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Substrate recognition in IncF conjugative T4SS F and R1

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Abstract

Bacteria have evolved conjugative type IV secretion systems (T4SS) to transport a nucleoprotein complex containing a relaxase and covalently bound single stranded DNA. To fulfil this function numerous protein-protein interactions take place to process and specifically select the substrate. This work gives an insight into the molecular specificity determinants of the IncF relaxase TraI and its selection by the coupling protein (CP) TraD.

Two translocation signals (TS A and TS B) for TraI of plasmid F and R1were mapped genetically that mediate independently from each other the transport of the protein by the T4SS. Furthermore a putative structure was found for the TS based on the already known crystal structure of RecD2. In this study I made single F to R1 amino acid exchanges in TS A and TS B and tested the resulting hybrid TS in an assay that shows the transport of the plasmid and the protein substrate to the recipient cell. The results showed that the exchange of single amino acid 626 in TS A from F to R1 leads to a specificity switch to the heterologous system. Three more TS A and TS B hybrids were tested and they showed no change in the specificity. Furthermore in TS B a single amino acid mutation of amino acid 1344 R to Q within a conserved cluster, which was found in both TS, was made leading to a reduced transfer of the TS.

Next I focused my investigations on the C-terminus of the TraD protein to gain insights about the special molecular features that might be important for the specific substrate selection. The CP of plasmid R1 and R100 share a prominent motif in this region that is not present in TraD of F. Using the same assay mentioned above I compared the protein translocation of F and R1 TraI through the R100 T4SS with the TraI translocation in the R1 and F system. The results of my experiments suggest that for the specific selection of the substrate other interaction partners than the CP C-terminus are needed.
Furthermore I used antiserum against TraD for a quantitative western blot analysis and I detected around 2300 TraD proteins per cell.

My experiments brought also evidence for a possible influence of the R1 partitioning locus on the conjugation machinery.

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1 Introduction

1.1 Type IV secretion systems

To transfer macromolecules across cellular membranes many bacteria have developed special secretion machineries. They can be assembled into six groups, type I to type VI (Thanassi & Hultgren, 2000; Henderson et al, 2004; Mougous et al, 2006).

This work focuses on the type IV secretion systems (T4SS). T4SS are trans-membrane multi protein complexes with the ability to transport both proteins and nucleoprotein complexes out of the cell. Many pathogenic bacteria use this ability of the T4SS for the infection of eukaryotic cells by direct cell to cell contact. Bacteria can also effect their environment, by the transport of effector molecules to the extracellular milieu, to enable colonisation of tissues and biofilm formation. Conjugative DNA transfer via T4S drives the spread of antibiotic resistances among other bacteria. This shows that T4SS are involved in many medically relevant processes and it is important to find out as much as possible about the way they function.

Based on their function, T4SS can be divided into three groups (Figure 1.1):

The first group transfers effector molecules, in the most cases proteins and in the case of *Agrobacterium tumefaciens* also DNA, directly into eukaryotic target cells. These toxins or virulence factors are part of the pathogenic mechanism of (thus far only Gram negative) bacteria and make survival in the tissue or the host cell possible (Alvarez-Martinez and Christie, 2009). For example *A. tumefaciens* is a phytopathogen that transports effector proteins (VirE2, VirE3, VirF proteins) and a nucleoprotein complex (T-DNA and VirD2) into plant cells (Christie, 1997). The VirB/VirD4 system of *A. tumefaciens* is also a very important model system for T4SS in gram-negative bacteria.

The second group uses the T4SS to release or absorb DNA to or from the extracellular environment instead of the donor cells (Chen et al., 2005; Hemilton et al., 2005). This group include the secretion machinery of *Neisseria gonorrhoeae* that

secretes naked DNA (Hamilton et al, 2005), and the DNA up taking systems from *Helicobacter pylori* and *Campylobacter jejuni* (Hofreuter *et al.*, 2001; Bacon *et al.*, 2000).

The third group are the conjugative systems. It is the largest and widespread group and is found in gram-negative and gram-positive bacteria and in some *Archaea* (Alvarez -Martinez and Christie, 2009). Bacterial conjugation is responsible for the spread of antibiotic resistances by the transfer of DNA into a recipient cell (De la Cruz & Davis, 2000; Christie, 2001). The conjugative T4SS consist of three components: The DNA transfer and replication proteins (Dtr) which initiate the enzymatic processing of the plasmid. The type IV coupling protein (T4CP) which works as a substrate receptor and recruits the substrate to the mating-pair formation proteins (Mpf) forming the translocation channel. The DNA is transferred as single stranded DNA (ss DNA) covalently bound to a relaxase. The relaxase is important for the recircularization of the plasmid within the recipient cell (Alvarez -Martinez and Christie, 2009; Dostal et al., 2010).

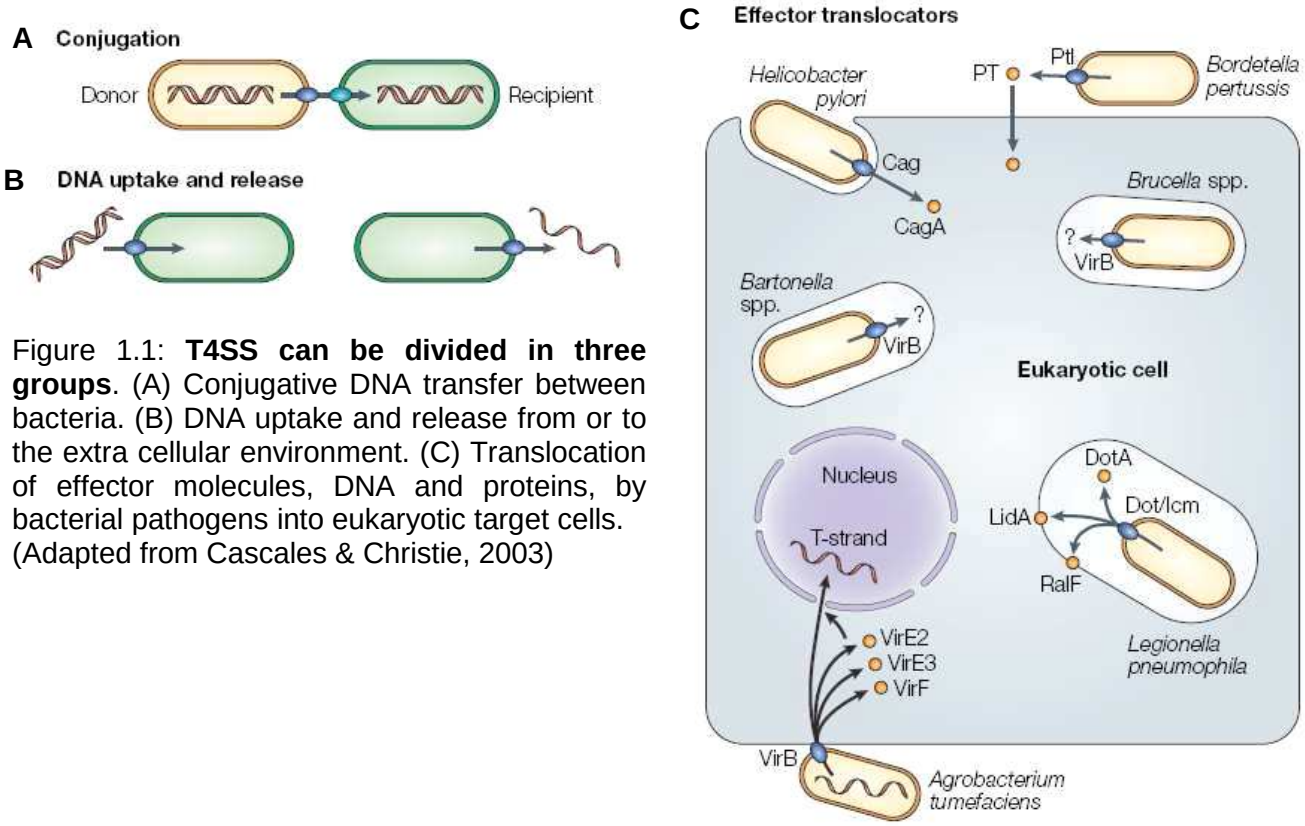

Figure 1.1: **T4SS can be divided in three groups**. (A) Conjugative DNA transfer between bacteria. (B) DNA uptake and release from or to the extra cellular environment. (C) Translocation of effector molecules, DNA and proteins, by bacterial pathogens into eukaryotic target cells. (Adapted from Cascales & Christie, 2003)

In this work I am concentrating on the last group, the conjugative T4SS. As mentioned before, this is the key mechanism of spreading antibiotic resistances. It would be a great medical benefit to know more about the recognition and recruitment of the DNA and protein substrate. The goal would be to manipulate or avoid the transport of these macromolecules. To achieve this goal as much as possible must be known about the components of the T4SS and their function. All proteins necessary for a functional T4SS are encoded on conjugative plasmids.

1.2 Conjugative plasmid

A classical plasmid is circular, extra-chromosomal double-stranded DNA (ds DNA) containing genes that are not essential for cellular function. Genes encoded by plasmids have replicative, self-maintenance and regulatory functions. Additionally to the genes that are absolutely necessary for the survival of the plasmid they harbour genes which offer a benefit to the host cell under certain conditions, e.g. a variety of antibiotic and heavy metal resistances, genes for the degradation of special organic substances or virulence genes.

Conjugative plasmids are large plasmids with a low copy number in most cases. They are essential for the horizontal gene transfer and the spread of genetic information. Therefore, they harbour all genes necessary for this transport and they are organised in a *tra* operon that encodes all components for a T4SS.

1.2.1 IncF-like plasmids

Plasmids with the same replication machinery or partitioning system were categorized into incompatibility (Inc) groups. Two plasmids of the same Inc group can not exist within one cell (Novick, 1987; Couturier et al, 1988).

All plasmids I was working with in this study belong to the IncF family, particularly to IncFI and IncFII. The transfer regions of these plasmids show a high homology compared to each other and some of the transfer genes are allelic (Frost et al., 1994). The host range of F-like plasmids is limited to the genera of *Enterobacteriaceae* (Carattoli, 2009).

All three plasmids I describe below are large in size (R1-16 around 40 kb, F and R100-1 around 100 kb) and low copy number plasmids (4 to 6 copies (Nordström et

al, 1980)). In the lab I used the derepressed versions of the plasmid with disrupted *finO* gene and constitutively expressed F-pili (Frost et al., 1994; Boyd et al., 1996).

IncFI

The F plasmid belongs to the IncFI family. This plasmid is also known as the sex factor, F factor or fertility factor which forms the F-pili for bacterial mating. To find out more about the genetics of mating it was used as one of the first model plasmids for conjugation. In 1994 the complete *tra* region of F was sequenced (Frost et al., 1994).

IncFII

Plasmid R1 and R100 are prominent members of the IncFII group. The R100 plasmid is also known as NR1 or R222. It is closely related to the R1 plasmid and it has almost the same genetic organisation but some specificities in the regulation of the transfer and replication are different. The two plasmids also differ in their encoded resistances. R1 originally encodes for kanamycin, streptomycin, sulfonamide, ampicillin and chloramphenicol resistance. The R100 has a tetracycline, chloramphenicol, streptomycin, sulfonamide and mercuric ion resistance (Womble & Rownd, 1988).

1.3 Conjugative DNA transfer of plasmid R1

In this section I will describe the current model for the conjugative transfer of plasmid R1. Figure 1.2 shows a schematic illustration of a R1 T4SS. It consists of three functional complexes: (i) the mating pair formation (Mpf) (Figure 1.2, grey), (ii) the T4CP TraD shown in Figure 1.2 in yellow and (iii) the DNA transfer and replication (Dtr) proteins or also called relaxosome proteins. For the initiation of conjugation a close cell to cell contact is necessary. The transfer of the conjugative plasmid can only be successful if the recipient cell harbours a plasmid belonging to a different Inc group or if the cell is plasmid free. With the obtained plasmid the recipient becomes a potential new donor and the spread of the plasmid can go on. As mentioned before, all genes required for this mechanism are encoded in the *tra* operon. It is an around 33 kb large region and contains about 40 genes for the T4SS.

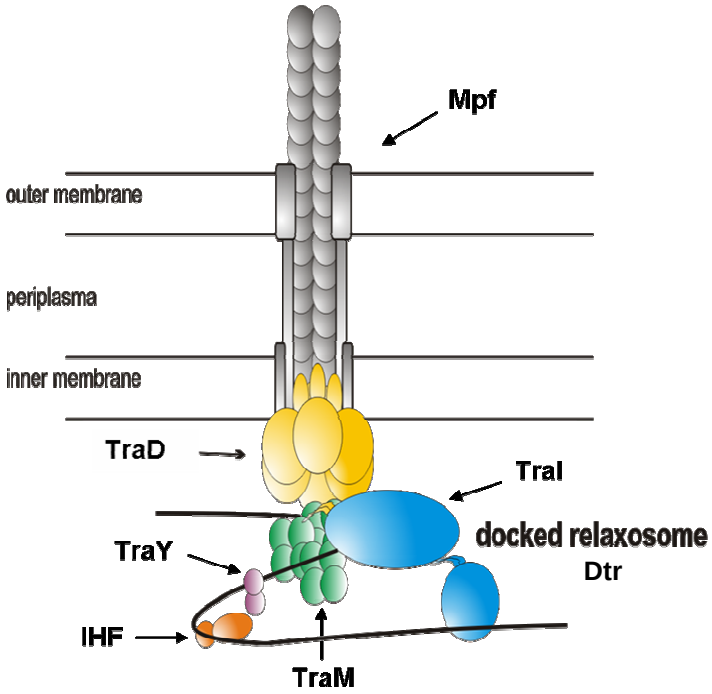

Figure 1.2: **Schematic illustration of a R1 conjugative T4SS**. The conjugative machinery is build up out of three functional components: The Mpf components, forming the membrane spanning channel (grey), the CP TraD (yellow) and the relaxosome. The relaxosome is a nucleoprotein complex including proteins TraI (blue), TraM (green), TraY (purple) and IHF (orange) and the DNA (black). (Provided by Silvia Lang)

Below I will describe the three functional complexes of the conjugative T4SS with special attention to the Dtr and the CP.

1.3.1 Mpf

The mating pair formation complex (Figure 1.2, grey) fulfils two important roles in conjugation. It forms the pilus which is necessary for the direct contact to other bacteria and it builds up the cell envelop spanning multi protein complex as transport channel for the substrate.

1.3.2 Dtr: The Relaxosome

The DNA is transferred by the T4SS as a single strand. For processing of the plasmid a nucleoprotein complex, called the relaxosome has to assemble at the origin of transfer (*oriT*). The relaxosome proteins include a relaxase, necessary for DNA cleavage, and some auxiliary proteins which support the relaxase. The auxiliary proteins influence the conformation of the DNA and make the *nic* site accessible for the relaxase. The processing of the plasmid starts with a specific cleavage of the

DNA at the *nic* site in the *oriT* (Lanka and Wilkins, 1995). In the case of R1 the relaxase is called TraI and the auxiliary proteins are TraM, TraY and the integration host factor IHF.

TraI

The TraI of F-like plasmids was originally described as *E. coli* DNA helicase I (Abdel-Monem & Hoffmann-Berling, 1976; Abdel-Monem et al., 1976). TraI of F and R1 are 97 % identical. This protein is essential for the DNA processing within the relaxosome complex for the conjugative transfer (Matson et al., 2001). TraI is a large 192 kDa protein that can be divided into multiple functional domains (Figure 1.3) including: (i) A transesterase domain also known as relaxase domain (nt. 1-309) shown in Figrue 1.3 in purple, (ii) a helicase domain (nt. 310-1505) coloured blue in Figure 1.3 and (iii) the C-terminal domain (nt. 1506-1757). The two catalytic domains do not overlap (Byrd et al., 2002) and both are necessary for a functional conjugative DNA transfer (Matson et al., 2001). It is unique for T4SS of plasmids belonging to the IncF and IncW groups that they encode a bifunctional protein with two catalytic functions.

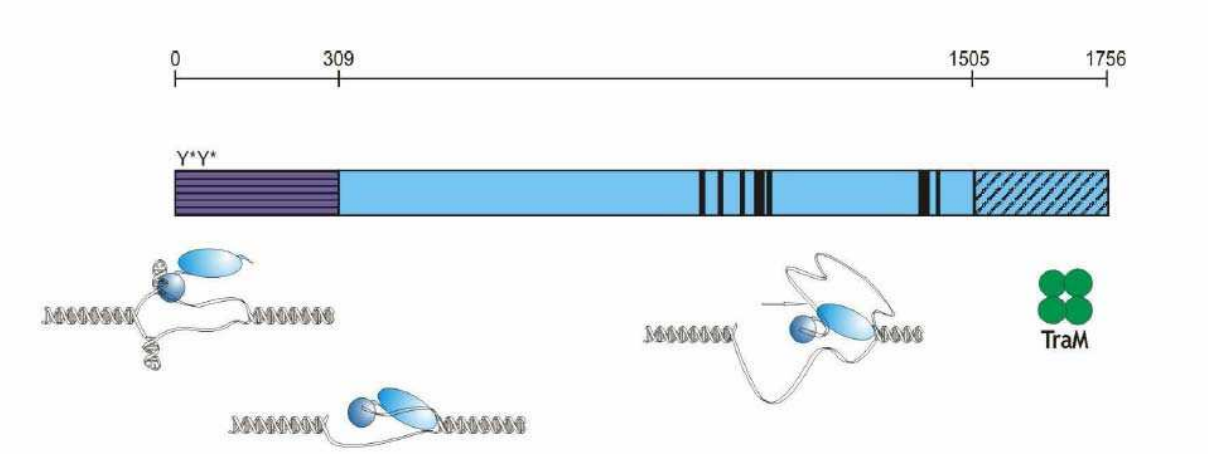

Figure 1.3: **Functional domains of TraI**. The N-terminal relaxase domain (residue 1-309, purple) nicks the plasmid and the C-terminal helicase domain (residue 309-1505, blue) unwinds it. The conserved helicase motives are indicated as black bars. The last 252 amino acids (striped) are binding sites for TraM.

The N-terminal relaxase domain binds specific to the *oriT* sequence around the *nic* site (Stern & Schildbach, 2001) and cleaves the DNA strand reversible at a specific

phosphodiester bond at the *nic* site in a Mg^{2+} dependent manner (Matson & Morton, 1991; Reygers et al., 1991). TraI alone cleaves supercoiled plasmid DNA *in vitro* and it has been shown that the auxiliary proteins as well as the T4CP TraD enhance the activity (Nelson *et al*., 1995; Howard *et al.*, 1995; Mihajlovic *et al.*, 2009). After the transesterification reaction the nicked strand remains covalently bound with the 5' end to the relaxase via phosphotyrosyl linkage (Byrd & Matson, 1997). The free 3' end is connected to the protein through a noncovalent linkage (Matson et al., 1993; Sherman & Matson, 1994). During the conjugational transport the DNA remains covalently linked to the relaxase domain of TraI and the protein is transferred to the recipient cell (Lang et al., 2010). After the transfer the relaxase religates the plasmid (Draper et al., 2005).

The helicase activity of TraI requires ATP hydrolysis. Duplex unwinding from the plasmids *oriT* by the TraI helicase occurs with a 5' to 3' direction (Abdel-Monem & Hoffmann-Berling, 1976) at high speed around 1100 b/sec (Sikora et al., 2006) and a high processivity of more than 850 bp of unwinding (Kuhn et al., 1979; Lahue & Matson, 1988). The conserved helicase motifs (Gorbalenya &Koonin, 1993) are located between residue 990-1505, but the whole domain is necessary for helicase function (Byrd et al., 2002). It has been shown that TraM, IHF and TraD stimulate helicase activity of TraI (Sut et al., 2009). A DNA binding site was found within the helicase motif by Dostal and Schildbach (Dostal & Schildbach 2010). It is less sequence specific than the one in the relaxase domain and maybe required for ss DNA transfer (Dostal & Schildbach, 2010). The C-terminal 225 amino acids of TraI bind to ss DNA (Guogas et al., 2009) and to the auxiliary protein TraM (Ragonese et al., 2007).

TraM

TraM is a 14 kDa cytoplasmatic protein that binds to its binding sites *sbmA* and *sbmB* within the *oriT* cooperatively as a tetramer (Verdino et al., 1999). The binding of TraM to the DNA leads to a bending of the DNA by about 50° (Fekete & Frost, 2000). *traM* expression is autoregulated by the binding of TraM to the *sbmB* binding site near this promoter (Fu et al., 1991; Schwab et al., 1991). The *sbmA* binding site near the *nic* site is located between the two binding sites for IHF and is important for the DNA transfer (Schwab et al., 1991). The presence of TraM is essential for the DNA

transfer (Karl et al., 2001) and it positively regulates the expression of the *tra* genes by promoting the *traJ* transcription (Polzleitner et al., 1997). It has been shown that TraM interacts with the TraD coupling protein (Disque-Kochem & Dreiseikelmann, 1997; Beranek et al., 2004; Lu & Frost, 2005; Lu et al., 2008) as well as with TraI (Ragonese et al., 2007). TraM enhances the TraI nicking activity *in vivo* and *in vitro* (Howard *et al*., 1995; Kupelwieser *et al.*, 1998; Mihajlovic *et al*., 2009) and the TraI helicase activity *in vitro* (Sut et al., 2009).

