems (Figure 3b,c). We present maps of the protein interactions that build these two F-type T4SSs and demonstrate that multiple protein components are likely interchangeable within these interaction networks.

### 4 | EXPERIMENTAL PROCEDURES

#### 4.1 | Bacterial strains and growth conditions

All bacterial strains are listed in supplementary material Table S1. *N. gonorrhoeae* MS11 was grown on GC chocolate agar plates with VCAT (EO labs.) at 5% CO2 at 37°C or in GCBL liquid medium containing 0.042% NaHCO3 and Kellogg’s supplement (Kellogg et al., 1963) with aeration at 37°C. *E. coli* were grown in LB medium (Bertani, 1951) at 37°C or 30°C. Antibiotics were used at the following concentrations: ampicillin (100 µg/ml), kanamycin (50 µg/ml), and streptomycin (50 µg/ml).

#### 4.2 | Construction of plasmids

Chromosomal DNA from *N. gonorrhoeae* MS11 was isolated from liquid overnight cultures using the GenElute Bacterial Genomic DNA kit from Sigma following the recommendation of the manufacturer except that approx. 8–9 ml of overnight culture was used for each preparation (rather than 1.5 ml) to compensate for a low OD600 in the overnight cultures. *N. gonorrhoeae* genes were amplified with *N. gonorrhoeae* MS11 chromosomal DNA as a template using primers 3–43, 67, 68, 71, and 72 (supplementary material Table S2). Genes encoded by the F-plasmid were PCR amplified with cell lysates of *E. coli* JM101 as a template using primers 44–64 and 75–78 (Table S2). The PCR products were digested with restriction enzymes cutting the restriction sites underlined in Table S2 and cloned into pKT25 (Karimova et al., 2001), pKTM25 (this study), p25N (Claessen et al., 2008), pUT18C (Karimova et al., 2001), pUTM18C (Ouellette et al., 2014), or pUT18 (Karimova et al., 2001) digested with the same restriction enzymes. Cloning into pCOLADuet-1 and pET22b were done with the primers indicated in supplementary material Tables S1 and S2 using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs.) according to the manufacturer’s instruction All plasmids are listed in supplementary material Table S1. The structure of all plasmids was confirmed by DNA sequencing. The sequences of the *N. gonorrhoeae* MS11 genes were found to match the sequence derived from Accession no. CP003909.

#### 4.3 | Construction of *N. gonorrhoeae* mutants, subcellular fractionation, and western blotting

The traH+ gene from *N. gonorrhoeae* strain MS11 was cloned into pMR100 to add the 3x-FLAG tag in-frame with the TraH+ coding sequence, creating the intermediate pAY25. The resulting traH+FLAG3 gene was subcloned from pAY25 into pMR68 to place it under transcriptional control by the anhydro-tetracycline-inducible promoter and locate it between gonococcal genes *iga* and *trbP*. The resulting plasmid, pAY27, was then used to insert the traH+FLAG3 construct onto the gonococcal chromosome in wild-type *N. gonorrhoeae* strain MS11 or its derivatives lacking traK+ (MR535, Ramsey et al., 2014), traG+ (PK186, Kohler et al., 2013), or atlA+ (PK127, Kohler et al., 2007). To FLAG3-tag TraH+ at the native locus, a fragment of DNA downstream of the traH native site was cloned in pAY25, creating pAY28. pAY28 was used to transform MS11, generating AY529. Gonococci were transformed as previously described (Ramsey et al., 2015). The expression of TraH+FLAG3 was induced with 0.2 ng/ml anhydro-tetracycline. Subcellular fragmentation and western blots were performed essentially as described before (Ramsey et al., 2014, 2015). For the western blots approximately 5 µg of protein from each fraction was subject to SDS-PAGE.

#### 4.4 | Bioinformatic analyses

For in silico localization studies, we used the SignalP 4.1 Server with the default setting for Gram-negative bacteria (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011), the TatP 1.0 server (http://www.cbs.dtu.dk/services/TatP/) (Bendtsen et al., 2005), and TOPCONS server (http://topcons.cbr.su.se/pred/reference/) (Tsirigos et al., 2015). Amino acid identity between the protein from...
4.5 | Determination of interactions and measurements of β-galactosidase activity

The initial interaction screening was done after the co-transformation of T18 and T25 encoding plasmids into E. coli BTH101, plating on LB agar plates with appropriate antibiotics, 0.5 mM IPTG and 40 μg/ml X-gal and incubated for 40–48 hr at 30°C. In the absence of functional complementation between T18 and T25, the colonies are white, while they are blue when functional complementation occurs. As a negative control, we used BTH101 cells co-transformed with vectors containing no inserts. For confirmation of positive interactions, the cells expressing a T18- and a T25-tagged protein were grown overnight at 30°C in LB with appropriate antibiotics. The overnight cultures were diluted with 0.9% NaCl and dilutions were spotted on LB agar plates with appropriate antibiotics, 0.5 mM IPTG, and 40 μg/ml X-gal and incubated for 40–48 hr at 30°C.

For β-galactosidase assays, cells were grown overnight at 30°C in LB with appropriate antibiotics and 0.5 mM IPTG and β-galactosidase activities were measured as described by Miller (1972).

4.6 | Protein expression and co-purification

TraV N, TraV f, and TraW N were overproduced in E. coli BL21(DE3) while TrbC f was overproduced in both E. coli BL21(DE3) and E. coli Origami2(DE3). For TraV N and TraV f expression, the N-terminal lipobox was replaced with a pelB signal sequence for periplasmic expression while his-tags were added in the C-terminal end. The entire reading frame of TraW N was expressed while E. coli TrbC f was expressed without the signal sequence since the attempt to express TrbC f with the signal sequence gave a low level of expression. Cells of E. coli BL21(DE3) carrying either pET22bTraV N, pET22bTraV f, pCOLATraW N, or pCOLATrbc f constructs or E. coli Origami2(DE3) carrying pCOLATrbc N were grown in LB at 37°C to an OD 600 of approx. 0.4. Overproduction was induced by the addition of IPTG to a final conc. of 1 mM and incubation was continued for 3 hr at 37°C. The cells were harvested by centrifugation and the cell pellets were frozen (−20°C). After one round of freezing and thawing, partial binding of TraV N and TraV f to nickel resin was observed (Figure S4) and samples treated this way were used for co-purification and control experiments. For control experiments, cells carrying expression plasmids were replaced with cells carrying pCOLADuet-1 or pET22b. For resin binding, samples were applied to 0.5 ml of washed and equilibrated Ni-NTA agarose beads (QIAGEN, Hilden, Germany) and incubated overnight at 4°C, with mixing. The beads were washed with 50 mM NaH 2PO 4, 300 mM NaCl, 20 mM imidazole at pH 8. Subsequently, bound proteins were stepwise eluted with 1 ml of 25 mM NaH 2PO 4, 150 mM NaCl, 125 mM imidazole at pH 8 and 1 ml of 50 mM NaH 2PO 4, 300 mM NaCl and 250 mM imidazole (Figure S4). Equal amounts of the eluted sample from co-purification and control experiments were analyzed by SDS-PAGE.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Koch B, Callaghan MM, Tellechea-Luzardo J, Seeger AY, Dillard JP, Krasnogor N. Protein interactions within and between two F-type type IV secretion systems. *Mol Microbiol.*, 2020,00:1–16. https://doi.org/10.1111/mmi.14582