5 DISCUSSION

Type IV secretion is a common secretory pathway which has become increasingly important since the discovery of its involvement in pathogenicity of a growing number of bacterial species. Human pathogens like *Helicobacter pylori* and *Bartonella henselae* use type IV secretion systems (T4SS) for delivery of effector molecules that cause disease of the colonized eukaryotic host cells. T4SS also account for the spreading of antibiotic resistances among bacteria by means of bacterial conjugation. The investigation of the detailed mechanisms of T4SS is therefore of central interest for modern health care and health management.

Despite the extensive research on this area (reviewed in ref. 19), many functional aspects of T4SS remain unclear. A set of proteins that participate in the formation of a membrane-spanning complex and in pilus synthesis is conserved in these secretion systems (18, 79, 112). Additionally, a membrane protein that does not directly join into the latter functions is required: the TraG-like protein (coupling protein). To provide insights into the function of TraG-like proteins, the genetic determinants and the biochemical properties of four members of this family originating from different type IV secretion systems were analyzed: TraG, TrwB, TraD and HP0524. These proteins belong to conjugative DNA transfer systems of plasmids RP4, R388 and F and to the pathogenicity-related type IV secretion system of the gastric pathogen *H. pylori*, respectively.

5.1 Sequential and structural conservation of TraG-like proteins

Sequence comparison of TraG-like proteins of conjugative DNA transfer systems and of other T4SS showed that TraG-like proteins contain two conserved domains harboring five central motifs (Figure 4.2). Sequence interpretation and alignment of the sequences to the structural information obtained from the crystal structure of truncated TrwB (TrwBΔN70) enabled the identification of supposedly conserved structural features.

The two sequence domains conserved throughout the family of TraG-like proteins harbor the structural unit that has been identified as the nucleotide binding domain (NBD) of TrwB (30, 31). The core of this structure, the nucleotide binding pocket, is formed by structural elements comprised in sequence motifs I, II and III. The nucleotide binding pocket is closely neighbored by a loop structure that is located in motif V. Sequence motif IV harbors structural elements that do not belong to the nucleotide

binding pocket but lie on the inside of the channel formed in TrwBΔN70 hexamers (Figure 5.1).