TraY

TraY of the R1 T4SS is a small protein (around 10 kDa) that belongs to the ribbon-helix-helix DNA binding protein family and normally forms dimers (Bowie & Sauer, 1990; Nelson & Matson, 1996). On the F plasmid, the protein has two different binding sites. One binding site for TraY lies within the *oriT* region between the TraM and IHF binding sites and the second near the P_y promoter where it up-regulates the expression of the *tra* genes (Nelson et al., 1993; Silverman & Sholl, 1996). The binding of TraY to the DNA induces a bend of about 50° (Luo et al., 1994) and the nicking activity of TraI is stimulated by TraY *in vivo* and *in vitro* (Nelson *et al*., 1995; Karl *et al*., 2001; Csitkovits & Zechner, 2003; Mihajlovic *et al*., 2009)

IHF

IHF, the integration host factor is a heterodimeric protein around 20 kDa of size and it plays an important role as an architectural factor that bends DNA and makes it accessible for protein interaction for example in DNA replication or transcriptional regulation (Landy, 1989). It was shown by X-ray crystal structure analysis that IHF bends the DNA around 160° by binding to the minor g roove of the DNA (Rice et al., 1996). The binding and bending is a stepwise process by first binding the straight DNA followed by bending (Sugimura & Crothers, 2006). The F plasmid has two IHF binding sites, *ihfA* and *ihfB*, near the *nic* site (Tsai et al., 1990). IHF stimulates the TraI nicking and helicase activity *in vitro* (Howard *et al*., 1995; Csitkovits *et al*., 2004; Mihajlovic *et al*., 2009; Sut *et al*., 2009) and it is essential for nicking *in vivo* (Kupelwieser et al., 1998). It was also shown that IHF and TraY must be properly positioned and oriented toward each other in the relaxosome complex for DNA cleavage and transfer (Williams & Schildbach, 2006).

1.3.3 TraD – The coupling protein

The T4CP connects the Dtr complex with the Mpf complex. It plays an important role in the recognition of the substrate and its hand off to the T4S channel (Cabezon et al., 1997; Hamilton et al., 2000; Cascales & Christie, 2004). The T4CP of IncF-like plasmids is called TraD and belongs to the TraG protein family, which includes also the CP TrwB from plasmid R388, TraG from RP4 and VirD4 from *A. tumefaciens*.

TraD is an inner membrane protein, in monomeric form around 86 kDa, with DNA binding ability (Panicker & Minkley, 1992). The protein has conserved WalkerA and B motives for NTP hydrolysis and it has been shown that it binds DNA non-specifically, with ss DNA as preferred substrate (Schroder *et al.*, 2002; Schroder & Lanka, 2003). That the NTP binding site of the CP is necessary for DNA transport (Balzer et al., 1994) and DNA binding activity (Moncalian et al., 1999) has been shown for other T4SS. Sanja Mihajlovic from our lab showed that a point mutation in the Walker A or B motive of R1 TraD also disrupts the DNA transfer by the R1 T4SS (Dissertation S. Mihajlovic, 2009).

The model for the structure of TraD within the T4SS is a homohexameric ring that forms a pore within the inner membrane. This model was based on microscopy analyses of the full length TrwB (Hormaeche et al., 2002) and the crystal structure of TrwBΔN70 (Figure 1.4) lacking the first 70 amino acids (Gomis-Rüth et al., 2001; Gomis-Rüth et al., 2002).

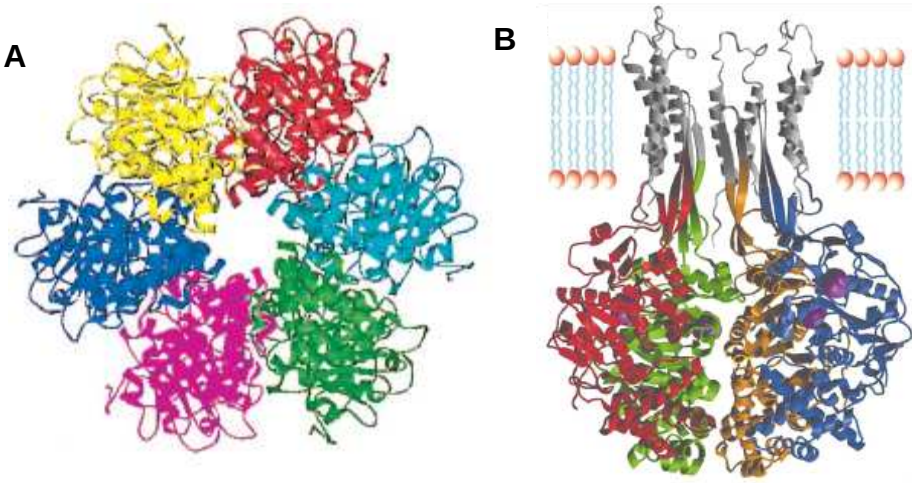

Figure 1.4: **Crystal structure of TrwBΔN70**. (A) Hexameric ring structure with a pore in the middle. Each monomer is illustrated in a different colour. (B) Side few with added trans-membrane domain (grey). (Adopted from Gomis-Rüth et al., 2001)

Experiments with mutated TraD lead to the model that TraD monomers within the membrane interact with its N-terminal region followed by the binding of the C-termini in a back-to-front manner (Haft et al., 2007). For the soluble TraDΔN130 it has been shown in our lab that it forms monomers, trimers and hexamers *in vitro* (Dissertation S. Mihajlovic, 2009).

The CP interacts with other proteins from the T4S channel and the relaxosome. The C-terminus of TraD interacts with the relaxosome protein TraM very specific (Disqué-Kochem & Dreiseikelmann, 1997; Beranek et al., 2004; Lu et al., 2008). By two hybrid analysis it has been demonstrated in our lab that TraD also interacts with TraI (Diploma thesis B. Klug, 2009).

Sastre and colleagues showed that the C-terminus of TraD is very important for its specificity of the relaxosome. The removal of even a small part of the C-terminus (last 37 amino acids) broadens the range of mobilizable relaxosomes but leads to a reduced transfer of its own (Sastre et al., 1998).

1.4 T4SS protein substrates and their translocation signals (TS)

The purpose of T4SS is typically to transport molecules out of the cell. DNA uptake systems are also known (Hofreuter *et al.*, 2001; Bacon *et al.*, 2000).
Different pathogens transfer effector proteins to eukaryotic cells. These protein substrates can vary in size from the small 22 kDa VirF from *A. tumefaciens* to the 142 kDa large CagA from *H. pylori*. The proteins can also be transported as oligomers like the hexameric *Bordetella pertussis* toxin. In conjugative T4SS and the VirB/D4 T4SS of *A. tumefaciens* the protein substrate is a relaxase covalently bound to ss DNA. Until now it is not fully understood how the T4CP recognise its substrate, but it is very likely that specific recognition features, called translocation signals (TS), on the proteins mediate this process. The knowledge about protein TS is summarised in Figure 1.5 and the following section.

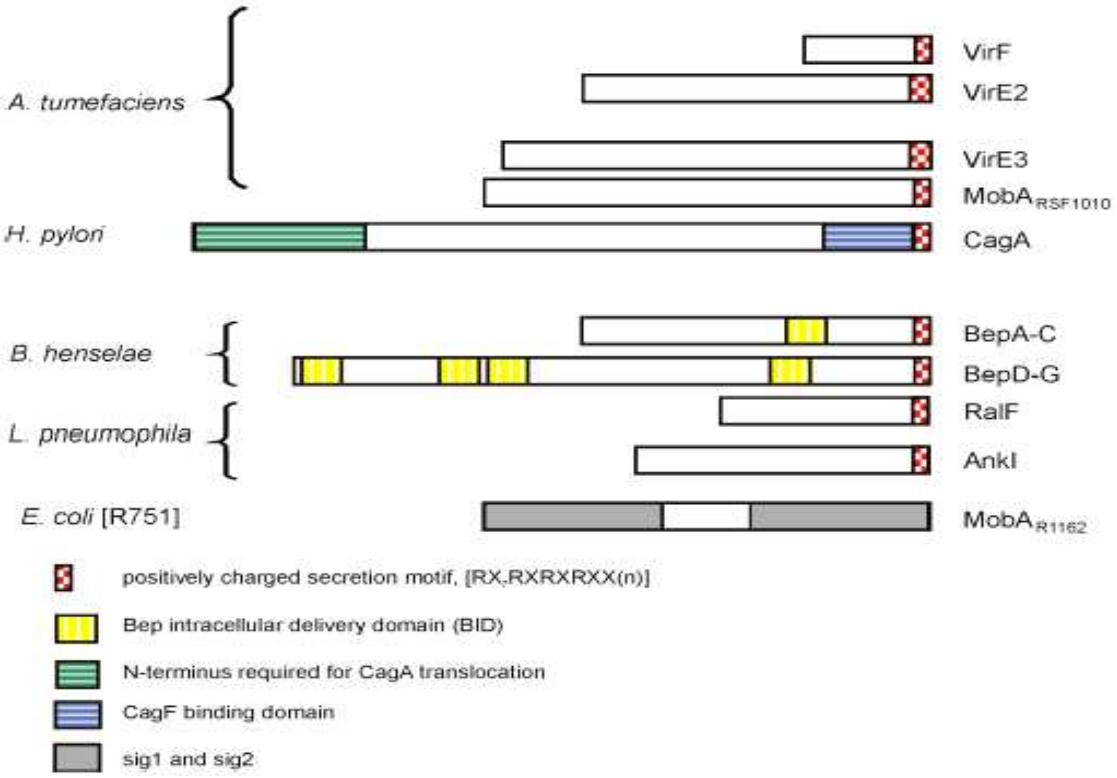

Figure 1.5: **Summary of determined TS of T4SS protein substrates**. The tested T4SS is shown at the left side. The substrate proteins with its identified TS are illustrated. The signals are described below. (Provided by Silvia Lang)

1.4.1 C-Terminal Signals

Very simple TS are C-terminal positively charged or hydrophobic motives not longer than 50 bp. The TS of the *A. tumefaciens* effector molecules VirF, VirE2 and VirE3 are located at the C-terminus shown in Figure 1.5 in red (Vergunst et al., 2000; Vergunst et al., 2003; McCullen et al., 2001). The TS contains a conserved positively charged arginine-proline-arginine motif, which is assumed to bind to complementary charged residues within the CP VirD4 (Vergunst et al., 2005).

For the RalF protein which is transported through the Dot/Icm system of *Legionella* it was shown that a hydrophobic residue in the last 20 amino acids (Figure 1.5, red) is necessary for translocation (Nagai et al., 2005). The crystal structure of VirE2 and RalF have been solved and the C-terminal domain shows a disordered structure that is solvent exposed (Amor et al., 2005; Dym et al., 2008).

Two substrates for the VirB T4SS of *Brucella spp*, called VceA and VceC, were recently identified by de Jong and colleagues (de Jong et al., 2008). For the VirB T4SS until now no CP was found that could recognise the substrate, but for both

proteins the last 20 amino acids, containing a positively charged motive, are required for the transfer. Furthermore it was shown that VceC was transferred by the heterologous Dot/Icm system of *L. pneumophila* (de Jong et al., 2008).

1.4.2 Intrinsic signals

The C-terminal TS are important but not sufficient for the transport of some protein substrates.

The substrates of *Bartonella spp*, the *Bartonella*-translocated effector proteins (BepA-G) need a positively charged C-terminus (Figure 1.5, red) as well as at least one internal Bep intracellular delivery (BID) domain (Figure 1.5, yellow) for translocation (Schulein et al., 2005).

A C-terminal positively charged motive (Figure 1.5, red) as well as the N-terminus (Figure 1.5, green) is important for the transfer of CagA, a substrate from *H. pylori* (Hohlfeld et al., 2006). Additionally to the presence of both ends of the protein the secretion chaperone CagF has to bind to the C-terminus of CagA (Figure 1.5, blue) for successful transfer (Pattis et al., 2007).

In these cases, both TS must be present on the protein for successful translocation. It is not possible to separate them.

1.4.3 TS of relaxases

The IncQ plasmid R1162 and the nearly identical plasmid RSF1010 are broad host-range plasmids. Relaxase $MobA_{R1162}$ consists of two independent catalytic domains, an N-terminal nickase (Scherzinger et al., 1993) and a C-terminal primase (Lin and Meyer, 1987; Honda et al., 1991). Parker and Meyer mapped two TS in $MobA_{R1162}$, called sig1 and sig2 (Figure 1.5, grey), associated with the two catalytic domains. It was shown that both TS are independently active within the full length protein. For the conjugative transport of $MobA_{R1162}$ by the R751 T4SS both TS and the auxiliary protein $MobB_{R1162}$ are required (Parker & Meyer, 2007).

Relaxases from broad host-range T4SS can be transported from different conjugative and effector T4SS without a covalently linked ss DNA (Draper et al., 2005; Parker & Meyer, 2007; Schulein et al., 2005; Vergunst et al., 2005). For the translocation of $MobA_{RSF1010}$ in the VirB/VirD4 system of *A. tumefaciens* it has been shown that the

last 50 amino acids (Figure 1.5, red) are sufficient to mediate the transfer (Vergunst et al., 2005). The N-terminus and MobB was not required.

Preliminary data TS of TraI

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Molecular recognition determinants for type IV secretion of diverse families of conjugative relaxases

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Two minimal TS for TraI

Using a triparental mating experiment Sanja Mihajlovic demonstrated that TraI is translocated to the recipient strain via conjugation. Due to that finding she established the Cre-recombinase assay for translocation (CRAfT) (Vergunst et al., 2000; Vergunst et al., 2005) to detect the minimal translocation signals (TS) of the protein. Therefore she tested deletion mutants of R1 TraI in the CRAfT and identified two minimal TS, called TS A and TS B (Fig. 1.6, grey). TS A extends from residue 530 to 816 and TSB from 1255 to 1564.

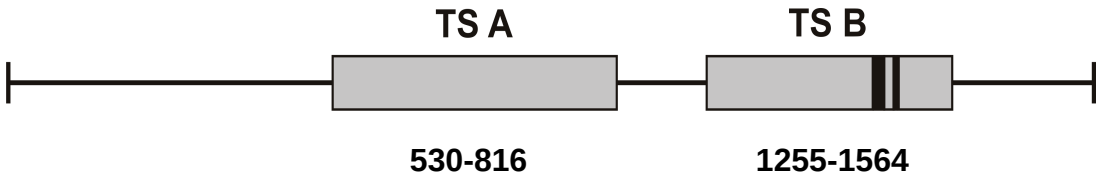

Figure 1.6: **Schematic illustration of the TraI protein and its translocation signals**. TS A extends from residue 530 to 816 and TS B from 1255 to 1564. The black stripes in TS B indicate conserved helicase motives within the TS. The full length TraI protein is 1756 amino acids long.

The possibility that these regions represent DNA binding motives was excluded by the mapping of the single-strand DNA binding activities of R1 and F TraI (Dostal & Schildbach, 2010).
The same mapping was made with the closely related and 97 % identical TraI protein of the F plasmid. Silvia Lang found two similar minimal TS also spanning the residues 530 to 816 (TS A, F) and residues 1255 to 1564 (TS B, F).

Compared with already known recognition domains from other protein substrates of T4SS the TS from TraI have unique features:

- They are not located at the C- or N-terminus of the protein.
- Both can be translocated independently from each other.
- They are much longer and more complex than, for example, the around 50 bp positively charged C-terminus of *A. tumefaciens* substrates.

In a CRAfT the Cre fused TS A and TS B were tested in the heterologous T4SS. The translocation of the TS was not detectable. The experiment showed that both TS of R1 and F TraI are specific for their cognate T4SS.

TS of TraI have a conserved recognition structure

Due to the fact that there are two TS and that they work independently for translocation, it was conceivable that they are structurally related. A comparison with already known structures using GenTHREADER (Jones, 1999; McGuffin & Jones, 2003) suggested an N-terminal truncated mutant of RecD2 helicase from *Deinococcus radiodurans* (Saikrishnan et al., 2008) as the best matching structure for TS A and TS B. The alignment displays about 20 % similarity (10 % sequence identity) for TS A and TS B to RecD2. The overlapped structures are shown in Figure 1.7 in black. This model represents a TraI protein recognition feature.

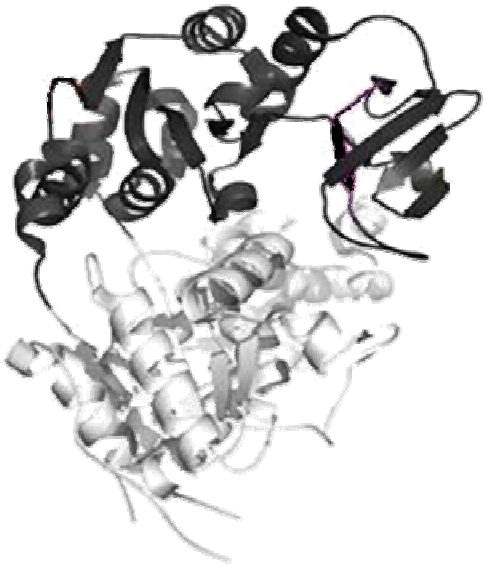

Figure 1.7: **Structural overlay of RecD2 and TSs of TraI**. The conserved regions of TS A and TS B compared with the structure of RecD2 are coloured black. The grey parts are RecD2 structures not overlapping with the TSs.

TS modules are conserved in relaxase families

Using PSI-BLAST (Altschul et al., 1997) partial sequences of TS A and TS B were searched in the known relaxase families. Out of the data a maximum likelihood tree was created. The TS was found in relaxases belonging to the MOB_Q and MOB_F groups (Garcillan-Barcia et al., 2009). Within the MOB_Q, MOB_{F1}, MOB_{F11} and MOB_{F13} groups only a single TS was found and in MOB_{F12} and other MOB_{F1} relaxases two TS were found (Gracillan-Barcia et al., 2009). That means that an internal gene duplication event took place.

The lengths and patterns of the tree branches for the duplicated TSs seem very similar, with a little bit longer branches for TS A. That means that there is more diversification for TS A.

1.5 Prokaryotic DNA-partitioning systems

During cell division it is indispensable for the cell that newly replicated plasmids are distributed accurately to the daughter cells. This mechanism guaranties genetic stability of low copy number plasmids and ensures that at least one copy is present in each cell after division.

All components necessary and sufficient for this purpose are encoded in a segregation operon, also called the *par* operon (Gerdes & Molin, 1986). In general three components are encoded: i) a centromer-like region on the DNA, ii) the

upstream gene encodes an ATPase and iii) the downstream gene a DNA binding protein that binds to the centromer-like region (Gerdes et al., 2000).

The partitioning systems can be classified into two types according to the nature of their ATPase (Gerdes et al., 2000).

Type I

The ATPase of this family is a Walker-type ATPase (Koonin, 1993). A member of this type I family is the partitioning system of the well characterized plasmid F (Gerdes et al., 2000). The partitioning locus of F is called *sop* for stability of plasmid (Ogura & Hiraga, 1983).

Type II

The ATPase of the type II family belongs to the actin/hsp70 superfamily of ATPases (Bork et al., 1992). Prominent members of this family are the partitioning systems of plasmid R100 and R1 (Gerdes et al., 2000). In this study I was working with the partitioning system of the R1 plasmid. Its function is described in detail below.

1.5.1 ParMRC complex of the R1 plasmid

In R1 the *par* locus encodes ParM and ParR, two *trans*-acting proteins, and a *cis*-acting centromere-like *parC* region (Gerdes & Molin, 1986; Dam & Gerdes, 1994), as shown in Figure 1.8, A.

parC

The 160 bp *parC* (C stands for centromer-like) region lies upstream directly in front of *parM* and *parR*. Transcription is started from a single P_{parA} promoter within the *parC* region (Min et al., 1991; Jensen et al., 1994). The promoter is flanked by two sets of five 11 bp direct repeats (iterons) (Jensen et al, 1994). These 10 iterons are necessary to ensure plasmid stability, the incompatibility towards other *parA* stabilized plasmids and a functional auto regulation by ParR (Dam & Gerdes, 1994; Jensen et al, 1994; Breüner et al., 1996)

ParR

The 13.3 kDa DNA-binding protein ParR (R stands for repressor) binds to the 10 iterons of the *parC* region cooperatively as a dimer (Dam & Gerdes, 1994; Møller-Jensen et al, 2003). ParR binds with its N-terminus to the DNA and with the C-terminus to ParM (Salje & Löwe, 2008). It was shown that the 10 ParR dimers form a helical scaffold with the centromer-like *parC* DNA region wrapped around (Møller-Jensen et al., 2007). This nucleoprotein complex forms a rigid clamp, enclosing the end of a ParM filament because of its higher affinity for ATP-bound ParM (Salje & Löwe, 2008).

In addition to its function as partitioning protein, ParR also works as a transcriptional repressor. By ParR binding to the DNA the P_{parA} promoter is repressed (Tabuchi et al, 1992; Jensen et al, 1994). That is important because an overproduction of ParR protein leads to a destabilization of the *par* containing plasmid (Dam & Gerdes, 1994). Consequently the expression of *parM* is also repressed. This regulation system ensures that only an appropriate amount of both Par proteins is present in the cell (Jensen et al., 1994).

ParM

ParM (M stands for motor) is a 37.5 kDa ATPase that is structural homolog to eukaryotic actin (Møller-Jensen et al, 2002). Like F-actin, ParM forms helical filaments (Møller-Jensen et al, 2002) out of two parallel protofilaments (Van den Ent et al, 2002). Despite this similarity these proteins show some differences. ParM builds up filaments 300-times faster than actin and the filaments show no polarity and grow on each end with the same rate (Garner, 2008). In contrast to actin they form left-handed filaments (Orlova et al., 2007; Popp et al., 2008). This conformational structure is important because of the binding to the right-handed ParRC clamp (Salje &Löwe, 2008). The ParM filaments exhibit dynamic instability on both ends driven by hydrolisation of ATP. ParM-ATP has a much lower dissociation rate than ParM-ADP (Garner et al, 2004; Garner, 2008). The ATP cap on both ends of the filaments is important for its stability (Garner et al., 2004). The specific binding of ParM to the ParRC complex (Jensen & Gerdes, 1997) protects the filaments from disassembly (Garner et al, 2007) and so they can form stable filaments from cell pole to cell pole (Møller-Jensen et al, 2002). Once the ends are stabilized filaments growth pushes

newly synthesized plasmids to opposite sites of the cell before cell division (Figure 1.8, B) (Møller-Jensen et al, 2003).

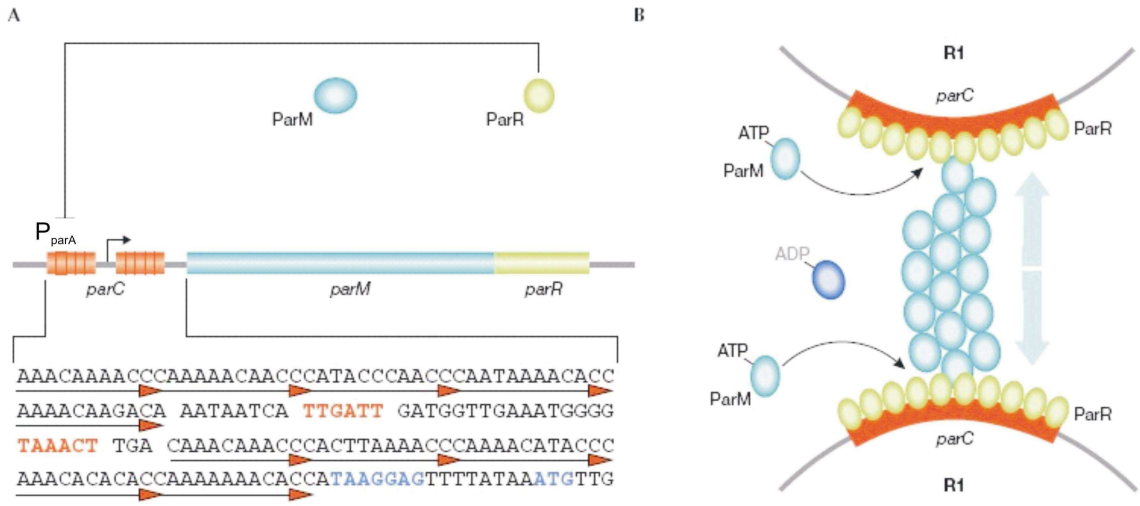

Figure 1.8: **Partitioning locus of plasmid R1**. (A) Transcription of the proteins ParM and ParR starts from the *parA* promoter that lies inside the *parC* region. The promoter is flanked by two times five 11 bp repeats. ParR binds direct as a dimer to those repeats. By ParR binding the promoter is repressed. (B) ParR binds to *parC* of the plasmid. This complex protects ParM filaments from disassembling. ATP bound ParM forms stable filaments and push the plasmids to the opposite poles before cell division. (Adopted from de la Cueva-Mendez & Pimentel, 2007)

<u>Preliminary data ParM and conjugation machinery</u>

Previous experiments in our group brought some unexpected results which indicate a connection between the plasmid partitioning system of R1 and the conjugation machinery.

Monika Nuk made the first observation during her studies on plasmid induced biofilm formation of *E. coli*. To find genes outside of the *tra* operon, which have an influence on biofilm formation, she performed mutagenesis of R1-16 with a miniTn5Cm transposon. One of the three R1-16miniTn5Cm mutants, with reduced biofilm formation, had an insertion of the transposon within the *par* locus and disrupted the *parM* open reading frame. This mutant was called R1-16miniTn5CmE5 and had 2.6-fold less biofilm compared to the R1-16 wild type. The assumption that the reduced biofilm is a consequence of defective conjugation could be excluded by a conjugation assay. The result shows the same conjugation frequency as wild type (Dissertation M. Nuk, 2008).

The strain was tested for R17 phage infection and it showed absolute immunity. This RNA phage requires for a conjugative pilus, a functional T4CP and the lytic transglycosylase p19 for successful infection (Bayer et al., 1995). Sandra Raffl from our lab showed that a functional relaxosome complex (*oriT* and *nic* site, TraM, TraY and TraI) is also required for R17 phage infection (unpublished data).
Furthermore Barbara Klug made a two hybrid analysis and observed an interaction of ParM and TraD (Diploma thesis B. Klug, 2009).
Christian Gruber from our lab used supercoiled *oriT* DNA and purified protein to perform a relaxase assay with TraI of R1 and additional ParM. The presence of ParM stimulates the nicking of the TraI relaxase two fold (unpublished Data).

1.6 Aims of this study

The purpose of this study was to get more insight into the molecular features of specific protein interactions between the IncF relaxase TraI and the CP TraD for substrate recognition. Furthermore I investigated the recent discovered connection between the R1 partitioning system and the conjugation machinery.

Aim I: Molecular specificity determinants in TraI TS

The specific transfer of R1 and F TraI is mediated by two TS, called TS A and TS B. With the exception of 2 amino acids in TS A and 9 in TS B the signals of F and R1 are sequence identical. Furthermore a conserved structure for both TS was found and proposed as a model for TraI TS structure.
Single amino acid exchanges and the well established CRAfT were used to determine if the variant amino acids in the TS are responsible for specific recognition by the cognate T4SS. The same experimental approach was used to test the predicted structure for the TS.

Aim II: Molecular specificity determinants of TraD for TraI recognition

The CP is known to act as a substrate receptor for the T4SS (Cascales & Christie, 2004). TraD of F, R1 and R100 showed some promising distinguishing features at the C-terminus, which could be responsible for specific TraI recognition.

Aim III: R1 partitioning system and T4SS connection

Recent experiments of our group showed a surprising connection between the partitioning system and the T4SS of R1. The use of a parM knock out strain and the ParM protein in the CRAfT brought new details of the interconnection of these two systems.

Aim IV: Western blot analysis

A part of my work was to test three antisera against the T4SS proteins TraD, TraY and TraI for their specificity and dilution range. The goal was to find out for which experimental approaches they can be used and if it is possible to determine the number of protein per cell via a quantitative western blot.

2 Materials and Methods

2.1 Material

2.1.1 Bacterial strains

Table 2.1: *Escherichia coli* strains used in this study

E. coli Strain	Description	References
DH5α	*endA1 recA1 gyrA96 thi-l hsdR17 supE44 λ- relA1deoR Δ(lacZYA- argF)- U169 φ80dlacZΔ(M15)*	Woodcock et al., 1989
MS411	*Ivlg rfp50 thi*	M. Schembri; DTU, Denmark
MS614	Sm^R, *ilvG, rfb-50, thi, rpsL*	M. Schembri; DTU, Denmark
CSH26Cm::LTL	Tc^R, CSH26 *galK::cat::loxP-tet-loxP*	Lang et al., 2010
CSH26Cm::LKL	Km^R, CSH26 *galK::cat::loxP-km-loxP*	Lang et al., 2010
DY330	W3110 *ΔlacU169 gal 490 ts λcl857 Δ(cro-bioA)*	Yu et al., 2000
BL21DE3	Cm^R, E. coli B F- ompT hsdS(rB-mB-) dcm+ Tetr gal l (DE3) endA Hte [argU IleY leuW Camr	Joel F. Schildbach; Johns Hopkins University

2.1.2 Plasmids

Table 2.2: Conjugative plasmids

Conjugative Plasmids		
Name	Description	Reference
R1-16	Km^R, IncFII, *fin-*	Goebel et al., 1977
R1-16Δ*parM*	Km^R, IncFII with site specific *parM* null mutation	This study
pOX38	Km^R, IncFI, derivative of F	Chandler & Galas, 1983
R100-1	Tc^R, Cm^R	Womble & Rownd, 1988
R100-1Δ*cat*	Tc^R	This study
R1-16Δ*traD*	Km^R, IncFII, *fin-* with site specific *traD* null mutation	Klug B.

Table 2.3: Vectors

Vectors		
Name	Description	Reference
CFP B	Amp^R; Cre fusion plasmid; *cre* from phage P1 cloned into the NheI and SalI site of pBR322	Parker & Meyer, 2007
CreTraI(3-1756)	CFP B with R1 *traI* encoding residue 3 to 1756	Lang et al., 2010
CreTraI(530-816)	CFP B with partial R1 *traI* encoding residue 530-816	Lang et al., 2010
CreTraI(1255-1564)	CFP B with partial R1 *traI* encoding residue 1255-1264	Lang et al., 2010
CreTraI F	*Kpn*I-*Sal*I fragment from p99I+ cloned into CFP B	Lang et al., 2010
CreTraI F (530-816)	CFP B with partial F *traI* encoding residue 530-816	Lang et al., 2010
CreTraI F (1255-1564)	CFP B with partial F *traI* encoding residue 1255-1564	Lang et al., 2010
CreTraI F TSA T757S	CFP B with partial F *traI* encoding residue 530-816 and an exchange from residue T at position 757 to S	This study
CreTraI F TSA L626H	CFP B with partial F *traI* encoding residue 530-816 and an exchange from residue L at position 626 to H	This study
CreTraI F TSB E1382V	CFP B with partial F *traI* encoding residue 1255-1564 and an exchange from residue E at position 1382 to V	This study
CreTraI F TSB H1283N	CFP B with partial F *traI* encoding residue 1255-1564 and an exchange from residue H at position 1283 to N	This study
CreTraI TSB N1283H	CFP B with partial R1 *traI* encoding residue 1255-1564 and an exchange from residue N at position 1283 to H	This study
CreTraI TSB-R1344Q	CFP B with partial R1 *traI* encoding residue 1255-1564 and an exchange from residue R at position 1344 to Q	This study
CreTraI F TSB R1344Q	CFP B with partial F *traI* encoding residue 1255-1564 and an exchange from residue R at position 1344 to Q	This study
CreParM	CFP B with *parM*	This study

CreParMK123A	CFP B with *parM* and a K123A mutation	This study
CreParMS39A	CFP B with *parM* and a S39A mutation	This study
CreParMD170E	CFP B with *parM* and a D170E mutation	This study
CreParR	CFP B with *parR*	This study
CreParRR6S	CFP B with *parR* and a R6S mutation	This study
CreTraI (3-1756) Sm	Sm^R; CreTraI(3-1756); *bla* gene replaced with *aadA* from pAH144 (Haldimann & Wanner, 2001)	Lang et al., 2010
pMS119HE	Amp^R, Ptac expression vector	Strack et al., 1992;
pMS119HEParM	pMS119HE with *parM*	Kirchberger P.
pMS119HEParMK123A	pMS119HE with *parM* and a K123A mutation	Kirchberger P.
pMS119HEParMD170E	pMS119HE with *parM* and a D170E mutation	Kirchberger P.
Codon usage plus	Provides rare t-RNA	Joel F. Schildbach; Johns Hopkins University
pET21cTraY	pET21 plasmid with 227 bp wild type *traY* gene of plasmid R1drd19	Joel F. Schildbach; Johns Hopkins University

2.1.3 Oligonucleotides

Table 2.4: Oligonucleotides for single amino acid exchanges. Red letters: restriction site; green letters: stop codon; underlined: mutated amino acid. Genbank Accession numbers: *traI* F, BAA97974; *traI* R1, AAQ98619.

Exchange	Primer Sets	Sequence 5' – 3'	Position on *traI*
TraI TSA T757S	Set 1: TraI_SFw5 + TraI_S757Rev	CACAGGCAGGGAGGCCGGTTCAGC	nt. 2280-2256
	Set 2: TraI_SRev8 + TraI_S757Fw	GCTGAACCGGCCTCCCTGCCTGTG	nt. 2256-2280
	Set 3: TraI_SFw5 + TraI_SRev8	ATAGTAGGTACC-GTACAGGTCCTGATAACC CATGTAGTCGACTTA-GGAATACAGCCGGAC	nt. 1588-1605 nt. 2448-2434
TraI TSA L626H	Set 1: TraI_SFw5 + TraI_L626HRev	GTCACCTCCGGGTGTCCGAGCACG	nt. 1889-1865
	Set 2: TraI_SRev8 + TraI_L626HFw	CGTGCTCGGACACCCGGAGGTGAC	nt. 1865-1889
	Set 3: TraI_SFw5 + TraI_SRev8	ATAGTAGGTACC-GTACAGGTCCTGATAACC CATGTAGTCGACTTA-GGAATACAGCCGGAC	nt. 1588-1605 nt. 2448-2434

Exchange	Primer Sets	Sequence 5' – 3'	Position on *traI*
TraI TSB E1382V	Set 1: TraI_SFw12 + TraI_E1382VRev	CCGGGGCGAATCACCCGGGTCTGCTG	nt. 4157-4131
	Set 2: TraI_SRev11 + TraI_E1382VFw	CAGCAGACCCGGGTGATTCGCCCCGG	nt. 4131-4157
	Set 3: TraI_SFw12 + TraI_SRev11	ATAGTAGGTACC-GGCAAAGAGCAGGTCATG CATGTAGTCGACTTA-TTTCACCCGTCCTTC	nt. 3763-3780 nt. 4692-4678
TraI TSB H1283N	Set 1: TraI_SFw12 + TraI_H1283NRev	CGTCCCGATTTGTCTCCC	nt. 3854-3837
	Set 2: TraI_SRev11 + TraI_H1283NFw	GGGAGACAAATCGGGACG	nt: 3837-3854
	Set 3: TraI_SFw12 + TraI_SRev11	ATAGTAGGTACC-GGCAAAGAGCAGGTCATG CATGTAGTCGACTTA-TTTCACCCGTCCTTC	nt. 3763-3780 nt. 4692-4678
TraI TSB N1382H, R1	Set 1: TraI_SFw12 + TraI_N1283HRev,R1	CATCCGGGTGTTTCTCC	nt. 3854-3837
	Set 2: TraI_SRev11 + TraI_N1283HFw,R1	GGAGAAACACCCGGATG	nt. 3837-3854
	Set 3: TraI_SFw12 + TraI_SRev11	ATAGTAGGTACC-GGCAAAGAGCAGGTCATG CATGTAGTCGACTTA-TTTCACCCGTCCTTC	nt. 3763-3780 nt. 4692-4678

Exchange	Primer Sets	Sequence 5' – 3'	Position on *traI*
TraI TSB R1344Q	Set 1: TraI_SFw12 + TraI_R1344QRev	GAAGCGCATCTGGTCACCGGT	nt. 4020-4041
	Set 2: TraI_SRev11 + TraI_R1344QFw	ACCGTTGACCAGATGCGCTTC	nt. 4041-4020
	Set 3: TraI_SFw12 + TraI_SRev11	ATAGTAGGTACC-GGCAAAGAGCAGGTCATG CATGTAGTCGACTTA-TTTCACCCGTCCTTC	nt. 3763-3780 nt. 4692-4678
TraI TSB R1344Q, R1	Set 1: TraI_SFw12 + TraI_R1344QRev	GAAGCGCATCTGGTCGCCGGT	nt. 4020-4041
	Set 2: TraI_SRev11 + TraI_R1344QFw,R1	ACCGGCGACCAGATGCGCTTC	nt. 4041-4020
	Set 3: TraI_SFw12 + TraI_SRev11	ATAGTAGGTACC-GGCAAAGAGCAGGTCATG CATGTAGTCGACTTA-TTTCACCCGTCCTTC	nt. 3763-3780 nt. 4692-4678

Table 2.5: Oligonucleotides for *parM* and *parR* cloning: red sequence: restriction sites; green sequence: stop codon. Genbank Accession number: ParA locus, X04268; *parM* nt. 183 to 1145 and *parR* nt. 11451 to 1448 from the ParA locus.

Name	Sequence 5' – 3'	Position
ParM_SFw	ATAGTAGGTACC-TTGGTATTCATTGATGACG	*parM* nt. 3-22
ParM_SRev	GCAATCGTCGACTTA-ATTACCTATGAGATACATACCGT	*parM* nt. 960-937
ParR KpnI Fw	ATAGTAGGTACC-ATGGACAAGCGCAGAACC	*parR* nt. 3-21
ParR R6S KpnI Fw	ATAGTAGGTACC-ATGGACAAGCGCAGCACC	*parR* nt. 3-21
ParR SalI Rev	GCAATCGTCGACTTA-TTAATTTATTAGCTTCATCGC	*parR* nt. 353-332

Table 2.6: Oligonucleotides for colony PCR and sequencing. Genbank Accession numbers: *traI* F, BAA97974; *traI* R1, AAQ98619; ParA locus, X04268; *parM* nt. 183 to 1145 from the ParA locus.

Name	Sequence 5' – 3'	Position
CFPFw	GGATAGTGAAACAGGGGCA	CFPB, nt. 1228-1246
CFPRev	AAATGACCCAGAGCGCTGC	CFPB, nt. 1424-1409
TraISeqFw3	GTACGGGAACAGGCCATA	*traI*, nt. 1810-1827
TraISeqRev7	TCACCCTCAGTCGCTCG	*traI*, nt. 2161-2145
TraISeqRev8	AGTCATGGTCACCTCAGG	*traI*, nt. 1896-1879
ParM1Fwseq	CCGGTAAGCGAAGTGGATA	*parM*, nt. 274-292
ParM2Fwseq	GTCTCTCTGGTTACATCTGC	*parM*, nt. 595-614
TraI SeqRev 10	CACTTAATCCGGCAATCGC	*traI*, nt. 985-967
Pms1	GCGTCAGGCAGCCATCGGAAG	pMS119HE plasmid
PmsReverse	CCGCTTCTGCGTTCTGA	pMS119HE plasmid

Table 2.7: Oligonucleotides for gene replacement. Genbank Accession numbers: ParA locus, X04268; *cat* R100, AP000342.

Name	Sequence 5' – 3'	Position
LoxFw	GAGAAAAAAATCAC-TATAACTTCGTATAG	*loxP*-sites (underlined) + homologous sequence *cat* of CSH26Cm
LoxRev	TATCAACGGTGGTATATCC-GGATAACTTCGTATAA	*loxP*-sites (underlined) + homologous sequence *cat* of CSH26Cm
ParMLoxFw	CCCAAAACATACCCAAACACACACCAAAAAAACACCATAAGGAGTTTTATAA-TATAACTTCGTATAG	*loxP*-sites (underlined) + homologous sequence ParA locus nt. 131-182
ParMLoxRev	GTTTGATTTACATCTGGATTTAGTTTGAAGGCAATGGTTCTGCGCTTGTCCATCA-GGATAACTTCGTATAA	*loxP*-sites (underlined) + homologous sequence ParA locus nt. 1200-1146
Ucas	CAAGAATTGCCGGCGGAT	pT809.gb, nt. 1-18
Dcas	GGTATTTCACACCGCATAGC	pT809.gb, nt. 81-62
ParC_Fw	CCCAAAAACAACCCATACCC	ParA locus, nt. 20-45
ParR_Rev	TCAGCAGCTCACATAAAAGG	ParA locus, nt. 1337-1318
CmLox_Fw	TTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCA-TATAACTTCGTATAG	*loxP*-sites (underlined) + Homologous sequence from *cat* R100, nt. 1-50
CmLox_Rev	ATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCA-GGATAACTTCGTATAA	*loxP*-sites (underlined) + homologous sequence from *cat* R100, nt. 660-610

2.1.4 Media

LB media were autoclaved for 20min at 121℃

LB medium:	10g/l	peptone
	5g/l	yeast extract
	5g/l	NaCl

LB agar medium:	10g/l	peptone
	5g/l	yeast extract
	5g/l	NaCl
	15g/l	agar agar

2.1.5 Antibiotics

Table 2.8: Used antibiotics.

Antibiotic	Stock concentration	End concentration
Ampicillin, Amp	100 mg/ml	100 µg/ml
Chloramphenicol, Cm	20 mg/ml in 96% EtOH	10 µg/ml
Kanamycin, Km	40 mg/ml	40 µg/ml
Streptomycin, Sm	50 mg/ml	25 µg/ml
Tetracycline, Tc	16 mg/ml in 70% EtOH	8 µg/ml

2.1.6 Buffers and Solutions

Agarosegel:

50 x TAE (Tris-acetate-EDTA):	2 M Tris-Acetate
	50 mM EDTA pH 8

50 x TBE (Tris-borate-EDTA):	890 mM Tris (Fluka)
	890 mM Boric acid
	2.5 mM EDTA

DNA 10x Loading Dye:	1 % SDS
	50 % glycerin
	1 x TBE
	0.05 % bromophenol blue (Bio Rad)
	0.05 % xylene cyanole (Bio Rad)

SDS-Page and Western Blot:

2 x FSB:	60 mg/ml DTT
	60 mg/ml SDS
	0.4 mg/ml bromphenol blue
	340 µl/ml glycerol
	53 µl/ml Tris pH 6.8
10 x Running Buffer:	30,25 g/l Tris
	144 g/l Glycin
	10 g/l SDS
Coomassie staining solution:	0.25 % Coomassie Brilliant Blue R250
	50 % Ethanol
	10 % Acedic acid
	40 % H_2O
Destaining solution:	30 % Ethanol
	10% Acedic acid
	60 % H_2O
CAPS:	10 mM CAPS
	10 % Methanol
	pH 11 with NaOH

10 x TST:	1.5 M NaCl
	1 % Tween 20
	0.5 M Tris
	pH 7.5 with HCl

Miniprep:

Lysis Buffer I:	25mM Tris-HCl, pH 7.5
	50mM Glucose
	10mM EDTA
Lysis Buffer II:	1M NaOH
	1% SDS
3 M potassium acetate:	3 M CH_3COOK
	5 M glacial acetic acid

2.2 Methods

2.2.1 *E.coli* growth and storage

Growth conditions

E. coli strains were grown at 37℃ in LB media on a shaker (180 rpm). For strains that contain an antibiotic resistance or a plasmid, the selective antibiotic was added. The temperature sensitive strain DY330 was grown at 30℃.