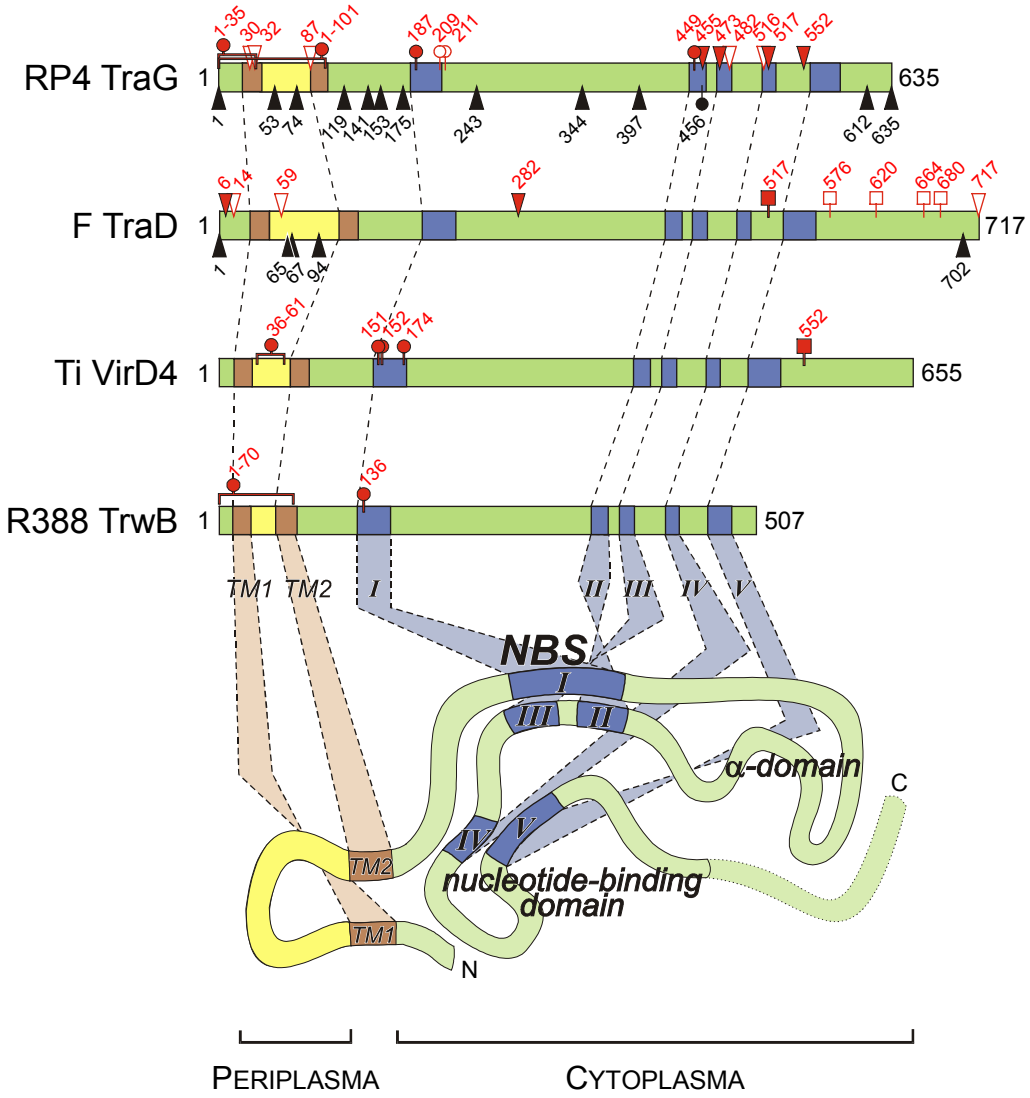

Figure 5.1. Domain structure of TraG-like proteins and DNA transfer activity of defined mutants. The domain structures of RP4 TraG, F TraD, Ti VirD4 and R388 TrwB are aligned. Protein domains are labeled as follows: cytoplasmic domains (green), periplasmic domains (yellow), transmembrane segments (*TM*, brown) and conserved sequence motifs I-IV (blue). The topology of the membrane anchor has been determined for TraD (53), TraG [this work and (87)] and VirD4 (22) and was predicted for TrwB from the calculated hydrophobicity profile of the amino acid sequence. Mutation derivatives of TraG-like proteins that have so far been characterized are indicated as follows: arrowheads [31-codon in frame insertion mutants or His_6-fusions (amino- or carboxyterminal)], circles (point mutants or deletions) and squares (introduction of a stop codon). The DNA transfer activity of each mutant is marked by red filling (abolished), red outlining (significantly reduced) and black filling (slightly reduced to normal activity). A schematic representation of the structure of TraG-like proteins is drawn below. It is based on the information provided by the crystal structure of the truncated TrwB derivative TrwBΔN70 lacking the first 70 residues (31). The nucleotide binding domain contains the conserved sequence motifs I-V, whereas the α-domain is less conserved. The nucleotide binding site (NBS) is located at motifs I, II and III. The length of the C-terminal domain (downstream of motif V) varies most significantly between the different TraG-like representatives. Data on the conjugation frequencies of the different mutants were assembled from the indicated references: TraG (4, 43, 87), TraD (43, 53, 83, 87); VirD4 (48).

The membrane topologies of TraG-like proteins TraD (F) and VirD4 (Ti) have been determined previously (22, 53). In the present work, the topology was also determined for RP4 TraG, using the same method that has been used for topology analysis of F TraD. For this, TraG mutants with in frame insertions containing a trypsin-sensitive motif were generated (4.2). The mutant proteins were assayed for trypsin sensitivity of their periplasmic domain in a whole-cell trypsinization assay of spheroplasted cells expressing the mutant proteins. By this means, the periplasmic domain (trypsin-sensitive insertion mutants) and the cytoplasmic domains (trypsin-resistant insertion mutants) of TraG were identified. The determined allover topology of TraG was reminiscent of the topologies determined for TraD and VirD4: a short cytoplasmic N-terminal sequence is followed by a periplasmic domain of approximately 40 residues and a large cytoplasmic C-terminal domain (Figure 5.1).

The insertion mutants of TraG that were generated for topology analysis and the derivatives constructed for purification (His_6-TraG, TraG-His_6, His_6-TraGΔ1, and His_6-TraGΔ2) were also phenotypically characterized. The transfer activity phenotype of each mutant was determined in mating experiments. The mating assay consisted of a complementation system involving two plasmids: one encoding the entire operon required for conjugative transfer with exception of *traG* and one encoding a copy of each *traG* insertion mutant (*traG::i31*, Table 4.1). Along with the mutant phenotypes analyzed in a previous study (4), the new set of mutant phenotypes enables the identification of functionally relevant determinants of TraG. Additionally, data from mutational analysis of other TraG-like proteins, including F TraD, Ti VirD4 and R388 TrwB are available. The complete set of data is summarized in Figure 5.1. Here, the sequential determinants are mapped to the structural and topological determinants of the proteins, providing a detailed overview on the relationship between sequence, structure and function of TraG-like proteins.