Cultures on LB agar plates were grown over night on 37℃ or 30℃.

Storage conditions

For short time storage (not longer than two weeks) the strains were stroke out on a LB agar plate, with appropriate antibiotics added if necessary, and stored at 4℃.
The strains were stored at -80℃ for long time stor age. Therefore an overnight culture was mixed in a ratio 1:1 with 40% glycerol and frozen.

2.2.2 PCR Techniques

Phusion Polymerase

Amplification of genes or DNA fragments needed for cloning was made with PCR. The *Pfu* DNA Polymerase from Fermentas was used because of the 3' to 5' proofreading activity. All primers used are listed in Section 2.1.3. The annealing temperature was set 5℃ below the lowest melting te mperature of the primers. Extension time was adjusted to the length of the template. Fermentas proposes 2min/kb. The reaction mix composition and the conditions for PCR are listed in Table 2.9.

Table 2.9: Standard reaction mix and PCR conditions for *Pfu* DNA Polymerase usage.

Reaction Mix	
Components	µl
Water, nuclease free	Variable
10X *Pfu* Buffer with MgSO4	5 µl
dNTPMix (2mM)	5 µl
Fw Primer (10pmol/µl)	5 µl
Rev Primer (10pmol/µl)	5 µl
Template DNA 50ng	Variable
Pfu DNA Polymerase (2.5u/µl)	0.5 µl
Total Volume	50 µl

PCR conditions			
Step	Temperature ℃	Time	Cycles
Initial denaturation	95℃	3 min	1
Denaturation	95℃	30 sec	35
Annealing	Tm – 5℃	30 sec	
Extension	72℃	2min/kb	
Final extension	72℃	10 min	1
Store at	4℃		

Mutagenesis with Two-Step PCR

To exchange a single amino acid a two-step PCR was made.

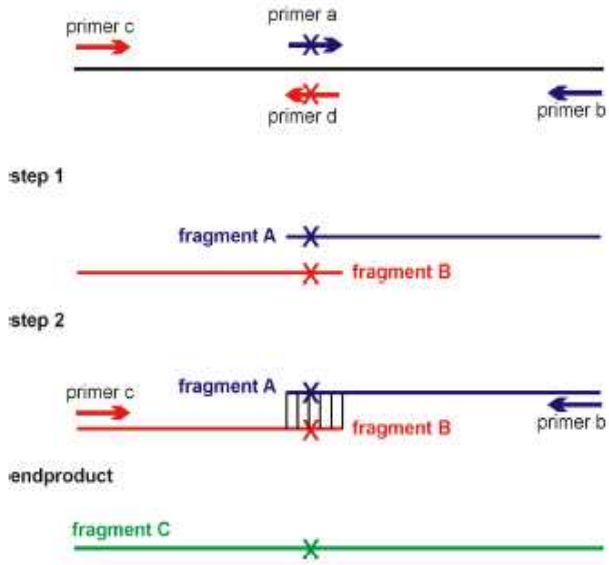

Figure 2.1: **Schematic illustration of the Two-Step PCR method**. In step 1 primer set 1 (primers c and d; red arrows) and primer set 2 (primers a and b; blue arrows) were used to create two PCR fragments A and B with the desired point mutation. The point mutation is indicated with a cross. The regions around the mutations are homologue and anneal during the PCR reaction in step 2. Primer set 3 (primers c and b) was used to amplify fragment C (green) that contains the exchanged single amino acid. (Adopted from Silvia Lang)

For the first step primer pairs set 1(Figure 2.1, red arrows) and set 2 (Figure 2.1, blue arrows) listed in Table 2.4 were used, in two independent PCR reactions, to create PCR products with the proposed point mutation (Figure 2.1, fragment A and B).

Table 2.10: Standard reaction mix and PCR conditions for the first step.

Reaction Mix	
Components	µl
Water, nuclease free	Variable
10X *Pfu* Buffer with MgSO4	5 µl
dNTPMix (2mM)	5 µl
Fw Primer Set1/2 (10pmol/µl)	5 µl
Rev Primer Set1/2 (10pmol/µl)	5 µl
Template DNA 50ng	Variable
Pfu DNA Polymerase (2.5u/µl)	0.5 µl
Total Volume	50 µl

PCR conditions			
Step	Temperature ℃	Time	Cycles
Initial denaturation	95℃	3 min	1
Denaturation	95℃	30 sec	35
Annealing	Tm – 5℃	30 sec	
Extension	72℃	2min/kb	
Final extension	72℃	10 min	1
Store at	4℃		

For the second step the products from the PCRs with primer set 1 and 2 were used as templates. The sequences, containing the point mutation, are homologous so that they can anneal and act as primers. During 10 cycles the polymerase fills up the template strains.

Table 2.11: Standard reaction mix and PCR conditions for the first 10 cycles of the second step.

Reaction Mix	
Components	µl
Water, nuclease free	Variable
10X *Pfu* Buffer with MgSO4	5 µl
dNTPMix (2mM)	5 µl
PCR product 1 50ng	Variable
PCR product 2 50ng	Variable
Pfu DNA Polymerase (2.5u/µl)	0.5 µl
Total Volume	40 µl

PCR conditions			
Step	Temperature ℃	Time	Cycles
Initial denaturation	95℃	3 min	1
Denaturation	95℃	30 sec	10
Annealing	Tm – 5℃	30 sec	
Extension	72℃	2min/kb	
Final extension	72℃	10 min	1

After 10 cycles of amplification 5 µl from each primer of set 3 (Table 2.4) were added to the reaction mix. The PCR was continued for 35 cycles

Table 2.12: PCR conditions for the last 35 cycles of the second step.

PCR conditions			
Step	Temperature ℃	Time	Cycles
Initial denaturation	95℃	3 min	1
Denaturation	95℃	30 sec	35
Annealing	Tm – 5℃	30 sec	
Extension	72℃	2min/kb	
Final extension	72℃	10 min	1
Store at	4℃		

Colony PCR

After transformation the clones were screened for the plasmid with colony PCR. Therefore a colony was picked from the selection plate and resuspended in 20 µl of water. The suspension was cooked at 95℃ for 10 min to lyse the cells and afterwards centrifuged for 1 min at 5000rpm. For PCR 1 µl of the lysed cell suspension was added to the reaction mix as template.

To check for the plasmid and the right orientation of the insert *Taq* Polymerase from Fermentas was used because this step does not require a proofreading activity of the polymerase.

Table 2.13: Standard reaction mix and PCR conditions for colony PCR.

Reaction Mix	
Components	µl
Water, nuclease free	14 µl
10x ThermoPol Buffer	2 µl
dNTPMix (2mM)	1 µl
Fw Primer (10pmol/µl)	1 µl
Rev Primer (10pmol/µl)	1 µl
Template	1 µl
Taq DNA Polymerase (5u/µl)	0.05 µl
Total Volume	20 µl

PCR conditions			
Step	Temperature ℃	Time	Cycles
Initial denaturation	95℃	1 min	1
Denaturation	95℃	30 sec	25
Annealing	Tm – 5℃	30 sec	
Extension	72℃	1min/kb	
Final extension	72℃	5 min	1
Store at	4℃		

2.2.3 DNA preparation and modification

Agarose gel electrophoreses

To separate DNA fragments 1% agarose gels prepared with 1x TAE or 1,2% agarose gels prepared with 1xTBE were used. For visualization of the DNA 0,05 µg/ml ethidium bromide was added. The gels were run in 1xTAE or 1xTBE Buffer.

To estimate the size or the concentration of the DNA fragments a DNA standard marker (Gene Ruler^{TM} 1KbDNA ladder or Gene Ruler^{TM} 100bp DNA ladder) was put on the gel together with the samples. The gel was visualized under UV light.

Plasmid isolation with a Miniprep Kit

Smaller plasmids like CFPB or pMS119HE were isolated with the QIAprep Spin Miniprep kit (Quiagen). The isolation was made as described in the manual of the manufacturer. The final elution step of the plasmid was made with 30 µl of nuclease free H_2O.

Plasmid isolation with phenol/chlorophorm extraction

Bigger plasmids like the R1-16 (around 40kb) were isolated with phenol/chlorophorm extraction.

Cells from a 4 ml ONC were harvested at 5000 rpm for 10 min. The pellet was resuspended in 200 µl of chilled lysis buffer I and 200 µl of freshly prepared lysis buffer II. The mixture was incubated at room temperature for 5 min and accordingly 150 µl of chilled 3 M potassium acetate was added. The sample was centrifuged for 10 min at 13 000 rpm at 4℃. Meanwhile 2 ml tubes c ontaining 400 µl water saturated phenol and 300 µl chlorophorm were prepared. After centrifugation the supernatant was added to the phenol/chlorophorm mixture and vortexed. After centrifugation for 5 min at 5000 rpm at room temperature the aqueous phase was transferred into a new reaction tube. By adding 1 ml of 96% ethanol, incubation for 30 min at -20℃ and centrifugation for 30 min at 13 000 rpm at 4℃ the DNA was precipitated. After removing the supernatant the DNA pellet was washed with 70% ethanol by centrifugation for 15 min at 13 000 rpm at 4℃ and dried by air at 37℃. The DNA was dissolved in 30 µl ddH_2O with 3 µl of DNase free RNase A (1 mg/ml) over night at 4℃.

Purification of PCR products

Before restriction digest PCR products were purified after gel electrophoreses. Therefore the gel slice with the DNA fragment inside was cut out. The QUIquick Gel extraction kit (Quiagen) was used to extract the PCR product out of the gel. The kit was used as intended by the provider. The final elution step was made with 30 µl of nuclease free water.

Restriction digest

The purified PCR products were digested with two restriction enzymes: *KpnI* and *SalI* (Fermentas). Double restriction was possible because both enzymes required the same 1x *BamHI* Buffer (Fermentas). The whole purified PCR product (500-1000ng) was digested. A 30 µl reaction mix was made, containing 25 µl of the PCR product, 3 µl 1x *BamHI* Buffer and 1µl (10 u/µl) of *KpnI* and *SalI* .

The restriction mixture was incubated over night at 37℃. The next day, the reaction was inactivated by heating up to 80℃ for 20 min. T he whole restriction digest was loaded on an agarose gel and purified via gel extraction as described above. Dephosphorylation was not necessary because of the two different restriction enzymes.

Ligation

The concentration of the insert for the ligation was calculated with the formula below:

$$\text{Insert concentration} = \frac{\text{Vector ng x Insert kb}}{\text{Vector Kb}} \times \frac{3}{1} \text{ (Insert/Vector ratio)}$$

Usually 100 ng of the cut vector was used for ligation. Reaction mixture with 15 µl volume was made containing 100 ng of the vectore, the calculated concentration of the insert, 1.5 µl 10x T4 ligase buffer, 1 µl T4 DNA ligase 1u/µl (Fermentas). The ligation was incubated over night at 16℃. Before t he transformation into electrocompetent *E. coli* cells the mixture was desalted by dialyzing against ddH_2O with a nitrocellulose filter (Millipore) for 1 hour at room temperature.

2.2.4 Electrotransformation of *E. coli*

Making electrocompetent cells

In 50 ml of fresh LB media an ONC was diluted 1:50. The culture was grown at 37℃ on a shaker (180 rpm) to an OD_{600} of 0.5 to 0.7. When it has reached the desired OD_{600} the culture was cooled down on ice for 10 min. Afterwards the cells were harvested at 5000 rpm for 6 min. The cell pellet was resuspended in 50 ml ice cold 10 % glycerol and centrifuged for 6 min at 5000rpm at 4℃. The supernatant has to be removed completely. This washing step was repeated once with 20 ml ice cold

10 % glycerol. The final washing step was made with 10 ml ice cold 10 % glycerol at 5000 rpm at 4℃ for 3 min. The pellet was resuspend ed in 500 µl ice cold 10% glycerol. The electrocompetet cells were used immediately or aliquots of 90 µl were made and frozen at -80℃.

Electrotransformation

Electrocompetent cells were transformed with plasmids, PCR products or desalted ligation mixture. Therefore 40 µl of electrocompetent cells were mixed with 10 – 300 ng of plasmid or the ligation mix and transferred to a cold electroporation cuvette. In the electroporator (Eppendorf 2510) the cells were pulsed with an 1800 volts shock. The time constant should be around 5. Directly after electroporation 1ml LB media was added and the suspension was incubated at 37℃ for 30 – 60 min. After the incubation, the mixture was diluted with LB or 0.9 % NaCl 10^0 - 10^{-3} and 100 µl were plated on selection plates containing the appropriate antibiotics to screen for the up taken plasmid. The plates were incubated over night.

The transformation rate can be determined as transformants/µg DNA.

2.2.5 Gene replacement by homologouse recombinaton (RED system)

Preparation of linear DNA fragments

First the *tet* cassette with the *loxP* sites was amplified out of the CSH26Cm::LTL strain with a colony PCR using the *Pfu* polymerase. Therefore the Lox Primers listed in Table 2.7 on position 1 and 2 were used.

Standard PCR conditions with *Pfu* polymerase were used to amplify the linear DNA fragments for transformation and homologous recombination (Figure 2.2, A.) The primers (Table 2.7, position 3, 4 and 9, 10) are composed of two sequences: An approximately 50 nucleotides long part which is homologous to sequences flanking the gene that should be replaced and the LoxFw and LoxRev primers for the amplification of a marker cassette.

The knock out was made to use it in the CRAfT. It was necessary to remove the selection marker in the end. This was achieved by the antibiotic resistance flanking *loxP* sites.

Induction and electrotranformation of DY330 cells

An ONC was diluted 1:50 in 50 ml fresh LB media in a 250 ml flask. The culture was incubated at 30℃ with shaking (180 rpm) until it reache an OD_{600} of 0.5 – 0.7. For induction, the flask was placed into a water bath at exactly 42℃ for 15 min (max. 20 min). This step activates the λ recombination genes *exo*, *bet* and *gam* (Yu et al., 2000). Immediately after the flask was placed into a mixture of water and ice and it was shaked until it has cooled down. From this point on it is very important that the induced culture is always kept on ice. Total time on ice should be min. 10 min and max. 40 min.

After induction the DY330 cells were made electrocompetent. The culture was transferred into a 50 ml Sarstedt tube and centrifuged for 8 min at 7000 rpm at 4℃. The supernatant was removed and the pellet was resuspended in 1 ml of ice cold 10 % glycerol and transferred to a 1.5 ml tube. The cells were spinned down for 1 min at 13 000 rpm at 4℃. This washing step was repeated three times. After the final washing step the pellet was resuspended in 100 µl ice cold 10 % glycerol and can be used for transformation (Figure 2.2, B.).

Transformation was performed as described above. It is important to employ at least 100 ng of linear DNA PCR product, better 300 – 500 ng, for successful transformation and homologous recombination. After 60 min of incubation at 30 ℃, 100 µl of the mixture was plated undiluted on a selection plate. The rest was spinned down, the pellet was resuspended in 100 µl LB and plated on a selection plate. The plates were incubated two days at 30℃.

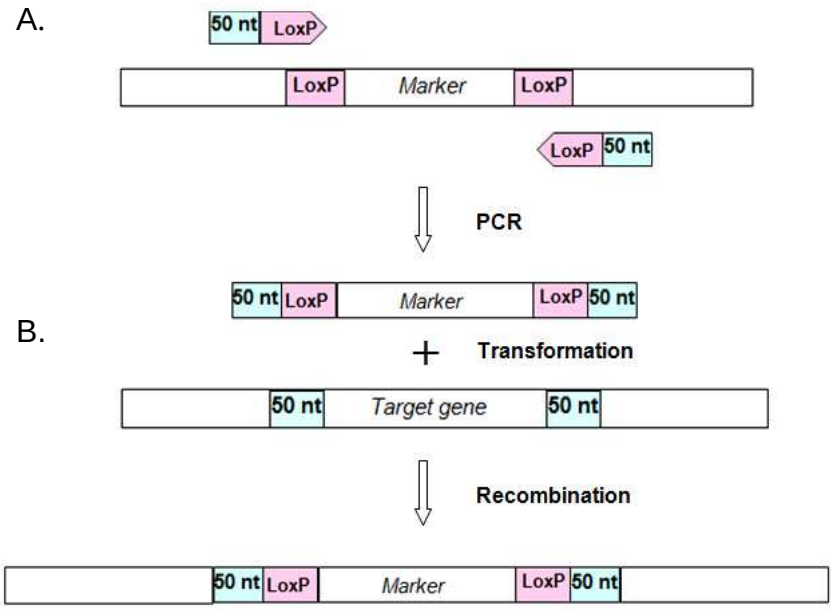

Figure 2.2: **Schematic strategy for gene replacement via homologous recombination**. The procedure consists of two steps. (A.) Preparation of linear DNA fragments containing a marker and 50 nucleotides homologous to the target gene at the 5' and 3' end. (B.) Transformation of the linear DNA fragment into induced DY330 cells. The λ recombination genes replace the target gene.

2.2.6 Cre Recombinase Assay for Translocation (CRAfT)

The CRAft was developed by Vergunst and colleagues to detect protein translocation and DNA transfer simultaneously (Vergunst et al., 2000, Vergunst et al., 2005). Silvia Lang established this method in our lap. Figure 2.3 shows a schematic illustration of the CRAfT.

The donor strain MS614 harbours the conjugative plasmid (Table 2.2) and a CFP B plasmid containing the protein of interest fused to a Cre recombinase (Table 2.3).

The recipient strain CSH626Cm::LTL harbours a chromosomal marker cassette for protein transfer detection. This cassette consists of a chloramphenicol (Cm) resistance interrupted by a tetracycline (Tc) resistance which is flanked by two *loxP* sites. Cre catalyses the recombination at the *loxP* sites and the switch from Cm to Tc resistance indicates the protein transfer from the donor to the recipient.

For the CRAfT in the R100 background CSH26Cm::LKL was used as recipient strain because of the tetracycline resistance on the R100-1 plasmid. In this strain the Cm resistance is interrupted by a kanamycin resistance.

100 µl of the donor ONCs were harvested and centrifuged at 5000 rpm for 6 min. The cell pellet was resuspended in 1 ml of 0.9 % NaCl. In 900 µl fresh LB medium the donors were diluted to an OD_{600} of 0.005 and incubated in a water bath for 1 h at 37℃. Meanwhile 4 ml of the recipient strain were harvested by centrifuging at 5000 rpm for 6 min and the pellet was resuspended in 1 ml 0.9 % NaCl. After 1 h of incubation, the donors reach an OD_{600} of 0.02. A 10x excess of the recipient was added to the donors and incubated at 37℃ in a water bath for 2 h 30 min. After the incubation time, the conjugation was stopped by vortexing for 1 min and cooling down on ice.

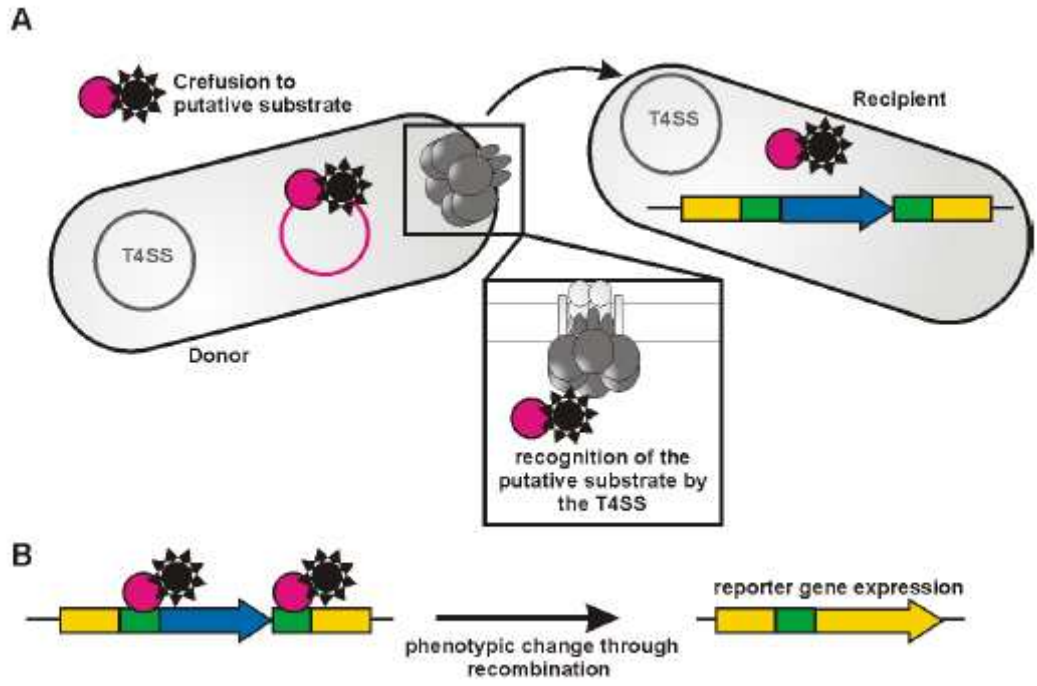

Figure 2.3**: CRAfT as a tool for the detection of conjugation and protein translocation**. A) Donor strain harbors a conjugative plasmid (grey) and a CFP B plasmid expressing the protein of interest (black star) fused to the Cre recombinase (pink circle). The recipient cell contains a resistance marker gene (yellow bars) interrupted with a second resistance (blue bar) flanked by *loxP* sites (green bars). The protein fused to Cre is recognized by the T4SS of the donor cell (black box) and transferred to the recipient. B) In the recipient cell Cre catalyses the recombination at the *loxP* sites. This leads to a detectable resistance switch. (Adopted from Silvia Lang)

Serial dilutions in 0.9 % NaCl of the suspension were made for plating on selection plates. The antibiotic concentrations for the selection plates can be found in Table 2.8. Recipients were selected by plating on LB agar plats containing tetracycline. Donors were identified by selecting on streptomycin or streptomycin and ampicillin

resistance. The Transconjugants were selected by plating on LB agar plates with kanamycin and X-Gal (50 µg/ml). Selection for the recombinants was made with chloramphenicol plates.
The selection for the CRAfT in the R100 background varies from the description above. Donors were selected with tetracycline or tetracycline and ampicillin plates and transconjugants with tetracycline and X-Gal plates. To screen for the recombinants chloramphenicol plates were used.
The conjugation frequency was calculated as transconjugants per donor and the protein translocation frequency as recombinants per donor.

The Cre recombinase activity, and on that way indirectly the stability of the fusion protein, was tested by direct transformation of 10 ng plasmid into the recipient strain. After 2 h 30 min incubation at 37℃ the suspension was diluted and stroke out on chloramphenicol and ampicillin plates. The transformation frequency per ng of DNA and the recombination frequency per ng of DNA were calculated.

2.2.7 Western Blot

Rabbit immunization with TraD

The TraDΔN130 protein was prepared by Sanja Mihajlovic. The protein concentration for immunization should be around 1 mg/ml. For immunization 625 µg/ml TraD was used and filled up with 1x PBS to 100µl. Three aliquots for the boosts were made and frozen at -20℃ until use. Before the immunization Georg Wäg added 1.5 to 2 fold of the sample volume Freund's adjuvant and a water in oil emulsion was made. The end volume for immunization should not be more than 1 ml. The injection was made subcutaneaously between the shoulder blades. After four weeks the second boost injection was made.

TraD anti serum preparation

Three weeks after the second boost injection 25 ml of blood from the rabbit were taken by Georg Wäg. The blood was incubated 2 h at room temperature to allow blood clotting. After detaching the coagulated blood from the Sarstedt tube wall it was centrifuged 30 min, 3000 rpm at 15℃. The blood pla sm was transferred into a 15 ml

Sarstedt tube and 1 mg/ml 10 % EDTA pH 7.8 and 0.02 % NaN_3 were added. The antiserum was stored at -4℃.

Sample preparation

Purified proteins were mixed with 10 µl 2x FSB and cooked at 95℃ for 5 minutes.

For the preparation of protein extracts, a culture was grown to an OD_{600} between 0.5 and 0.7. Volumes complying with OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1 ml were harvested, and centrifuged for 5 min at 5000 rpm. Pellets were mixed with 1x FSB and denatured at 95℃ for 10 min. The samples ware centrifuged for 1 min at 5000 rpm and the protein extract in the supernatant was loaded on the gel.

SDS-Gel electrophoreses

A discontinuous SDS page was used to separate the proteins for western blot analysis.

A 4.5 % stacking gel was used to load the samples and a 12.5 % running gel where the proteins were separated according to their size.

The running gel was prepared as indicated in Table 2.14. To avoid that the gel dries-out and the surface makes waves 200 µl of buthanol were dropped on the top. Polymerization was finished after 3 hours. The gels were stored in running buffer at 4℃. Before use, the gel was washed with water to rinse the buthanol.

Table 2.14: Running gel mixture for 1 middle gel

12.5 % Running gel (7 ml)	
Components	Volumes
ddH_2O	2.3 ml
1.5 M Tris/HCl pH 8.8 (low gel buffer)	1.7 ml
30 % Acrylamide/Bis-acrylamide 37.5:1 (Rotiphorese gel 30 -ROTH)	3 ml
10 % SDS	72 µl
10 % APS	19.2 µl
TEMED	6.2 µl

The stacking gel was prepared freshly as in Table 2.15 described. A comb was stuck in to form the slots for sample loading before it polymerizes.

Table 2.15: Stacking gel mixture for 1 gel

4.5 % Stacking gel (2ml)	
Components	Volumes
ddH_2O	1.15 ml
0.5 M Tris/HCl pH 6.8 (upper gel buffer)	0.5 ml
30 % Acrylamide/Bis-acrylamide 37.5:1 (Rotiphorese gel 30 -ROTH)	0.3 ml
10 % SDS	20 µl
10 % APS	20 µl
TEMED	4 µl
0.5 % Bromphenole blue	0,8 µl

Together with the samples a marker was loaded on the gel (Page Ruler^{TM} Prestained Protein Ladder, Fermentas). The camber was filled up with 10x running buffer. The electrophoreses was performed at 16 mA until the blue front ran out or longer according to the size of the protein.

Blotting

To activate the membrane (Immobilon-P transfer Membrane, Millipore) it was equilibrated in methanol for 1 min and afterwards stored in CAPS buffer until use. The stacking gel was removed from the running gel. It is necessary to mark the membrane before blotting to be assured on which site of the membrane the proteins were transferred. The gel and the membrane were assembled between two filter papers and sponges in a clamp. To transfer the proteins to the membrane it is important that there are no air bubbles between the membrane and the gel. The tank of the blotting chamber was filled up with CAPS buffer and the blotting “sandwich” was placed into the chamber. Blotting was performed at 240 mA and under spinning. The time depends on the size of the protein: TraY 1h, TraI relaxase and TraD 1h 30min and TraI full length 2h.

Blocking

After blotting, the membrane was blocked with 50 ml 1x TST containing 10 % dried milk powder (blotting grade) over night on a shaker at 4℃.

Immunoblotting

The primary and secondary antibodies were diluted in 1x TST containing 10 % dried milk powder. The dilution of the primary antibody depends on the quality of the antiserum (1:1000 to 1:40000). The secondary anti rabbit antibody was always diluted 1:10000. The dilutions can be reused up to 5 times.

After the blocking, the TST milk powder mixture was poured off. The membrane was incubated for 60 min with the first antibody at room temperature on a shaker. Washing of the membrane was made 3 times for 10 min with 1x TST. After the washing step the blot was incubated with the secondary antibody for 60 min at room temperature on a shaker. The three washing steps were repeated.

The detection of the western blot was made with chemiluminescence reaction of the ECL system from Amersham as prescribed.

Quantitative western blot analysis of TraD

For a quantitative western blot 10 µl of protein extract from lysed cells complying with OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1 ml were run on a gel together with a titration serial of purified TraD protein. Blotting to the membrane and immunoblotting was performed as described above. After the detection, the intensity of the cell lysate bands were quantified with Image J software and compared with the protein standard to calculate the TraD protein amount in the cell extract. Using viability counting the number of colony forming units per OD_{600} unit was determined.

3 Results

3.1 Molecular specificity determination in TraI TS

3.1.1 Sequence alignment of TS A and TS B

Silvia Lang showed that the minimal TS of TraI are translocated independently from each other and that they are specific for their cognate T4SS. This leads to the hypotheses that TS A and TS B must have something in common, because of the independent translocation, and that there are differences within the TS for each plasmid that support specific recognition by the cognate T4SS.

The features shared by TS A and TS B were visualized by the structural overlap with the already known structure of RecD2. The predicted structure fits for both TS.

The question for the differences was answered by a sequence alignment of TSA and TSB from R1 and F using ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2).

The alignment of the TS from F and R1 in Figure 3.1 shows that there are only two amino acid variations in TSA and nine in TSB (red residues in Figure 3.1).

A comparison of the amino acid sequence of TSA and TSB showed not much similarity but a well conserved cluster was found in both TS of F and R1 marked in Figure 3.1 with a yellow box.

A) TS A (residue 530 to 816 of TraI)

```
R1    QVLITDSGQRTGTGSALMAMKDAGVNTYRWQGGEQRPATIISEPDRNVRYARLAGDFAAS 60
F     QVLITDSGQRTGTGSALMAMKDAGVNTYRWQGGEQRPATIISEPDRNVRYARLAGDFAAS 60
      ************************************************************

R1    VKAGEESVAQVSGVREQAILTQAIRSELKTQGVLGHPEVTMTALSPVWLDSRSRYLRDMY 120
F     VKAGEESVAQVSGVREQAILTQAIRSELKTQGVLGLPEVTMTALSPVWLDSRSRYLRDMY 120
      ********************************** ************************

R1    RPGMVMEQWNPETRSHDRYVIDRVTAQSHSLTLRDAQGETQVVRISSLDSSWSLFRPEKM 180
F     RPGMVMEQWNPETRSHDRYVIDRVTAQSHSLTLRDAQGETQVVRISSLDSSWSLFRPEKM 180
      ************************************************************

R1    PVADGERLRVTGKIPGLRVSGGDRLQVASVSEDAMTVVVPGRAEPASLPVSDSPFTALKL 240
F     PVADGERLRVTGKIPGLRVSGGDRLQVASVSEDAMTVVVPGRAEPATLPVSDSPFTALKL 240
      *********************************************:**************

R1    ENGWVETPGHSVSDSATVFASVTQMAMDNATLNGLARSGRDVRLYS 286
F     ENGWVETPGHSVSDSATVFASVTQMAMDNATLNGLARSGRDVRLYS 286
      *********************************************
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B) TS B (residue 1255 to 1564 of TraI)

```
R1    LGKEQVMVPVLNTANIRDGELRRLSTWEKNPDALALVDNVYHRIAGISKDDGLITLQDAE 60
F     LGKEQVMVPVLNTANIRDGELRRLSTWETHRDALVLVDNVYHRIAGISKDDGLITLQDAE 60
      ****************************:: ***.************************

R1    GNTRLISPREAVAEGVTLYTPDKIRVGTGDRMRFTKSDRERGYVANSVWTVTAVSGDSVT 120
F     GNTRLISPREAVAEGVTLYTPDTIRVGTGDRMRFTKSDRERGYVANSVWTVTAVSGDSVT 120
      **********************.************************************

R1    LSDGQQTRVIRPGQERAEQHIDLAYAITAHGAQGASETFAIALEGTEGNRKLMAGFESAY 180
F     LSDGQQTREIRPGQEQAEQHIDLAYAITAHGAQGASETFAIALEGTEGNRKLMAGFESAY 180
      ********.******:********************************************

R1    VALSRMKQHVQVYTDNRQGWTDAINNAVQKGTAHDVLEPKPDREVMNAQRLFSTARELRD 240
F     VALSRMKQHVQVYTDNRQGWTDAINNAVQKGTAHDVFEPKPDREVMNAERLFSTARELRD 240
      ************************************:***********:************

R1    VAAGRAVLRQAGLAGGDSPARFIAPGRKYPQPYVALPAFDRNGKSAGIWLNPLTTDDGNG 300
F     VAAGRAVLRQAGLAGGDSPARFIAPGRKYPQPYVALPAFDRNGKSAGIWLNPLTTDDGNG 300
      ************************************************************