In summary, the mutational analysis of TraG-like proteins leads to the following conclusions:

1. Mutations in any of the conserved motifs I-V substantially attenuated or abolished the activity, consistent with these conserved regions playing an essential role in the mechanism of secretion.
2. Insertions into transmembrane segments were hardly tolerated. Thus, proper translocation of the periplasmic domain is required for transfer activity. Likewise,

deletion of the cytoplasmic N-terminus or of the entire membrane anchor resulted in transfer-deficient proteins.

3. In-frame insertions into the periplasmic domain were mostly tolerated although deletion of the entire periplasmic domain was not. This indicates that the periplasmic domain may have a strictly structural importance rather than an enzymatic function.
4. Insertions into the domain between TM2 and motif I did not significantly affect the transfer activity. Similarly, insertions into the α-domain were mostly tolerated. These domains can therefore be categorized as non-essential.
5. Truncations of the C-terminus (past motif V) of F TraD severely reduced but did not abolish F-transfer activity. Such truncations were however seen to alter the specificity of transfer, since the mutations resulted in a broadening of the range of mobilizable plasmids (83). Thus, the C-terminus of TraD is probably responsible for the specificity of interaction with the F plasmid relaxosome. The specificity determinant of the C-terminus is more or less pronounced in the different representatives of TraG-like proteins, which either promote transfer of a broad range of plasmids or are restricted to a very specified interaction with their own relaxosome. Consistently with this assumption, the sequence and the length of the C-terminus varies considerably among TraG-like proteins.

5.2 TraG-like proteins are oligomeric proteins that bind to DNA, nucleotides and relaxase but do not hydrolyze NTPs *in vitro*

TraG-like proteins originating from four different conjugation/ secretion systems were purified for biochemical analysis: TraG (RP4), TrwB (R388), TraD (F) and HP0524 (*H. pylori*). The full-length forms of TraG and TraD were purified as N-terminally His_6-tagged derivatives His_6-TraG and His_6-TraD. Additionally, deletion derivatives lacking the membrane anchor (TraGΔ2, TrwBΔ1, HP0524Δ1) and derivatives containing a point mutation in the putative nucleotide binding site (TraGK187T, TraGΔ2K187T) were purified. These proteins were analyzed for the putative nucleoside triphosphate hydrolase (NTPase) activity and the potential DNA-, nucleotide- and relaxase-binding ability. The biochemical properties of the TraG-like proteins thus analyzed are summarized in Table 5.1.

Table 5.1. Biochemical properties of TraG-like proteins and derivatives analyzed in the present work.

Protein	Transfer activity	Solu-bility	Oligomeri-zation	NTPase activity	Nucleotide binding	DNA binding	Relaxase binding
TraG	+[a]	+	+	–	NA[b]	+	+
TraGK187T	–	+	+	–	NA	+	+
TraGΔ1	–	–	NA	NA	NA	NA	NA
TraGΔ2	–	++	–	–	+	+	–
TraGΔ2K187T	ND[c]	++	–	–	+/–	+	–
TraD	+	+	+	–	ND	+	ND
TrwB[d]	+	+	+	–	+	ND	ND
TrwBΔ1/ TrwBΔN70[e]	–	++	–	–	+	++	ND
HP0524Δ1	ND	+/–	(+)[f]	–	NA	+	ND

[a] +, positive; –, negative; +/–, intermediate; ++, high/strong
[b] NA, not applicable
[c] ND, not determined
[d] studied by Hormaeche *et al.* (39)
[e] properties of TrwBΔN70 were studied by Moncalián *et al.* (64)
[f] with reservations (see text)

Oligomeric state

Full-length TraG and TraD had a pronounced tendency to form oligomers or aggregates, as determined by gel filtration and glycerol gradient centrifugation. Likewise, full-length TrwB behaved as a hexamer (39). In contrast, truncated derivatives TraGΔ2 and TrwBΔ1 behaved as monomers in solution. It is concluded that the membrane anchor of TraG and TrwB is responsible for intermolecular self-interactions. Whereas these interactions lead to TraG aggregation and TrwB hexamerization *in vitro*, they are probably important for the self-assembly of the protein in the cell membrane *in vivo*. Unlike TraGΔ2 and TrwBΔ1, truncated HP0524 (HP0524Δ1) behaved like a tetra- or pentamer upon gel filtration. It is however not possible to draw basic conclusions from the latter observation because of the protein's generally very restricted solubility.

NTPase activity

NTPase assays were carried out in the presence of Mg^{2+} with each of the purified TraG-like proteins. ATP- or GTP-hydrolysis was however not detected for any of the TraG-like proteins, regardless of the conditions applied. The conclusion is that, despite their sequence similarity to NTPases, TraG-like proteins do not possess NTPase activity, at least not under *in vitro* conditions. An additional factor may induce/stimulate these proteins to function as NTPases *in vivo*. Among the many possibilities, ssDNA, Ca^{2+} or

T4SS proteins of the VirD2- (relaxases), VirB4- or VirB11-type have hitherto failed to promote NTPase activity of TraG-like proteins *in vitro*. The finding that TraG-like proteins bind to nucleotides and that binding is inhibited by Mg^{2+} (see below) indicates that these proteins do not function as conventional ATPases, which usually require the presence of a divalent cation for hydrolysis.