R1    LRGFSGEGRVK 311
F     LRGFSGEGRVK 311
      ***********
```

Figure 3.1: **Amino acid sequence alignment of the TS from F and R1 TraI**. The alignment of the sequence of TS A and TS B was made with ClustalW2 program. In A) the comparison of R1 and F TS A and in B) the comparison of R1 and F TS B are shown. Variant amino acids are coloured in red. A conserved cluster in both TS of R1 and F is marked with a yellow box. Both non identical amino acids in TS A were exchanged and black circles mark the exchanged variant amino acids in TS B.

3.1.2 Position of variant amino acid on the predicted TS model

The next step was to find the position of the variant amino acids on the predicted TS structure.

First the two variant amino acids of TS A were localized. They are positioned on the opposite ends compared to the RecD2 structure in Figure 3.2 shown as red and yellow circles. They were compared with the positions of the 9 variant amino acids of TS B and a co-localization of a residue from TS A and TSB was found.

Amino acid 626 of TS A has a single position on the model, shown in red in Figure 3.2. The co-localized residues are 757 from TS A and 1382 from TS B are shown in yellow in Figure 3.2.

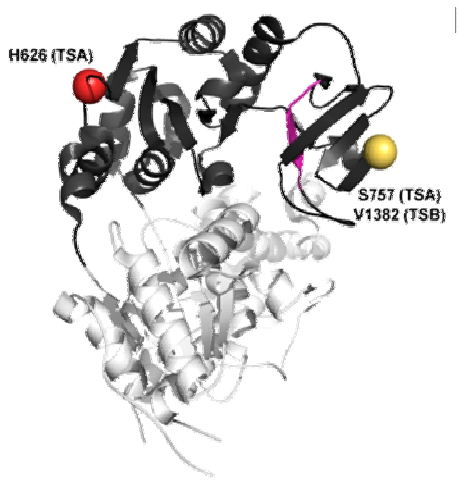

Figure 3.2: **Predicted structural overly of TSA and TSB with the structure of RecD2**. The structure of RecD2 from *D. radiodurans* is shown in grey. Structural parts of RecD2 conserved in TSA and TSB are colored in black. Position of amino acid 626 of TS A is shown in red and the co-localized residues 757 of TS A and 1382 of TS B are shown in yellow. The pink β-sheet represents a conserved cluster in TS A and TS B.

The position of the conserved cluster was also determined. It is positioned within a β-sheet coloured in pink in Figure 3.2 in both TS structure overlays with RecD2.

3.1.3 Construction of hybrid TS

As mentioned before, the transfer of minimal TS is specific for their cognate T4SS although the structural analysis predicted fold similarity for all TS of F and R1 TraI. There must be molecular specificity determinants within the TS that mediate this specific protein transport by the T4SS.

In this study, I first focused on defining the molecular specificity determinants of TS A and TS B for protein translocation. Promising targets are the variant amino acids in TS A (2 amino acids) and TS B (9 amino acids). The strategy was to exchange a variant amino acid in the TS from F to R1 and test the resulting hybrid TS in the CRAfT in both (F and R1) systems. If the exchanged amino acid is responsible for the specificity determination of the protein transfer during conjugation, than an alteration in the protein translocation frequency of the hybrid TS compared to the wild type TS should occur.

I decided to mutate both non identical amino acids (626 and 757) of TS A and amino acid 1382 of TS B, because of its position on the predicted model for the TS structure.

For the co-located amino acids 757 of TS A and 1382 of TS B (Figure 3.2, yellow) I expected that a mutation on this position in the TS should be tolerated. Due to the fact, that there is an existing natural variation of amino acids on this position it is not likely that it is involved in specific recognition of the TS and another mutation should not have a dramatic influence on the specificity.

The single positioned amino acid 626 of TSA (Figure 3.2, red) with no matching heterogenic amino acid of TSB is a promising candidate to be important for the specific translocation of the TS.

As a proof for this prediction and the proposed structural model for TS of TraI, I decided to mutate one of the variant amino acids of TS B that lies not on the overlapping structure with RecD2. I assumed that a mutation of residue 1283 should not have an influence on recognition and specificity if our structural model is correct.

I generated the hybrid TS with two-step PCR. During this special PCR method the amino acid is exchanged with special primers containing the desired mutation. Single amino acid exchanges from F to R1 of residue 626 and 757 in TS A and 1382 in TS

B were made. In the case of variant amino acid 1283 I exchanged the residue in both directions, F to R1 and R1 to F. The generated hybrid TS are listed in Figure 3.3.

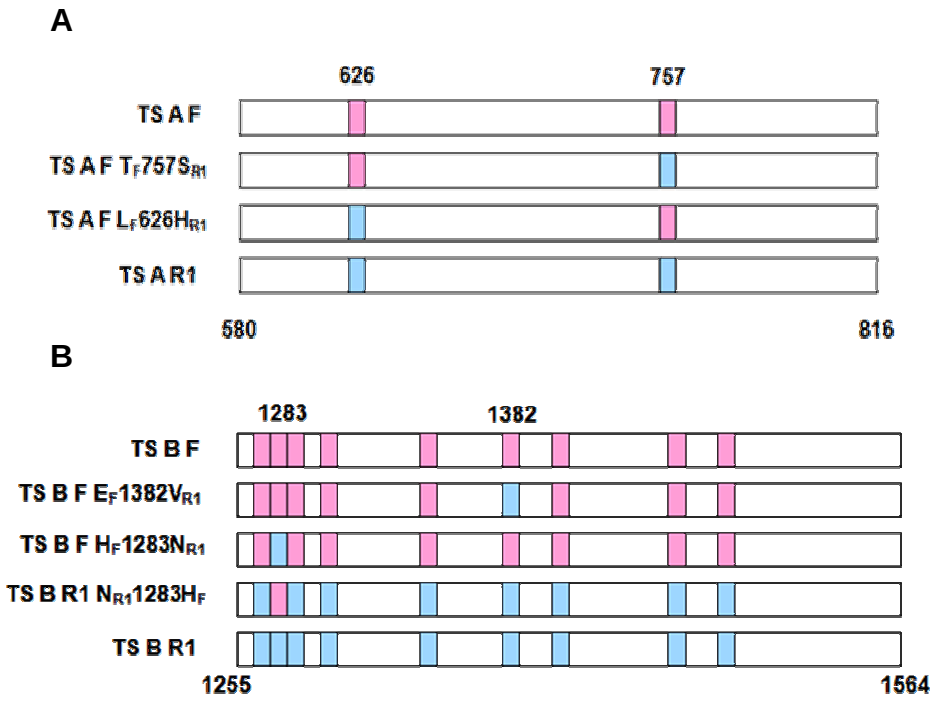

Figure 3.3: **Schematic illustration of generated hybrid TS**. By single amino acid exchange hybrid TS were produced. A) Both variant amino acids in TS A were exchanged and B) residue 1283 and 1382 were exchanged in TS B. Non identical amino acids from F are coloured in pink and from R1 in blue.

3.1.4 Variant amino acid 626: A molecular fidelity switch in TS A

To test the hybrid TS for its specificity I used the CRAfT developed by Vergunst and colleagues (Vergunst et al, 2000; Vergunst et al, 2005). Therefore the hybrids had to be fused with its N-terminus to the Cre-recombinase in the CFP B vector. Cre alone lacks features for the transport through the T4SS, but it is transported to the recipient when fused to the TS. The recipient harbours a Cm resistance gene that is interrupted with a Tc resistance gene flanked by *loxP* sites. Cre cut the *loxP* sites and this leads to a switch of the resistance from Tc to Cm and the protein translocation can be detected.

Before the use in the CRAfT the fusion protein was tested for its stability and the function of the Cre-recombinase. This was tested via transformation of 10 ng of the empty CFP B vector as control and the fusion protein containing plasmid to the

CSH26Cm::LTL recipient strain. Selection was made for the transformants and recombinants. The transformation rates for all tested plasmids was around 10^6 transformants/ng of DNA and the recombination rate was equal for all constructs at around 10^5 recombinants/ng of DNA. This test was made with all fusion proteins used in this work.

After I was sure that my constructs were functional, I tested the hybrid TS in the CRAfT for its protein translocation in the cognate and the heterologous background. The derepressed plasmids R1-16 of R1 and pOX38Km of F were used for the assay.

Figure 3.4 shows on the top a schematic illustration of the tested TS and their hybrids. The bars indicate the protein translocation frequency of the tested constructs. The upper panel illustrates the results in the R1-16 background and the lower in the pOX38Km background. In both panels the scale goes from the detection limit at 10^{-6} up to 10^{-3} recombinants per donors.

The conjugation frequencies of all donor strains were also tested. In the R1-16 and pOX38Km background all of the conjugation frequencies were in the normal range between 10^0 and 10^1 transconjugants per donors (data not shown).

As positive controls for the protein translocation the full length TraI (data not shown) and wild type TS A or TS B of the appropriate system were used. In the R1-16 background the transfer rate for both TS is around $3x10^{-5}$ recombinants per donors. In the pOX38Km background the translocation of TS A is around $3x10^{-4}$ and for TS B around $5x10^{-4}$ recombinants per donors. The empty CFPB plasmid (data not shown) and the TSA or TSB form the heterologous system were used as negative control. The transfer rate of all negative controls was under the detection limit of 10^{-6} recombinants per donor or not detectable.

As assumed the amino acid exchange TSA F T757S shows nearly the same protein translocation frequency as the wild type TSA in pOX38Km and could not be detected in the R1-16 background (Figure 3.4). The exchange TS B F E1382V reduces the translocation in the pOX38Km background to about 10^{-4} recombinants per donors,

but similar to TSA F T757S does not lead to detectable protein translocation in the heterologous system (Figure 3.4).

The amino acid exchange TS A F L626H from F to R1 leads to a specificity switch from the F to the R1 system (Figure 3.4, black bar). A transfer of the hybrid in the cognate system was not detectable. Moreover, a 12-fold gain of protein translocation in the heterologous system R1 appears. Compared with the protein translocation frequency of the wild type TS A from R1 the protein translocation of the hybrid TS A F L626H shows no statistically significant difference.

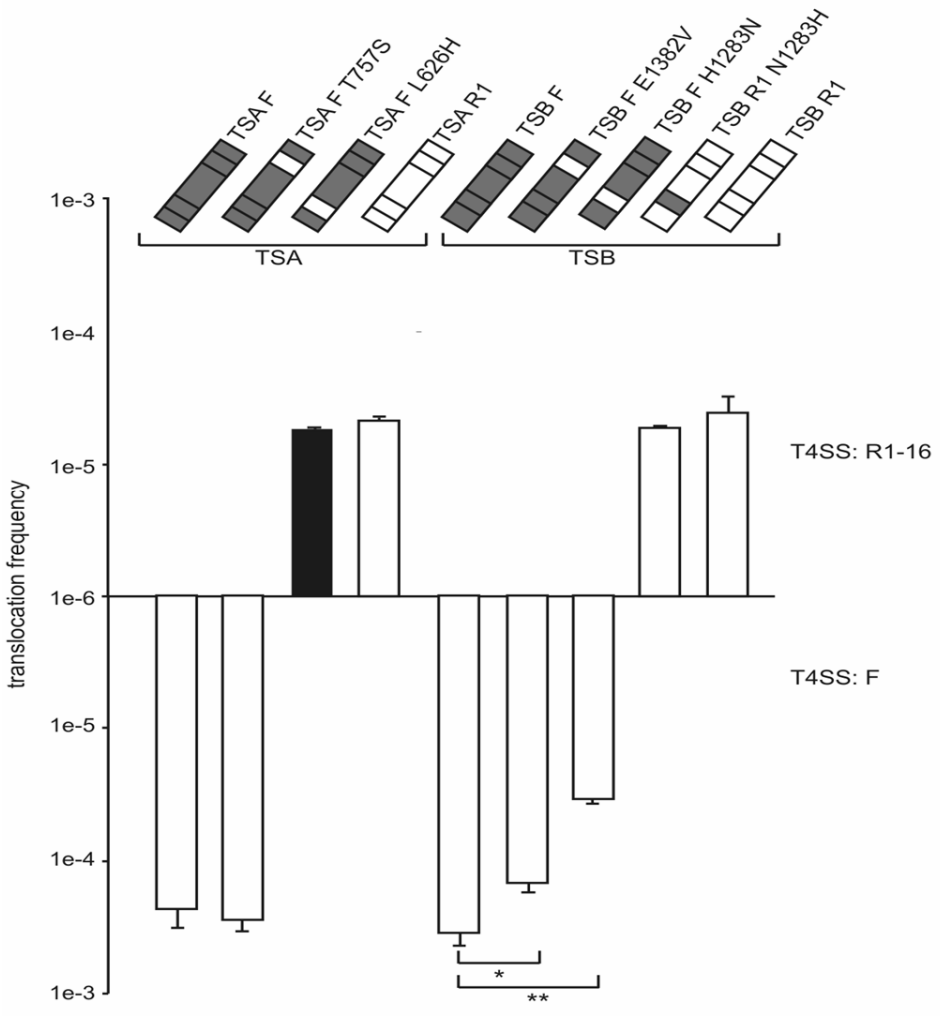

Figure 3.4: **Exchange of variant amino acid 626 from F to R1 in TSA switches fidelity**. The tested TS and hybrid TS are shown above. Grey stands for F and white for R1. The stripes illustrate the single amino acid exchanges. The upper panel shows the results of the assay in the R1-16 and the lower panel in the pOX38Km background. The translocation frequency is defined as recombinants per donors. All TraI fragments are tested in both systems for at least 3 times and are statistically significant compared to the negative control. The mean values and standard deviation are shown. The exchange of L626H in TSA from F leads to a specificity switch from F to R1 (black bar). (*, P<0.05; **, P<0.01)

As a proof for the predicted model of TS as a recognition motive I exchanged the variant amino 1283 of TS B. The exchange was made in F from F to R1 and in R1 from R1 to F. This hybrid pair was also tested in the CRAfT for its protein translocation. The exchange was tolerated in R1 and in F the translocation was reduced 10-fold. There was no detectable protein translocation in the heterologous system in both cases.

3.1.5 A conserved cluster influences translocation of TS

As I mentioned above, a conserved cluster was found in TS A and TS B. The conservation in both TS is a clear hind that this cluster could be important for correct recognition and translocation. Because of its location on the predicted model in a ß-sheet (Figure 3.2, pink ß-sheet) near the position of a variant amino acid in TS A and TS B it could also be an important structure giving feature.

To ask whether a mutation in this conserved sequence shows a loss or reduction of protein transfer, an amino acid exchange R1344Q in TSB from F and R1 was created with a two-step PCR (Figure 3.5).

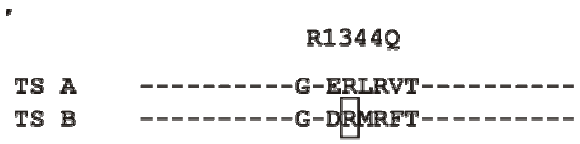

Figure 3.5: **Sequence alignment of the conserved cluster in TS A and TS B**. Within the conserved motif (G[E/D]R[L/M]R[V/F]T) an amino acid exchange R1344Q in TS B from F and R1 was made (marked with the black box).

I tested the mutated TS B R1344Q F and R1 in the CRAfT for its protein translocation. Figure 3.6 shows the result of the CRAfT. Black bars indicate the protein translocation frequency of the tested constructs. The scale starts at the detection limit at 10^{-6} and goes up to 10^{-2} recombinants per donors.

The conjugation frequencies for all donor strains were also tested (data not shown). In the R1-16 background all frequencies were as expected in the range between

10^0 and 10^1 transconjugants per donors. In the pOX38Km background only the conjugation frequency of the full length TraI containing donor showed a regular reduced frequency between 10^{-1} and 10^0 transconjugants per donors.

As positive controls for the protein translocation the full length TraI and the wild type TS B were used. TS B from R1 shows a protein translocation frequency about $3x10^{-5}$ (Figure 3.6, A) and TSB F about $4x10^{-4}$ (Figure 3.6, B) recombinants per donors. The empty CFP B vector was used as negative control and showed no protein transfer in both systems.

Figure 3.6, A shows the results in the R1-16 background. The protein translocation of the TS B R1344Q R1 was reduced about 25 % compared to wild type TS B R1. The result from the experiment in the pOX38Km background is shown in Figure 3.6, B. The transfer of TS B R1344Q F was near the detection limit ($3x10^{-6}$ recombinants per donors) compared to wild type TS B F.

It seems that the conserved cluster in the TS of TraI is important for its function.

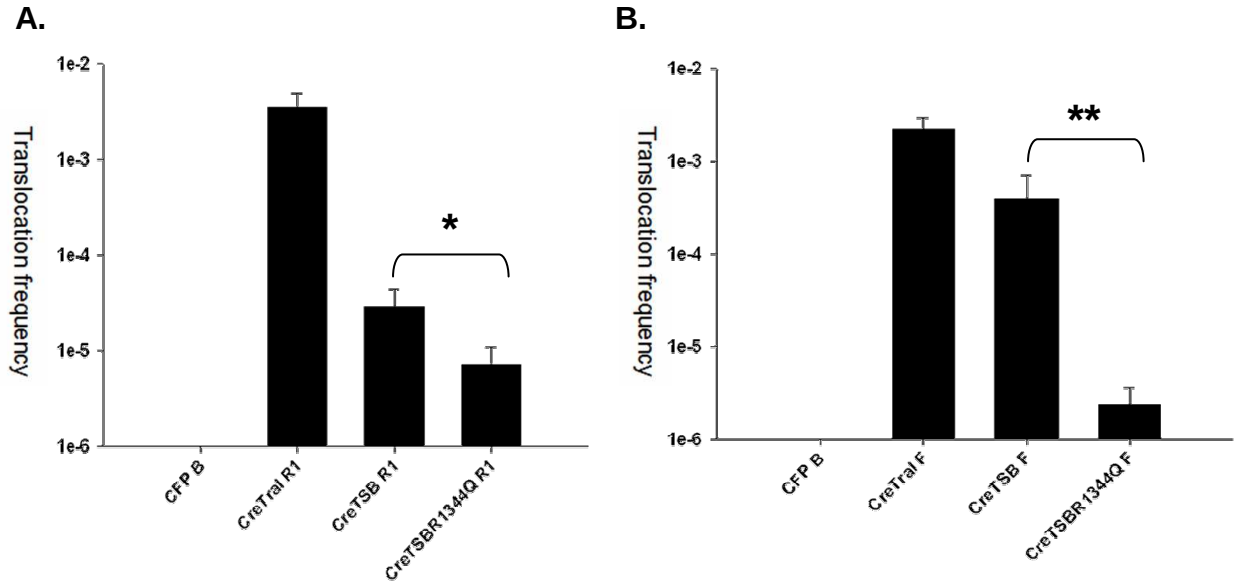

Figure 3.6: **Amino acid exchange in the cluster leads to a reduced protein translocation**. TSB with a R1344Q exchange was tested for protein translocation. (A.) CRAfT in the R1 background. (B.) CRAfT in the F background. In both systems the mutated TSB shows reduced protein translocation. Protein translocation frequency was calculated as recombinants per donors. The results are the mean values with standard deviations out of at least 3 experiments. All results are significantly different compared to the negative control.
(*, P<0.05; **, P<0.01)

3.2 Molecular specificity determinants of TraD for TraI recognition

It is supposed that the T4CP protein acts as a substrate receptor (Cascales & Christie, 2004). After we have found the molecular recognition signals of TraI with its putative structure, we concentrated on identifying the structural features of TraD are important for the specific recognition of TraI.

3.2.1 Remarkable differences in F and R1 TraD C-terminus

As a first step an alignment of TraD from R1 and F (95 % identical) was made using ClustalW2 program. The alignment of the amino acid sequence shows remarkable differences at the C-terminus: R1 TraD shows a sequence of PQQ repeats that does not exist in F TraD. On the other hand F TraD has a longer C-terminal tail.
To find out, if these differences are unique in R1 and F TraD a comparison with other T4CP was made. In the TraD of plasmid R100 we found another sequence of PQQ repeats like in R1 TraD (Figure 3.7, yellow box) and it also misses the longer C-terminal tail of F TraD (Figure 3.7, green box).

It was shown that the C-terminus of TraD is responsible for the specificity of F-plasmid conjugative transfer (Sastre et al, 1998).
I asked whether it is also responsible for the specific recognition of TraI in protein translocation and whether the plasmid specific differences in TraD mentioned above are responsible for plasmid specific protein recogniton.

I decided to test the protein translocation of TraI from F and R1 in the R100 background in the CRAfT. I expected three possible outcomes from the experiment:
(i) TraI of R1 should be translocated efficiently by R100 if the PQQ repeats present in R1 and R100 TraD are involved in the specific recognition of TraI.
(ii) For the case that F TraI is transported by R100 the PQQ repeats are not involved in the recognition process. If that happens we would have to concentrate on the similarities between the F and R100 T4CP and relaxosome proteins.
(iii) The third case would be that none of the heterologous TraI proteins are translocated by R100. That would mean that other molecular components of TraD or

maybe proteins of the relaxosome complex are involved in specific recognition of TraI.

Figure 3.7: **Sequence alignment of TraD from R1, R100 and F**. The yellow box represents the PQQ repeats in TraD of R1 and R100. The longer C-terminal tail of TraD of F is marked with a green box. Genbank Accession number: TraD R1, AAT85682; TraD R100, BAA78884; TraD F, AAC44181.

3.2.2 Disruption of R100 chloramphenicol resistance

In the CRAfT protein translocation can be detected because of the resistance switch in the recipient strain from kanamycin to chloramphenicol. To use the R100-1 plasmid (the derepressed R100 plasmid) in the CRAfT I first had to remove the chloramphenicol resistance from the plasmid.

The *cat* gene was replaced by homologous recombination. In the primers the identical sequences of *cat* are the first 50 bp from the 5'end and the last 50bp from the 3' end. As resistance marker for the insertion I chose kanamycin flanked by *loxP* sites (LKL cassette). The *loxP* sites ensure that the resistance can be removed after the disruption of the *cat* gene. Therefore the plasmid was isolated out of the DY330 strain after the homologous recombination and transferred into the DH5α strain together with the CFP B plasmid. In the DH5α strain the Cre-recombinase expressed from the CFP B cuts the *loxP* sites and removes the LKL cassette from the plasmid. Via conjugation the R100-1 plasmid was transported into MS411 strain.
The MS411 strain harbours a R100-1 plasmid containing only a tetracycline resistance and is now ready for use in the CRAfT together with the CSH26Cm::LKL recipient strain.

3.2.3 Transfer of R1 and F TraI in R100

The protein translocation of TraI from F and R1 by the R100-1 machinery was tested. The result is shown in Figure 3.8. Black bars indicate protein translocation frequencies and grey bars conjugation frequencies. The detection limit for the assay is at 10^{-6} recombinants or transconjugants per donors.

As positive control the transfer of TraI in the wild type system was detected. TraI F shows a protein translocation frequency of around $3x10^{-3}$ recombinants per donors and TraI R1 around $8x10^{-3}$. The empty CFP B vector in the R100-1 background was used as negative control and showed a transfer rate of 10^{-6} recombinants per donors.