Nucleotide-binding activity

Nucleotide-binding was assayed by measuring the fluorescence increase of fluorescent nucleotide-derivatives (TNP-nucleotides) upon binding. Due to technical requirements, nucleotide-binding was measurable only for truncated derivatives of TraG and TrwB. TraGΔ2 and TrwBΔ1 were shown to bind ATP with moderate affinity [K_d^{ATP}(TraGΔ2) = 0.34 mM and K_d^{ATP}(TrwBΔ1) = 0.13 mM]. Additionally, both proteins were found to bind ADP, with a similar affinity as was found for ATP-binding. Compared to TraGΔ2, mutant TraGΔ2K187T had a significantly reduced nucleotide-binding ability, which may account for the earlier reported transfer-defective phenotype of TraGK187T (4). Competition experiments revealed that other NTPs were able to displace protein-bound ATP and that the diphosphate moiety of nucleotides was the core structure required for binding. The presence of DNA markedly reduced ATP-binding of TrwBΔ1 and, reversibly, DNA-binding was inhibited by the presence of ATP (see below). This effect was less pronounced in case of TraGΔ2, whose DNA-binding capacity was merely lowered by the presence of nucleotides. Both proteins however strongly responded to the presence of Mg^{2+}, which significantly inhibited ATP- and ADP-binding of TraGΔ2 and TrwBΔ1.

Inhibition of ATP-binding (more specifically inhibition of TNP-ATP-binding) by Mg^{2+} has been reported previously for cation pumps K_{ATP} (102) and Ca^{2+}-ATPase (66). It was observed for Ca^{2+}-ATPase that low (10^{-5}-10^{-2} M) levels of Mg^{2+} induced an increase of ATP-binding, whereas higher (above 10^{-2} M) levels of Mg^{2+} lowered the affinity for ATP. These effects were proposed to be a consequence of the formation of Mg·ATP (K_d= 0.3 mM) and Mg_2·ATP (K_d= 20 mM), respectively, which are bound with differential affinity (50). Additionally, the protein was proposed to bind Mg^{2+} (K_d= 0.1 mM) and to undergo conformational changes upon Mg^{2+}-binding (66). In the competition experiment carried out with TraGΔ2, significant inhibition by Mg^{2+} occurred at concentrations as low as $5{\cdot}10^{-4}$ M (70% fluorescence reduction). Under these conditions, 61% of the TNP-ATP molecules form a binary complex with Mg^{2+}

(assuming $K_d^{TNP\text{-}ATP\cdot Mg} = K_d^{ATP\cdot Mg} = 0.3$ mM) and only 2.4% exist as a ternary Mg^{2+} complex (assuming $K_d^{TNP\cdot ATP\text{-}Mg_2} = 20$ mM). Whether Mg·TNP-ATP formation was the reason for the observed decrease in binding affinity, or whether direct Mg^{2+}-binding to TraGΔ2 induced TNP-ATP release through conformational changes remains to be assessed. Interestingly, the opposite effect of Mg^{2+} was observed for the non-catalytic binding sites of *E. coli* F1-ATPase: here, the presence of Mg^{2+} was absolutely required for TNP-ATP-binding (109).

DNA-binding activity

TraG-like proteins were assayed for binding of double-stranded DNA (dsDNA) in fragment retardation (fragment shift) experiments. TraG and TraD, as well as truncated derivatives TraGΔ2, TrwBΔ1 and HP0524Δ1 were shown to bind dsDNA. Sequence specificity for the *oriT* sequence was excluded in case of TraG and TraD. Competition experiments with single-stranded DNA (ssDNA) furthermore revealed that TraG, TraD and TrwB also bind to ssDNA and that ssDNA is the preferred DNA substrate. The dsDNA-binding activity of TrwBΔ1 and TraGΔ2 was quantified in a standardized assay. The binding activity of TrwBΔ1 was thereby shown to be threefold higher than the one measured for TraGΔ2 [K_d^{app}(TrwBΔ1) = 24 nM and K_d^{app}(TraGΔ2) = 75 nM]. Similarly to nucleotide-binding, dsDNA-binding of TrwBΔ1 and TraGΔ2 was observed to be inhibited by the presence of Mg^{2+}. Also, the presence of ATP and ADP reduced the DNA-binding activity of TrwBΔ1, indicating that DNA-binding was competed by nucleotide-binding. The interference between both binding activities and their common sensibility to Mg^{2+} further suggests that the DNA- and nucleotide-binding domains have a structural and functional overlap.

Relaxase-binding activity

Interactions between TraG and relaxase TraI have been postulated earlier (43, 72). Direct evidence for this interaction was now obtained by measuring the protein-protein interactions between purified TraG and TraI *in vitro*, using the surface plasmon resonance (SPR) technique (BIACORE®). Binding of TraI was shown to be tight and specific and to occur rapidly ($k_a = 10^5\ M^{-1}s^{-1}$). Mutant TraGK187T interacted with TraI to the same extent, indicating that the conserved residue K187 of motif I is not required for TraI-binding. Interaction analysis with truncated derivative TraGΔ2 revealed that removal of its membrane anchor prevented the interaction with relaxase. Thus, the N-

terminal membrane anchor of TraG (residues 1-102) is not only essential for TraG self-interactions, but also for TraG-relaxase interactions.

5.3 Functional dissection of DNA-, nucleotide- and relaxase-binding activities of RP4 TraG

Apart from characterizing the basic functions of TraG, the biochemical analysis of TraG and derivatives TraGK187T, TraGΔ2 and TraGΔ2K187T provided insights into the structure-function relationship of TraG. The isolated cytoplasmic domain of TraG was found to bind DNA as well as ATP and ADP, without requirement of the membrane anchor. The binding domains for DNA- and nucleotide-binding probably have a structural and functional overlap. The Walker A sequence signature at residue K187 is important for nucleotide-binding but is not essential for DNA- or relaxase-binding. Relaxase-binding however requires the presence of the membrane anchor, which is also needed for oligomerization. As is outlined below, the membrane anchor does probably not directly interact with TraI, but is a structural requirement needed for TraG-TraI interaction. The assignment of the biochemical functions of TraG to different protein domains is summarized in Figure 5.2.

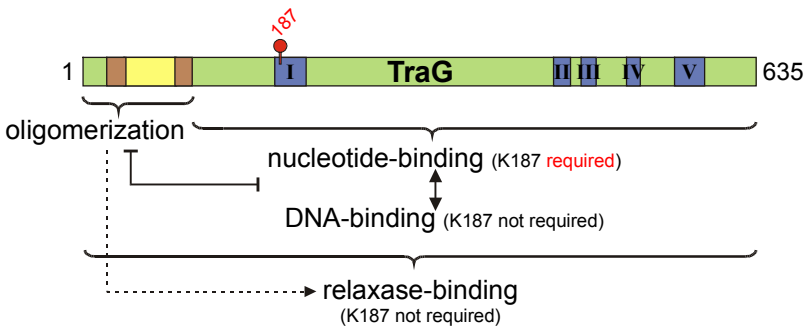

Figure 5.2. Functional domains of TraG. The domain structure of TraG is drawn schematically. Labels and numbers are as in Figure 5.1. Biochemical functions of each delimited domain are indicated. The arrows in the scheme have the following meanings: nucleotide- and DNA-binding activities interfere with one another; oligomerization is probably a prerequisite for relaxase-binding; nucleotide- and DNA-binding function independently from oligomerization.

Relation between oligomerization and relaxase-binding of TraG

The failure of truncated TraG to oligomerize may be related to its failure to interact with relaxase. It is conceivable that TraG assembly should occur prior to relaxase-binding since the protein needs first to be properly inserted into the membrane and to build its final putative pore-like architecture before binding to another bulky protein like relaxase. Another possible explanation for the defect in relaxase-binding of TraGΔ2 is

that the relaxase-interacting domain is situated in the deleted N-terminus. This N-terminus consists of a short cytoplasmic tail (residues 1-23) followed by a transmembrane segment, a periplasmic domain (residues 44-82) and a second transmembrane segment. After excluding the transmembrane segments, the periplasmic and cytoplasmic regions of the membrane anchor remain as possible domains for relaxase-interaction. Since relaxase is a cytoplasmic protein, the periplasmic domain of TraG is unlikely to play a role in relaxase-interaction. Thus, apart from the possibility that oligomerization of the protein *per se* is a requirement for relaxase-interaction, the short cytoplasmic domain preceding the first membrane segment may be required for this interaction, although it is probably too short to be a domain of its own.

The differential results provided by SPR analysis of TraG-TraI*, TraGΔ2-TraI* and TraI-TraGΔ2* interactions, where * indicates the immobilized ligand, demand an explanation. Whereas TraGΔ2 did not interact with immobilized TraI, free TraI did however interact with immobilized TraGΔ2. These results can be interpreted when assuming that oligomerization of TraG is a prerequisite for relaxase-binding, as was discussed above. The following model is proposed: immobilization of TraGΔ2 by covalent linkage to a solid surface, as in the experimental setup for SPR, may promote self-association of TraGΔ2 (Figure 5.3). The faint interaction measured for binding of TraG to immobilized TraGΔ2 (Figure 4.23) indeed indicates that TraGΔ2 has a low residual ability for self-interactions.