The protein translocation frequency of the R1 TraI in the R100-1 background was about $1x10^{-6}$ recombinants per donors. The F TraI in the R100-1 background showed a transfer rate around $7x10^{-6}$ recombinants per donors. Both TraI in the R100-1 background showed no statistically significant difference compared to the

translocation frequency of the negative CFP B control (10^{-6} recombinants per donors).

The conjugation frequency for all tested variants was as expected. For the full length TraI in the pOX38Km background around 10^{-1} transconjugants per donors and for the rest between 10^{0} and 10^{1} transconjugants per donors.

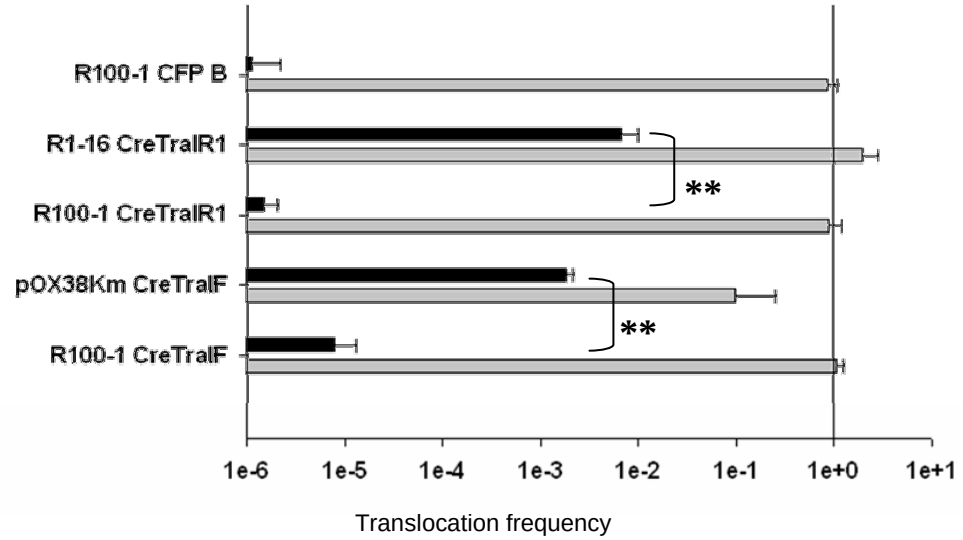

Figure 3.8: **Protein translocation of R1 and F TraI in R100-1 background**. Protein translocation (black bars) of R1 and F TraI in the R100-1 background is not statistically significant compared to the negative control. Conjugation frequency (grey bars) is in the expected range. Protein translocation frequency was determined as recombinants per donors and conjugation frequency as transconjugants per donors. The mean values and standard deviation out of 3 experiments are shown.
(**, P<0.01)

3.3 Conjugative transfer of proteins from the R1 partitioning system

3.3.1 Construction of R1-16Δ*parM*

The R1-16miniTn5CmE5 strain constructed by Monika Nuk was tested in the conjugation assay, but it was never tested whether the absence of ParM has an influence on protein translocation.

To find this out with a CRAfT it was necessary to create a new *parM* knock out strain for two reasons:
(i) The miniTn5Cm insertion lies within *parM*. The bi-cistronic genes *parM* and *parR* are under the control of the same promoter, and it is possible that *parR* was also effected by the disruption of the *parM* open reading frame.
(ii) The second reason for the new knock out was the chloramphenicol resistance marker in the miniTn5Cm transposon. Detection of the protein transfer in the CRAfT is possible because of a resistance switch in the recipient strain from tetracycline to chloramphenicol. With a chloramphenicol resistance in the donor strain it is not possible to detect the protein translocation.

The R1-16Δ*parM* strain was made via homologous recombination. The homologous sequences for the primers were chosen with special care to avoid an influence on the *parR* gene. 50 bp in front of the start codon of parM and the first 50 bp of parR, including the start codon were used as homologous sequences. As resistance marker I used a tetracycline cassette flanked by *loxP* sites (LTL cassette). After homologous recombination in the DY330 strain the R1-16Δ*parM*::LTL plasmid was isolated and transferred together with a CFP B plasmid into DH5α strain. The Cre-recombinase expressed from the CFP B plasmid cuts the *loxP* sites and removes the tetracycline marker. Via conjugation, the R1-16Δ*parM* plasmid without the LTL cassette was transferred into MS411 and MS614.

3.3.2 Protein translocation and conjugation is not effected

The R1-16Δ*parM* plasmid was tested in *E. coli* strains MS411 and MS614 because it was planned to make further assays with both strains. In the CRAfT the protein translocation of TraI and the conjugation was measured.
The results are shown in Figure 3.9. The black bars show protein translocation frequencies of the tested TraI and the grey bars the conjugation frequencies. The detection limit is at 10^{-6} recombinants per donors.

As positive controls the protein transfer of TraI and conjugation in the R1-16 background was measured. The empty CFP B vector was used together with both plasmids as negative control.

Like the R1-16miniTn5CmE5 strain, the R1-16Δ*parM* strain shows no significant difference in conjugation frequency (around 10^0 to 10^1 transconjugants per donors) compared to the R1-16 positive controls (Figure 3.9).

The protein translocation of all strains is in the range of 10^{-3} recombinants per donors and shows no statistically significant differences compared to each other.

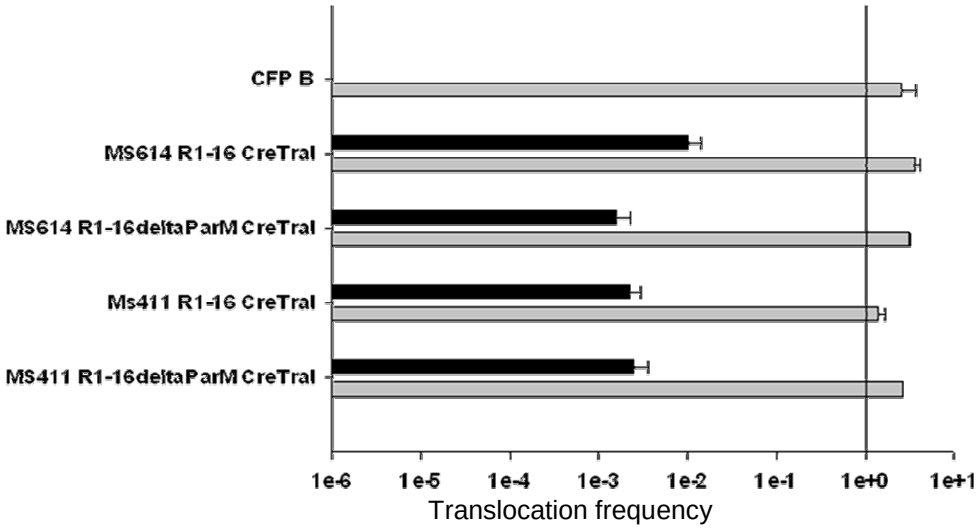

Figure 3.9: **R1-16Δ*parM* protein translocation and conjugation**. The CRAfT was performed with *E. coli* strain MS614 and MS411 carrying wild type R1-16 or R1-16Δ*parM* plasmid. The conjugation frequency is shown in grey and the protein translocation frequency in black. Conjugation frequency is defined as transconjugants per donor and protein translocation as recombinants per donor. All results are statistically significant compared to the negative control and show no statistically significance compared to each other. The mean values and standard deviation of at least 3 experiments are shown.

3.3.3 R17 phage infection of R1-16Δ*parM*

To compare the ability for R17 phage infection of R1-16Δ*parM* with the R1-16miniTn5CmE5 strain, Sandra Raffl performed a R17 phage infection assay. Surprisingly, different than the results with R1-16miniTn5CmE5, the R1-16Δ*praM* strain shows the same infection level as wild type R1-16.

3.3.4 ParM is translocated in the R1 system

The next approach was to test in the CRAfT if ParM is translocated. The experiment was made in the R1-1 and in the pOX38Km background. Black bars in Figure 3.10 represent protein translocation frequencies.

The conjugation frequencies for all donor strains were also tested (data not shown). In the R1-16 background all frequencies were as expected in the range between 10^0 and 10^1 transconjugants per donors. In the pOX38Km background only the conjugation frequency of the full length TraI containing donor showed a regular reduced frequency between 10^{-1} and 10^0 transconjugants per donors.

As positive control for the protein translocation the transfer of TraI in the cognate system was measured. The transfer frequency in R1-16 was around 10^{-2} and in pOX38Km around $2x10^{-3}$ recombinants per donors. The empty CFP B plasmid was used as negative control and shows no detectable protein translocation over the detection limit of 10^{-6} recombinants per donors.

The result of the CRAfT in Figure 3.10, A shows that ParM is translocated in the R1 system with a translocation frequency of around $6x10^{-5}$ recombinants per donors.

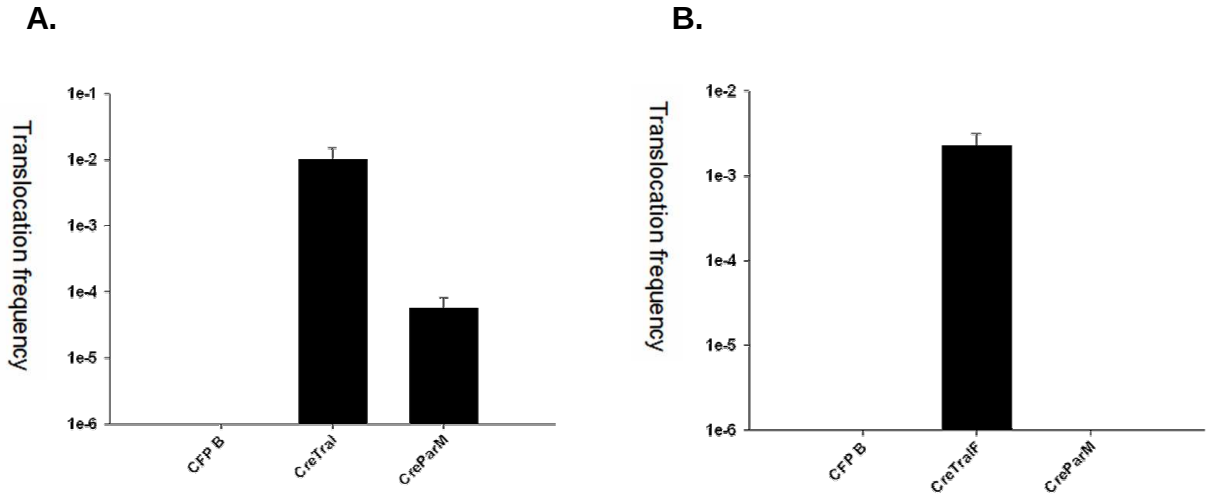

Figure 3.10: **ParM is translocated in the R1 system, but not in F**. ParM was tested for protein translocation in the R1-16 and the pOX38Km background. (A.) Results of the CRAfT in the R1 system. ParM shows protein translocation. (B.) CRAfT in the F system. No protein translocation of ParM was measured. Protein translocation was determined as recombinants per donors. The experiments were repeated 3 times. The results represent the mean values and standard deviation of the data. All data shown are statistically significant compared to the negative control.

To be sure that ParM was transferred because the parMRC system of R1 is specific for the R1 plasmid, ParM was also tested in the pOX38Km background for its protein translocation. The F system uses another family (Type I) of partitioning system than R1 (Gerdes et al., 2000). It is called sop for stability of plasmid (Ogura & Hiraga, 1983). Although the ATPases ParM and SopA fulfil similar functions during partitioning they show no homology (Gerdes et al., 2000; Hayes, 2000).
If the ParM translocation is specific for the R1 system it should not be transferred by the F T4SS. Figure 3.10, B shows that there is no detectable protein translocation of ParM in the pOX38Km background.

3.3.5 ParR binding and correct filament formation is required for efficient ParM translocation

The resulting question out of the results shown above is how the ParM protein is translocated. There are three possibilities:
(i) ParM is transported as free protein or filament
(ii) ParM binds to the DNA or TraI as single protein or filament and is translocated because of the binding
(iii) ParM filaments are transported because it is bound to the DNA via ParR

To answer these questions two more CRAfTs, one in the R1-16 and the other in the R1-16Δ*parM* background, were made with characterized *parM* mutants.
The ParMK123A lost the ability to bind to ParR-*parC* complex but it can be integrated into a filament of wild type ParM (Salje & Löwe, 2008). Additionally a ParMS39A mutant with a reduced ParR binding ability that can be integrated in a filament of wild type ParM was tested (Salje & Löwe, 2008).
ParMD170E is an ATPase deficient mutant acquired from K. Gerdes (Jensen and Gerdes, 1997). The ATP hydrolysis is responsible for the dynamic instability of the filaments (Garner et al., 2004). Without this function ParMD170E form hyperfilaments that seem to be static and they were also formed in the absence of ParR-*parC* complex (Møller-Jensen et al., 2002).

First I tested the three mutants for protein translocation in the R1-16 background. In this experiment wild type ParM proteins are provided from the R1-16 plasmid. Consequently all tested ParM mutants can be integrated into wild type filaments as

mentioned above. The result is shown in Figure 3.11. Black bars indicate protein translocation frequencies.

The full length TraI and ParM were used as positive controls. TraI was translocated at a rate about $8x10^{-3}$ recombinants per donors and ParM shows a protein translocation frequency around $5x10^{-5}$ recombinants per donors.
The empty CFP B vector was used as negative control and shows no detectable protein translocation over the detection limit of 10^{-6} recombinants per donors.
.
ParMK123A and ParMD170E mutants show reduced protein translocation compared to the wild type ParM. Translocation of ParMK123A is 5-fold reduced and ParMD170E 4-fold reduced compared to the $5x10^{-5}$ recombinants per donors of ParM. ParMS39A shows no statistically significant difference in protein translocation compared to the ParM positive control. It seems that ParR binding and correct filament formation is important for efficient ParM translocation.
The conjugation frequencies of all tested strains were as expected in the range between 10^0 and 10^1 transconjugants per donors (data not shown).

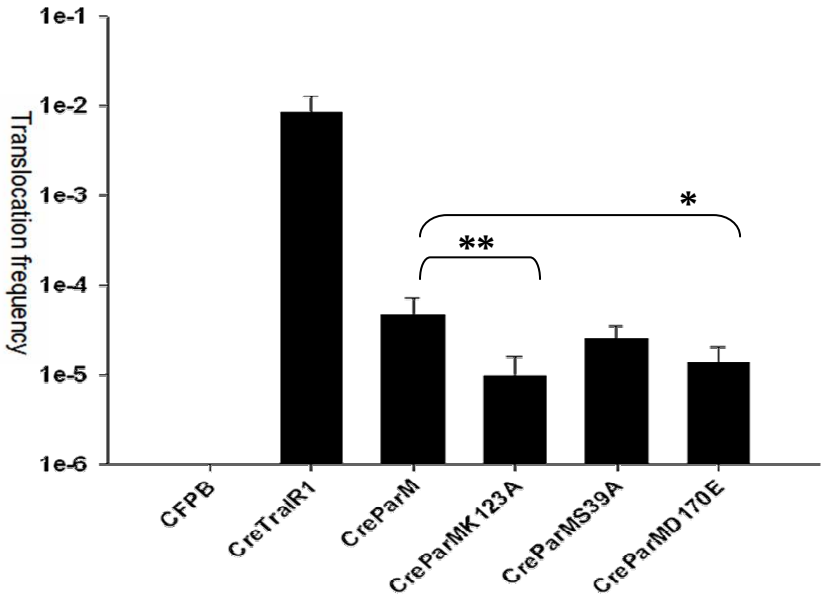

Figure 3.11: **Protein translocation of wild type ParM and mutated alleles in MS614 [R1-16]**. All three mutated ParM proteins are transferred in the CRAfT in the R1-16 background. The protein translocation frequency is in the cases of ParMK123A and ParMD170E lower compared to the wild type ParM. ParMS39A shows no difference to the positive control. All data shown are statistically significant compared to the negative control. The protein translocation frequency is determined as recombinants per donors. The results are the mean values out of 3 experiments and standard deviation. (*, $P<0.05$; **, $P<0.01$)

In the second CRAfT ParMK123A, ParMS39A and ParMD170E were tested in the R1-16Δ*parM* background for protein translocation (Figure 3.12). Without additional wild type ParM proteins from R1-16Δ*parM*, the ParMK123A proteins should be present as single molecules, because it has no possibility to bind to ParR and start filamentation, and ParM and ParMD170E as filaments. The same positive and negative controls were used as described above.

The conjugation frequency of all tested strains is as expected in the range of 10^0 to 10^1 transconjugants per donor (Figure 3.12, grey bars).

The full length TraI positive control was transferred around 10^{-3} recombinants per donors (Figure 3.12, black bar), but none of the tested ParM proteins showed a significant protein translocation compared to the negative control.

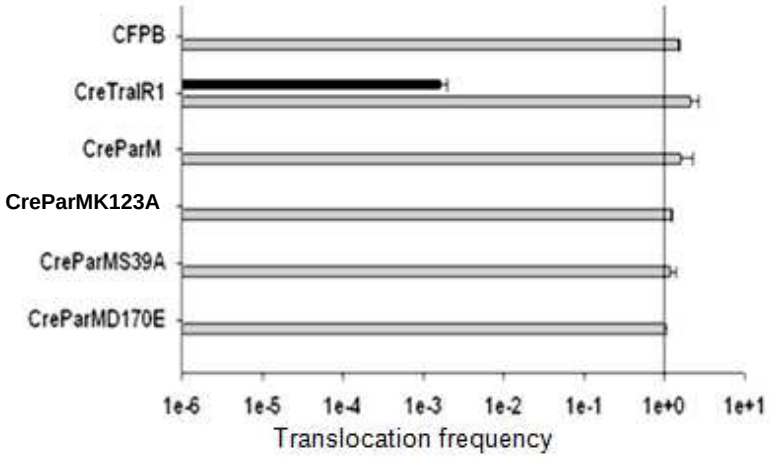

Figure 3.12: **Protein translocation of wild type ParM and mutated alleles in MS614 [R1-16Δ*parM*]**. None of the tested ParM proteins was transferred in the R1-16Δ*parM* background. The black bar shows the protein translocation frequency of the positive control. The transfer is statistically significant compared to the CFPB negative control. The conjugation frequencies, indicated as grey bars, of all strains are within the expected range. The figure shows the mean value of the results out of 3 experiments with standard deviation. Conjugation frequency was calculated as transconjugants per donors and the protein translocation frequency as recombinants per donors.

3.3.6 ParR translocation

The protein translocation of ParM123A, ParMS39A and ParMD170E in the R1-16 background showed that ParR binding could be important for the transfer. To test that hypothesis I decided to test ParR in the CRAfT.

Together with the wild type ParR protein a mutated ParRR6S, that is not able to bind to *parC* (Salje & Löwe, 2008), was tested for protein translocation in the CRAfT (Figure 3.13). Again TraI was used as positive control (Figure 3.13, black bar: around 10^{-2} recombinants per donor) and the empty CFP B vector as negative control (under 10^{-6} recombinants per donors). The conjugation frequencies of all tested donor strains were around 10^{0} transconjugants per donors as expected (Figure 3.13, grey bars).

As shown in Figure 3.13 the protein translocation frequency of ParR was under the detection limit of 10^{-6} recombinants per donors. ParRR6S showed a protein translocation frequency of $1x10^{-6}$ recombinants per donors and compared to the negative control there is no statistically significant difference (Figure 3.13, white bar).

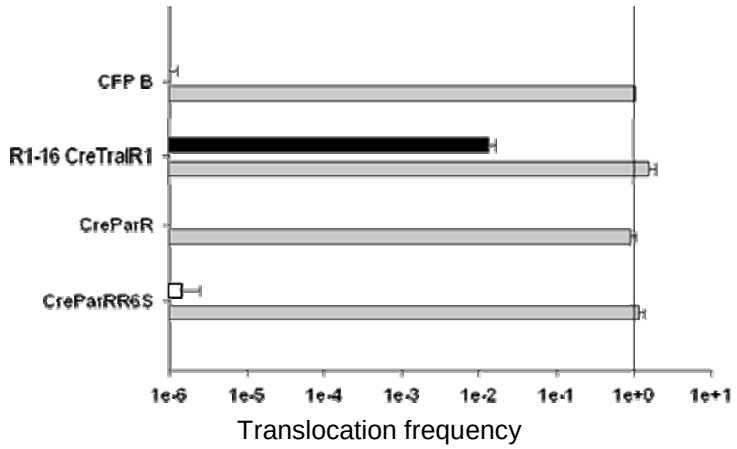

Figure 3.13: **Translocation of ParR and ParRR6S in the R1-16 background**. The conjugation frequencies are shown as grey bars. The black bar indicates protein translocation with statistically significant difference to the negative control and the white bar without. Both, ParR and ParRR6S were not transferred efficiently by the R1-16 system. The conjugation frequency is determined as transconjugants per donors and the protein translocation frequency as recombinants per donors. The results are the mean values out of 3 experiments.

3.4 Western blot analysis

Antibodies are used in many biochemical analysis and assays, like western blot or coimmunoprecipitation. After the immunisation with a protein the polyclonal antiserum is obtained out of the blood. In my case rabbits were immunised with TraI, TraY and

TraD. These antisera have to be tested by western blot for their ability to detect their antigen, the best dilution range and the exposure time. Afterwards it can be decided for which approaches they are suitable. It was the goal to make quantitative western blots to calculate how many TraI, TraY and TraD proteins exist in a single cell.

3.4.1 TraI relaxase antiserum

The rabbit was immunized with the purified relaxase domain of TraI (Fraction C3-9) by Marta Sut. The requirements to make a quantitative western blot are to detect the protein in purified samples as well as within protein extracts.

First I decided to find out what are the best concentrations of MS614[R1-16] protein extract to get a proper dilution series. I started to test protein extracts complying with cells of OD_{600} 0.8, 0.4, 0.2 and 0.1 in 1 ml. The samples were loaded on a 12.5 % sds-gel and electrophoreses was done at 16 mA per gel. A portion of the OD_{600} 0.8 sample remained in the slot, meaning that it is overloaded. Coomassie staining was performed over night at 4℃. The results in Figure 3.14, A show that the sample with OD_{600} 0.8 in 1 ml is too high concentrated (Figure 3.14, A lane 1) because no separated bands are visible.

I decided to start my protein extract dilution series out of cells with OD_{600} 0.4 in 1 ml and go down to OD_{600} 0.2, 0.1 and 0.05 in 1 ml. The sds-gel electrophoreses and coomassie staining was performed as mentioned above. The resulting protein extract dilution series is shown in Figure 3.14, B. The OD_{600} 0.4 (Figure 3.14, B lane 1) is still a little bit overloaded, but I decided that it represents a good upper limit of my titration series.

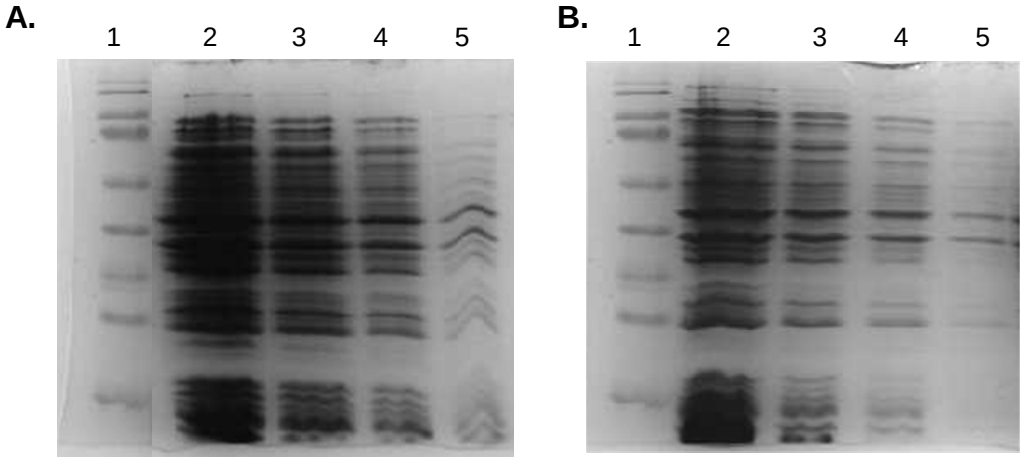

Figure 3.14: **Dilution series of MS614[R1-16] protein extract**. (A.): 1) Page Ruler™ Prestained Protein Ladder, 2-5) MS614[R1-16] protein extract OD_{600} 0.8, 0.4, 0.2 and 0.1 in 1ml. (B.) 1) Page Ruler™ Prestained Protein Ladder, 2-5) MS614[R1-16] protein extract OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1ml.

Next I tested whether I can detect the purified TraI relaxase in a western blot with the antiserum. As standard I used Page Ruler^{TM} Prestained Protein Ladder from Fermentas. For the detection of the TraI relaxase I used a titration series of 70, 60, 50, 40, 30, 20 and 10 ng of purified TraI relaxase. The remaining two slots I loaded with protein extracts from MS614 [R1-16] and MS614 [R1-16Δ*traI*] (OD_{600} 0.2 in 1ml). The idea was, to see a band for the full length TraI in the MS614[R1-16] lane that should not be there in the MS614 [R1-16Δ*traI*] lane. Further more I can test the antiserum for unspecific protein interaction in the protein extracts. Two gels were made, one for blotting and the other one for coomassie staining.