According to the model, immobilization of TraGΔ2 mimics membrane anchoring and stabilizes TraGΔ2 self-interactions, which on their turn enable complex formation between TraGΔ2 oligomers and relaxase. It is thus conceivable that immobilized TraGΔ2 can form TraGΔ2-multimers, whereas free TraGΔ2 in solution cannot. In this context, it is worth mentioning that the normally monomeric, truncated TrwB-derivative TrwBΔN70 was observed to oligomerize upon crystallization and electron microscopy (31). Similarly to TraGΔ2 immobilization to chip surfaces, the conditions for electron microscopy and for crystallization of TrwBΔN70 may also have supported the formation of protein oligomers that are normally too labile to exist in free solution.

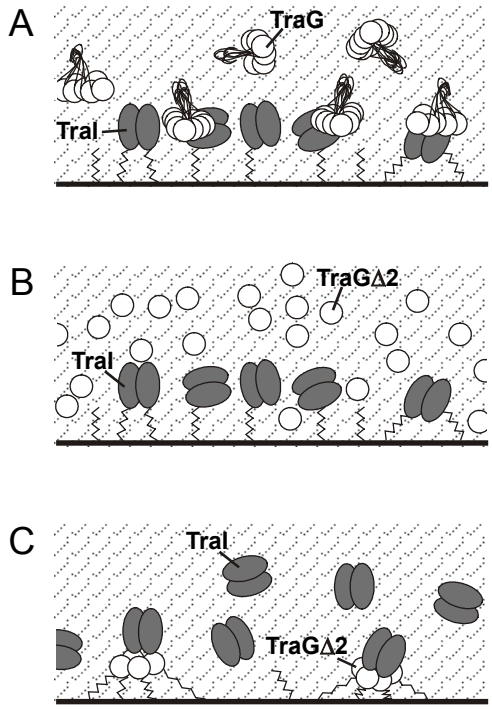

Figure 5.3. Model explaining the different interactions between TraG, TraGΔ2 and TraI. (A) Full-length TraG forms stable oligomers that interact with immobilized TraI. **(B)** TraGΔ2 is monomeric in solution and cannot interact with immobilized TraI. **(C)** TraI can interact with immobilized TraGΔ2, since immobilization and local concentration of TraGΔ2 allows the protein to multimerize.

5.4 The role of TraG-like proteins in type IV secretion systems

TraG-like proteins are transporters for delivery of specific substrates into the periplasmic space

T4SS function as active transporters for delivery of substrates destined for secretion. A question of central interest is how the energy for this transport is provided. Sequence analysis of T4SS-encoded proteins indicated that NTP hydrolysis may be the motor for type IV secretion, since three proteins with putative NTPase activity were identified. Apart from the TraG-like proteins, these include the VirB4-like and the VirB11-like proteins. Each of these proteins is an essential component of the studied T4SS. The proposed NTPase activity was confirmed *in vitro* for three proteins of the VirB11-family that were also seen to form hexamers (44, 45, 77). The crystal structures of HP0525 (*H. pylori*) and of its nucleotide-bound form furthermore suggested a role in export of substrates and/or a chaperone-like function in the assembly of the type IV secretion apparatus itself (84, 114). In contrast, purified forms of the VirB4-like proteins TrbE (RP4) and TrwK (R388) were found to lack NTPase activity. Still, mutation in the putative nucleotide binding site produced a transfer-deficient phenotype (75), indicating that nucleotide-binding is an essential function of VirB4-like proteins. The same effect was observed for TraG-like proteins, which equally lack NTPase activity *in vitro*. A specific conformation or an additional factor may be required for

these proteins to induce NTPase activity.

Several observations have however led to the conclusion that TraG-like proteins function as active transporters that route the substrate to be secreted through the inner membrane:

a) TraG-like proteins were found to bind DNA (64, 69, 87), which is the primary substrate of the T4SS used in bacterial conjugation.
b) TraG-like proteins interact with protein components of the relaxosome (25, 87), which is the protein-DNA complex that is formed for processing of the transfer-DNA in bacterial conjugation.
c) The crystal structure of the truncated TraG-like protein TrwBΔN70 showed that the cytoplasmic domain of TrwB forms a hexameric channel structure that probably protrudes through the inner membrane (31).

These findings lead to the hypothesis that TraG-like proteins form an inner membrane pore specifically recognized by the secreted substrates. A model illustrating the proposed architecture of the TraG-associated RP4 relaxosome is presented (Figure 5.4).

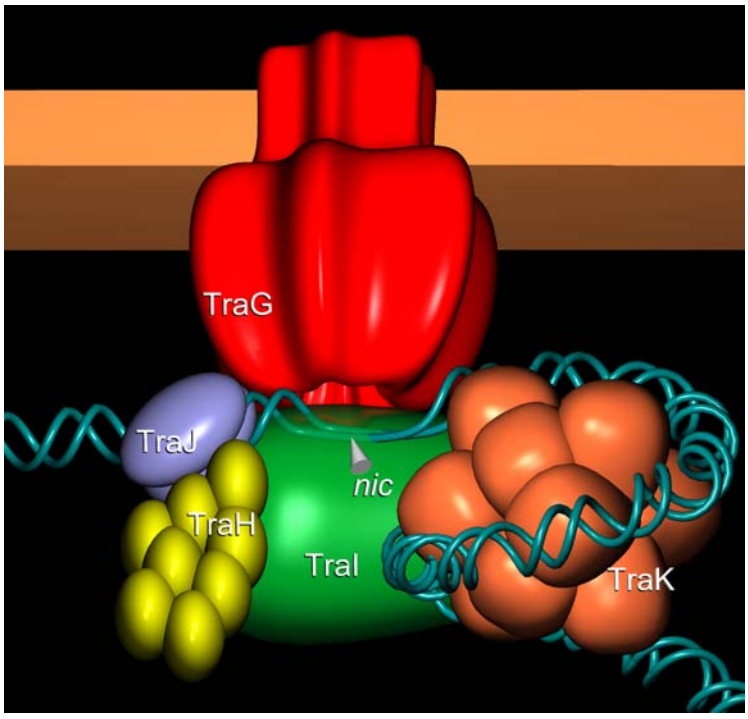

Figure 5.4. Proposed model for the RP4 relaxosome. TraG (red) is a membrane anchored, multimeric protein probably forming a pore-like structure that could serve as a channel for translocation of the transferred ssDNA (T-DNA). The relaxase TraI (green) and the plasmid DNA both bind to this TraG pore. TraI cleaves the *oriT* sequence of RP4 at the *nic* site and is covalently attached to the 5' end of the DNA single strand. TraJ (blue) binds to the sequence upstream of the *nic* site (*srj*) and is required for relaxase activity. TraH (yellow) is a homomultimer that stabilizes the TraI-TraJ-DNA complex, probably by bridging TraJ and TraI. TraK (orange) binds to a sequence downstream of the *nic* site and functions as a DNA chaperone, facilitating the formation of the TraI-DNA adduct.

In conclusion, it is proposed that TraG-like proteins either hydrolyze nucleotides themselves under inducing *in vivo* conditions that are not fulfilled *in vitro*, or regulate the activity of a different NTPase (like VirB11), by feeding it with nucleotides and/or discharging the products of hydrolysis. The fact, that purified TraG proteins do not hydrolyze but bind ATP as well as the product of its hydrolysis, ADP, supports the latter hypothesis. In this mechanism, release and binding of nucleotides could be triggered by Mg^{2+}. Thus, TraG-like proteins, which are known to bind to substrates of type IV secretion, are likely to be also involved in their active export.

Different substrates of T4SS are probably translocated by TraG-like proteins

The transported substrates of T4SS consist of a protein (like CagA of *H. pylori*) and/or of a protein complexed to DNA (like TraI-*oriT* of RP4). The *Agrobacterium tumefaciens* Ti-plasmid secretion system transports the VirD2-*T-DNA* complex, along with virulence-associated proteins VirE2 and VirF into plant cells. VirE2 and VirF translocation depends on the VirB/VirD4 transport system (related to the Mpf/TraG system of RP4) but does not require DNA transfer (103). Secretion of CagA by *H. pylori* is equally dependent on the corresponding Mpf/TraG-like system of *cag* PAI (27). It is conceivable that an analogous, Mpf/TraG dependent protein secretion exists in the conjugative transfer system of RP4. TraC, a functional analogue of VirE2, is transferred into recipient cells during RP4-mediated conjugation (76). Since TraC and VirE2 are cytoplasmic proteins lacking a signal sequence for secretion by the *sec* system (GSP system), TraG and VirD4 may possibly mediate their transport through the inner membrane.

The DNA-binding activity of HP0524 may be a relict from an ancestral conjugative DNA-transfer system

Genetic analysis has revealed that *hp0524* is absolutely required for infectivity of *H. pylori* (20, 27). Here, comparison of the biochemical properties of HP0524Δ1 to other TraG-like proteins of conjugative transfer systems revealed close similarities. In view of the fact that the 145 kDa CagA protein is the only known substrate for the type IV secretion system of *H. pylori*, the finding that HP0524Δ1 binds to DNA is remarkable. A conjugation-like mechanism for DNA transfer between *Helicobacter* strains was suggested earlier (47), although the genetic determinants have not been identified. Two predicted relaxases encoded by orfs *hp0996* and *hp1004* (3, 99) are possible candidates for interaction with HP0524. Thus, involvement of HP0524 in a DNA transfer system

cannot be excluded. On the other hand, type IV secretion systems of pathogens have most probably evolved from conjugative DNA transfer systems (112). Thus the DNA binding activity of HP0524 may be a residual activity from a TraG ancestor of a conjugative transfer system. Whether DNA is still actively transported by this secretion system, or whether it is even involved in pathogenicity, remains to be elucidated.

In some T4SS, the sec system has possibly substituted the function of TraG-like proteins

The putative translocation activity of TraG-like proteins appears to be limited to crossing the inner membrane. It is notable that the only type IV secretion system clearly lacking a TraG-homologue is found in *Bordetella pertussis*. The Pertussis toxin liberation system, Ptl, employs a two-step mechanism for secretion. Pertussis toxin subunits rely on the *sec* system (GSP system) for translocation through the inner membrane and the Ptl system is responsible for transition of the holotoxin across the outer membrane barrier (12). The activity of the Ptl transport system is limited to the outer membrane and is therefore distinct from the VirB prototype of type IV transporters, which convey substrates across both the inner and outer membranes. The notable absence of a *traG* homologue in *B. pertussis* implies that the putative ancestral TraG-like protein of this secretion system has been substituted by the *sec* system of the host during evolution.

Interactions between TraG-like proteins and components of the Mpf complex are uncertain

The membrane-spanning Mpf complex of T4SS is formed independently of the presence or absence of TraG-like proteins or other components of the relaxosome. However, both the Mpf- and the TraG-like proteins are essential components for type IV secretion. The functional connection between TraG-like proteins and the Mpf complex is uncertain. Physical interactions have been postulated (14, 32), but biochemical evidence for this hypothesis remains to be supplied. The periplasmic domain of TraG could possibly mediate interactions of this type. It was verified for two proteins of the Mpf-system of RP4 whether they might provide an interface with TraG: TrbB, the VirB11-like ATPase (43), and TrbE, the VirB4-like putative ATPase (75). Yet, neither TrbB-TraG nor TrbE-TraG interactions were detected by SPR or affinity chromatography (43, 75). Thus, direct evidence for a linkage between TraG-like proteins and components of the Mpf transfer machinery is still lacking. The investigation of protein-protein interactions with other components of the Mpf-system is

hampered by the limited solubility of Mpf proteins. These proteins seem to be functional only as a unit and, with exception of the VirB11 analogs, have not been shown to exert a biochemical activity *in vitro*.

It is also conceivable that there is no direct linkage between the Mpf-complex and the TraG-like protein at all. Maybe, both components take charge of virtually independent functions in type IV secretion. The primary function of the Mpf-complex consists of pilus production and establishing of a close contact with the recipient cell. This is thought to be the first step of the secretion process. As a probable second step, the transport of the secreted substrates into the periplasm is accomplished by the TraG-like protein. This transport may occur spatially separated from the Mpf complex, yet it must somehow be triggered by a signal of the Mpf-complex, mediating that a recipient cell has come into contact. The further steps for secretion of the substrates from the periplasmic space into the exocytoplasma or directly into a recipient cell remain unknown. Resolution of these challenging aspects will probably prove decisive to unraveling the mechanism of type IV secretion.

5.5 Perspectives

The current study has delivered insights into the mechanism of type IV secretion. The detailed biochemical characterization of a key component of this secretion system, the TraG-like transporter protein, provides a valuable basis for the search for inhibitors of type IV secretion. Screenings can be performed with *in vitro* assays like the TraG-TraI, TraG-DNA and TraG-nucleotide interaction. Verification of the inhibiting activity *in vivo* is accomplished by measuring the conjugation rate in mating assays. A T4SS inhibitor could suppress the pathogenicity of a large number of pathogens, as well as the spreading of antibiotic resistance genes through bacterial conjugation. Such inhibitors thus represent a powerful tool for health care and health management.

The detection of interactions between individual components of T4SS is decisive for unraveling their functional interplay, with the final perspective to understand the detailed mechanism of T4SS. One such an interaction was assessed in the present work. The TraG-like protein of RP4 was shown to interact with the relaxase TraI, thus bringing the substrate (the TraI-bound transfer DNA) into immediate proximity of its putative transport pore. The detection of other interacting partners of the TraG-like protein will be essential to trace the further route of the substrate after the probable passage trough the TraG-like transport pore.