The SDS-gel (12.5%) electrophoreses was performed at 16 mA per gel. The protein was transferred to the membrane for 1 hour 30 minutes at 240 mA. The membrane was blocked over night.

For immunoblotting, I tried several TraI relaxase antiserum dilutions from 1:1000 to 1:10000. The blot was incubated with each antibody for 1 hour with several washing steps between the primary anti TraI relaxase and secondary anti rabbit antibody. The exposure time was 15 minute.

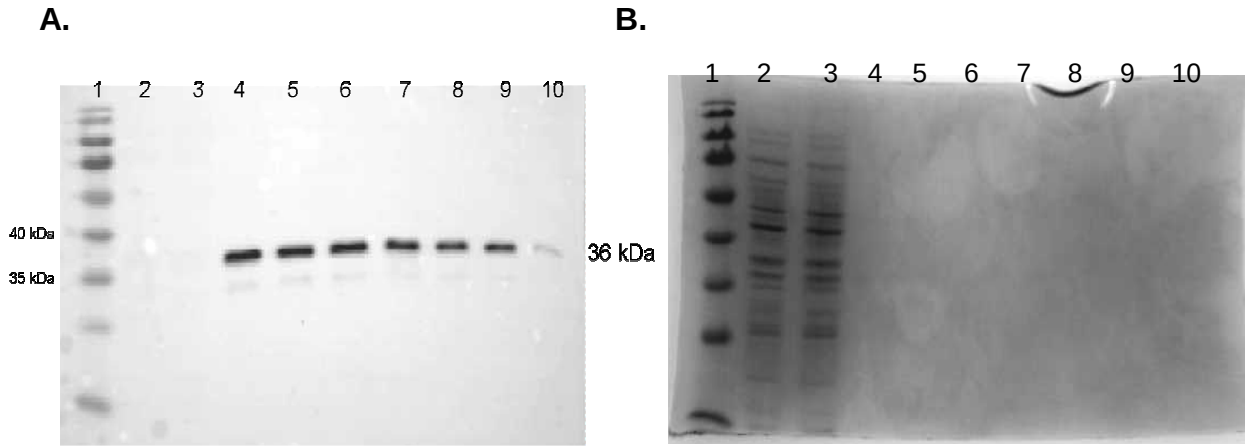

Figure 3.15.: **Western blot detection of purified TraI relaxase**.
(A.) Western blot: 1) Page Ruler^{TM} Prestained Protein Ladder, 2) MS614 [R1-16] protein extract OD_{600} 0.2 in 1ml, 3) MS614 [R1-16Δ*traI*] protein extract OD_{600} 0.2 in 1 ml, 4 - 10) 70, 60, 50, 40, 30, 20 and 10 ng purified TraI relaxase.
(B.) Coomassie gel: 1) Page Ruler^{TM} Prestained Protein Ladder, 2) MS614 [R1-16] protein extract OD_{600} 0.2 in 1ml, 3) MS614 [R1-16Δ*traI*] protein extract OD_{600} 0.2 in 1 ml, 4 - 10) 70, 60, 50, 40, 30, 20 and 10 ng purified TraI relaxase.

I obtained the best result for the detection of purified TraI relaxase with the antiserum dilution of 1:10000 (Figure 3.15). The purified TraI relaxase can be detected from 10 ng upwards with this dilution. Using ImageJ software I checked if the intensity of the

bands compared to the protein concentration shows linearity. Only a linear titration series can be used as standard for a quantitative western blot, given that it is possible to detect TraI within protein extract. Linearity was given from 10 to 50 ng TraI relaxase. Figure 3.16 shows the result of the linear regression. From 50 ng up the intensity reaches a plateau.

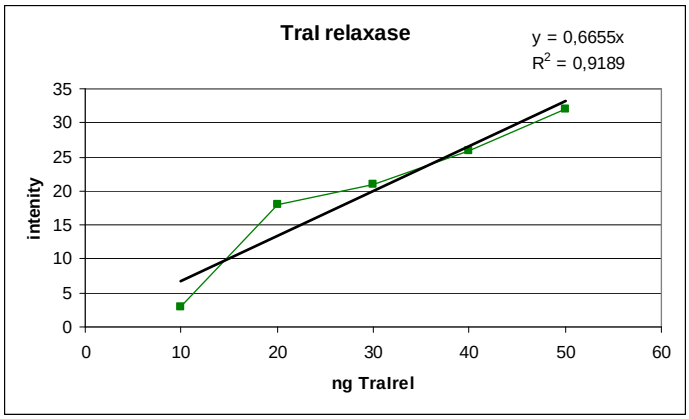

Figure 3.16: **Linear regression of TraI relaxase titration series**. The intensity of the bands was measured and compared to the protein concentration (green rectangles). The linear regression shows linearity from 10 to 50 ng TraI relaxase. The trend line is indicated in black. The linear equation and the coefficient of determination are shown.

No unspecific bands are visible in the protein extract lanes (Figure 3.15, A lane 2 and 3) on the western blot. It was also not possible to detect specific TraI full length bands in the protein extract of MS614[R1-16]. I assumed that the blotting time of 1 h 30 min, which was adjusted to the 36 kDa TraI relaxase, was too short for the 192 kDa full length TraI. The coomassie gel shown in Figure 3.15, B furnishes evidence for the presence of protein extract in lane 2 and 3.

The next approach was to test the antiserum for the detection of purified full length TraI and TraI within a protein extract.

On the gel I loaded the marker mentioned above, a titration series of purified full length TraI (80, 40, 20, 10 and 5 ng) and MS614[R1-16] protein extracts containing the proteins out of cells with an OD_{600} of 0.4, 0.2, 0.1 and 0.05 in 1 ml.

The SDS-gel (12.5%) electrophoreses was performed at 16 mA per gel. The protein was transferred to the membrane for 2 hour at 240 mA. The membrane was blocked over night.

Dilutions of the antiserum from 1:500 to 1:10000 were tested. The blot was incubated with each antibody 1 hour and exposed for 15 minutes.

The 1:1000 dilution of the antiserum showed the best result for the detection of the TraI protein (Figure 3.17). With a lower dilution too many unspecific background bands appears and with a higher dilution the signals become too weak. The purified protein can be detected down to 10 ng.
The TraI in the protein extracts shows only a very slight signal in the higher concentrated samples with OD_{600} 0.4 and 0.2 in 1 ml (Figure 3.17, arrow). Because of the weak band and high background in these lanes (Figure 3.17, lane 7 and 8) quantification is not possible. The unspecific signals are reduced in the lower concentrated protein extracts (Figure 3.17, line 9 and 10) but the signal for the TraI is absent. Higher concentrations of the cell extract would lead to overloaded bands with a very high background of unspecific bands.

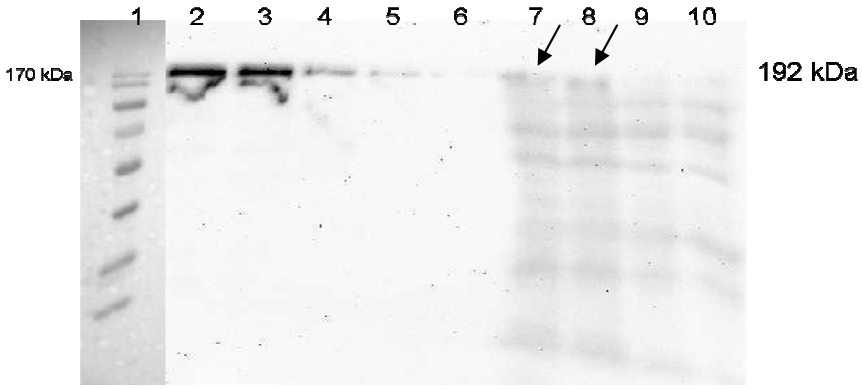

Fig. 3.17: **Western blot detection of TraI full length protein**.
1) Page Ruler^{TM} Prestained Protein Ladder, 2 – 6) 80, 40, 20, 10 and 5 ng purified TraI, 7 – 10) MS614 [R1-16] protein extracts OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1 ml. The arrows are pointing to detected TraI in the protein extracts of OD_{600} 0.4 and 0.2 in 1 ml.

As mentioned before, for a quantitative western blot a signal of the protein within a protein extract is needed to quantify it and compare it with the standard titration series. In the case of TraI the detected signal in the protein extract was too weak for quantification and so I stopped the TraI western blot experiments at this point.

3.4.2 TraY/lysozyme antiserum

The TraY protein (Fraction B765) for the immunization of the rabbit was contaminated with lysozyme (40% TraY and 60 % lysozyme; Dissertation M. Sut, 2009). This circumstance was recognized after the immunization was finished and the antiserum was prepared. It contains a mix out of TraY and lysozyme antibodies. It was my task to test, if it is possible to detect TraY with this antibody mix.

My first approach was to test the antiserum for the detection of the TraY/lysozyme mix and pure lysozyme. I used a 12.5% SDS-gels. As marker I loaded Page Ruler^{TM} Prestained Protein Ladder from Fermentas. I put on the gel a titration series of TraY/lysozyme mix (100, 200, 400 and 800 ng) and pure lysozyme (10, 20, 40 and 80 ng).

SDS-gel electrophoreses was performed with 16 mA per gel. The plotting time for the TraY was 1 hour. Blocking was made over night.

I tested several TraY/lysozyme antiserum dilutions from 1:500 to 1:1000. The incubation time for the primary and secondary (anti rabbit) antibody was 1 hour with several washing steps between. With my standard exposure time of 15 min I got too high background noise on the blot and so I extended the exposure time to 30 min.

The best results I got from the 1:1000 dilution of the serum. Lower dilutions showed too high unspecific background.

Figure 3.18 shows that the TraY/lysozyme mix can be detected in high concentrations over 200 ng. Detection of lysozyme is possible from 40 ng up. These results do not tell us if purified TraY can be detected with this antiserum.

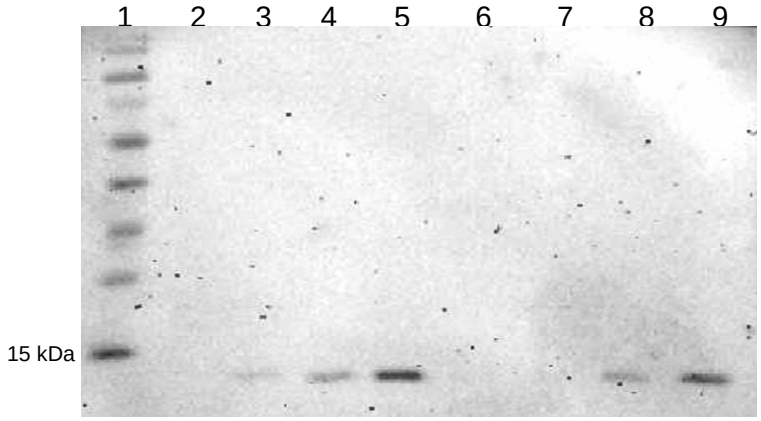

Figure 3.18: **Western blot detection of TraY and lysozyme**.
1) Page Ruler^{TM} Prestained Protein Ladder, 2 – 5) TraY/lysozyme mix 100, 200, 400 and 800 ng, 6 – 9) lysozyme 10, 20, 40 and 80 ng

I decided to try to remove the lysozyme antibodies to obtain anti serum only against TraY. Pure TraY bands are necessary for the quantitative western blot to make a titration series of TraY that can be quantified and compared with the TraY signals from the protein extract.

To remove the lysozyme antibodies, I added 100 µg/ml lysozyme to the serum and incubated for 30 min on ice to precipitate them. After centrifugation for 10 min, 13000 rpm at 4℃, I diluted the supernatant 1:1000. The same concentrations of TraY/lysozyme mix and pure lysozyme were used for a western blot as described above. The blot was incubated with the lysozyme treated antiserum. I expected that the lysozyme bands will clear up or disappear. The results showed that both bands, lysozyme and TraY/lysozyme mix, vanished (data not shown). The attempt to clean the antiserum from the lysozyme antibodies failed.

The next step was to test the antiserum for TraY detection in protein extracts. MS614 [R1-16] cells were lysed by cooking, and so no lysozyme should be in the samples. A dilution serial of protein extract was loaded (OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1ml). Electrophoreses and blotting was made as described above. The TraY/lysozyme antiserum was diluted 1:1000. No bands for TraY could be detected (data not shown).

I assumed that the TraY concentration in the MS614 [R1-16] protein extract is too low for detection and so I decided to test the protein extract from a TraY over expression strain BL21DE3[codon usage plus][pET21cTraY]. 1 ml of ONC was transferred to 100 ml fresh LB medium and let grow to an OD_{600} 0.5 at 37℃. Samples were taken to produce uninduced protein extract. The rest was induced with 1 mM IPTG and incubated for 3 h at 37℃.

The same standard mentioned above was used. Protein extracts out of uninduced and induced cells with OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1 ml were loaded. Additionally, 800 ng of TraY/lysozyme was put on the gel as positive control. Electrophoreses and blotting was made as described above. The TraY/lysozyme antiserum was diluted 1:1000.

The results shown in Figure 3.19, lane 2-5, confirm my assumption that the concentration of TraY within cells is too low to detect it with this antiserum. After the over expression TraY bands are visible in the protein extract (Figure 3.19 lane 6-9).

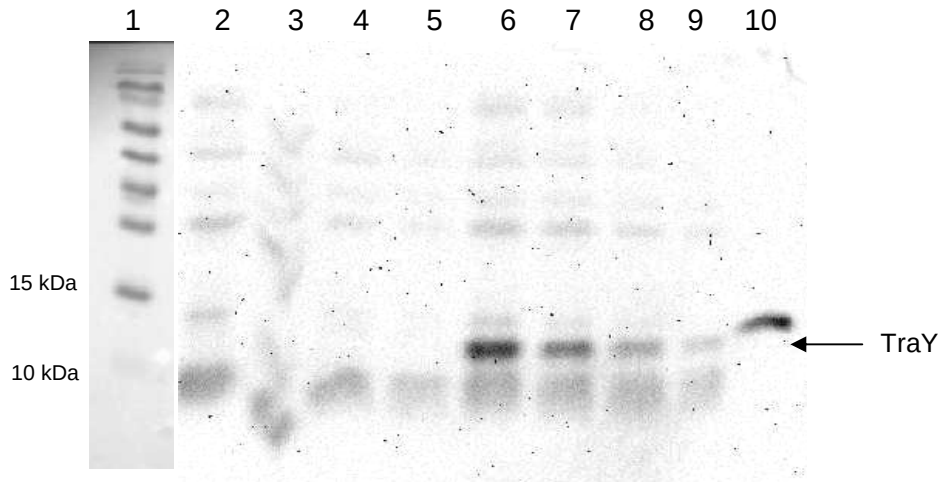

Figure 3.19: **Western blot detection of TraY in an over expression strain**.
1) Page Ruler^{TM} Prestained Protein Ladder, 2 - 5) BL21DE3 [codon usage plus] [pET21cTraY] uinduced protein extract OD_{600} 0.4, 0.2, 0.1 and 0.005 in 1 ml,
6 – 9) BL21DE3 [codon usage plus] [pET21cTraY] induced protein extract OD_{600} 0.4, 0.2, 0.1 and 0.005 in 1 ml, 10) 800 ng TraY/lysozyme

A quantitative western blot could not be made with this antiserum because of some reasons: It was not possible to clean the antiserum from the lysozyme anti bodies to create a titration series of TraY. The results in Figure 3.19 show that TraY can only be detected in protein extract from an over expression strain. The quantification of the bands was very difficult or impossible because of the high background.
For me it was not possible to make a quantitative western blot with this antiserum to find out how many TraY molecules in a single cell exist.

3.4.3 TraD antiserum

An antiserum against TraD was also tested. TraDΔN130 (Fraction A2DI) lacking the N-terminal 130 amino acid long transmembrane domain was prepared for the immunization by Sanja Mihajlovic (Mihajlovic S. et al, 2009).

To test whether TraD can be detected with the antiserum I loaded on the SDS-gel (12.5%) a titration series of purified 69 kDa TraD (TraDΔN130) protein (70, 60, 50, 40, 30, 20 and 10 ng).
The remaining two slots I loaded with protein extract out of MS614 [R1-16] and MS614 [R1-16Δ*traD*] cells with an OD_{600} of 0.1 in 1 ml. I wanted to test, whether I can detect TraD in the MS614[R1-16] protein extract. The MS614[R1-16Δ*traD*] lane

should not show a band for TraD. Further more I can see, if there are any non specific bands in the protein extract lanes.
Electrophoreses was performed at 16 mA per gel. The protein was transferred to the membrane for 1 hour 30 minutes at 240 mA. The membrane was blocked over night.
I tested antiserum dilutions from 1:1000 to 1:10000. The incubation time for the primary and secondary (anti rabbit) antibody was 1 hour with several washing steps between. Exposure time was 15 minutes.

With an antiserum dilution of 1:10000 strong bands were visible for the purified TraD protein and for TraD in the MS614 [R1-16] protein extract (Figure 3.20). The cell extract lanes show some unspecific bands and one of them in the MS614[R1-16Δ*traD*] cell extract lane was at the same size as TraD (Figure 3.20, arrow).

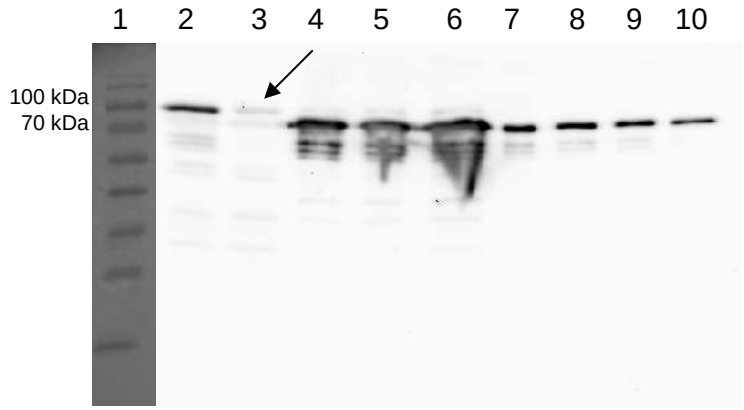

Figure 3.20: **Western blot detection of TraD**.
1) Page Ruler^{TM} Prestained Protein Ladder, 2) MS614[R1-16] protein extract, 3) MS614[R1-16Δ*traD*], 4 – 10) 70, 60, 50, 40, 30, 20 and 10 ng purified TraD. The arrow is pointing to an unspecific band at the same size as TraD.

To control if this unspecific band comes from a cross-reaction with a protein from the R1-16Δ*traD* plasmid or from the chromosome, I made a blot with the protein extract from MS614 [R1-16], MS614 [R1-16Δ*traD*] and MS614 without a plasmid, containing cells of OD_{600} 0.2 and 0.1 in 1 ml. To exclude that this band is strain specific for MS614 I also tested the protein extract of CSH26. I chose a higher and a lower concentrated protein extract of my series to see if the intensity of the unspecific band correlates with the amount of protein in the sample. If the unspecific signal comes

from a protein it should be less intense in the lower concentrated protein extract samples.

The resulting western blot is shown in Figure 3.21. The unspecific band appears in the protein extracts of MS614 [R1-16Δ*traD*] (Figure 3.21, lane 2 and 3) and MS614 (Figure 3.21, lane 6 and 7) strain without the plasmid. The band correlates with the protein concentration in the protein extracts. That shows the cross-reacting protein comes not from the plasmid, but from a protein of the chromosome. The band also appears in the protein extract of CSH26 (Figure 3.21, lane 8 and 9), meaning that it is not MS614 strain specific.

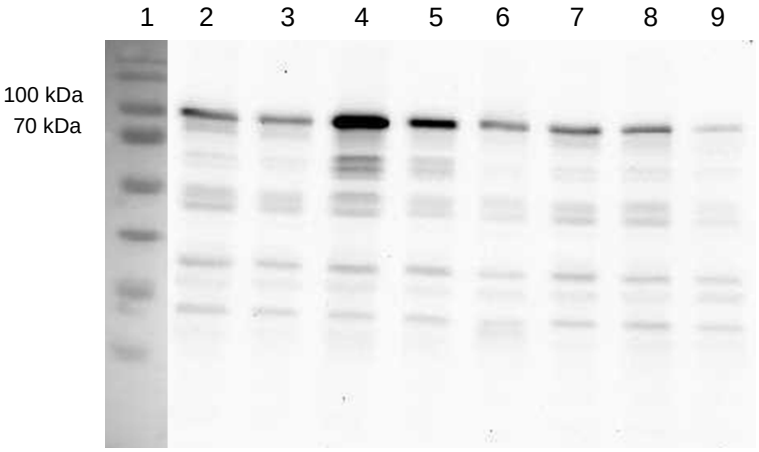

Figure 3.21: **Unspecific band comes from the chromosome**.
1) Page Ruler^{TM} Prestained Protein Ladder; 2 and 3) MS614 [R1-16Δ*traD*] protein extract OD_{600} 0.2 and 0.1 in 1 ml; 4 and 5) MS614 [R1-16] protein extract OD_{600} 0.2 and 0.1 in 1 ml; 6 and 7) MS614 protein extract OD_{600} 0.2 and 0.1 in 1 ml; 8 and 9) CSH26 protein extract OD_{600} 0.2 and 0.1 in 1 ml

Due to the fact, that I saw very intensive bands with a 1:10000 dilution of the TraD antiserum, I decided to test higher dilutions to eliminate the unspecific bands. If the band in the TraD knock out is really unspecific it should disappear together with the other unspecific bands. I incubated several blots with MS614 [R1-16] and MS614 protein extract from OD_{600} 0.4 and 0.05 in 1 ml with antiserum dilutions from 1:15000 to 1:40000. The highest and lowest concentrated protein extracts of my titration series were chosen to see, whether I can detect TraD in both samples with this high anti serum dilutions. I extended the exposure time to 30 minutes to be sure to detect even a very weak signal.
The unspecific band vanished at a dilution of 1:35000 and 1:40000 (Figure 3.22). The specific bands for TraD in the MS614[R1-16] protein extracts remains visible and

showed a good intensity. Now I had the chance to quantify the intensity of the TraD band in the protein extract lanes without the additional signal form the unspecific chromosomal band.

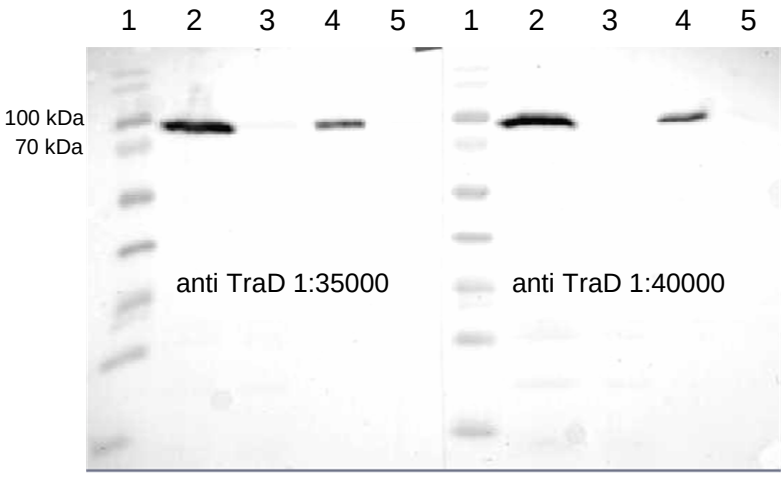

Figure 3.22: **Unspecific band from the chromosome vanishes**.
1) Page Ruler^{TM} Prestained Protein Ladder, 2) MS614 [R1-16] protein extract OD_{600} 0.4 in 1ml, 3) MS614 protein extract OD_{600} 0.4 in 1ml, 4) MS614[R1-16] protein extract OD_{600} 0.05 in 1ml, 5) MS614 protein extract OD_{600} 0.05 in 1ml

I tested both dilutions, whether it is possible to detect purified TraD protein titration series from 70 ng down to a concentration of 5 ng (data for the 1:40000 dilution is shown in Figure 3.23, A lane 2-6). For both dilutions it was possible to detect the lowest concentration. I used the 1:40000 TraD antiserum dilution for further experiments.

In summary I can say that the TraD antiserum is very competent. It can be used for assays with low concentrated purified TraD at a dilution of 1:10000 and for higher concentrations the dilution can be varied up to 1:40000.
For the work with protein extracts out of lysed cells I would recommend a dilution of 1:40000 for the antiserum. This high dilution prevent from unspecific bands which coincide with the TraD band.

3.4.3.1 Quantitative western blot TraD

Due to the fact that the TraD antiserum can be used for the detection of purified protein and to detect TraD within a protein extract, I had the chance to make a quantitative western blot to determine the TraD molecules per cell.

Several equal blots were made with a titration series of purified TraD protein (70, 50, 30, 20, 10 and 5 ng) as standard and MS614 [R1-16] protein extracts (OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1 ml) (Figure 3.23, A.).

The SDS-gel electrophoreses was performed at 16 mA per gel. The protein was transferred to the membrane for 1 hour 30 minutes at 240 mA. The membrane was blocked over night.

I used the antiserum dilution of 1:40000. The incubation time for the primary and secondary (anti rabbit) antibody was 1 hour with several washing steps between. Exposure time was 30 minutes.

Using ImageJ software the intensity of the purified TraD bands was measured and a linear regression was made (Figure 3.23, B.). The same procedure was made with the protein extract titrations (Figure 3.23, C.).

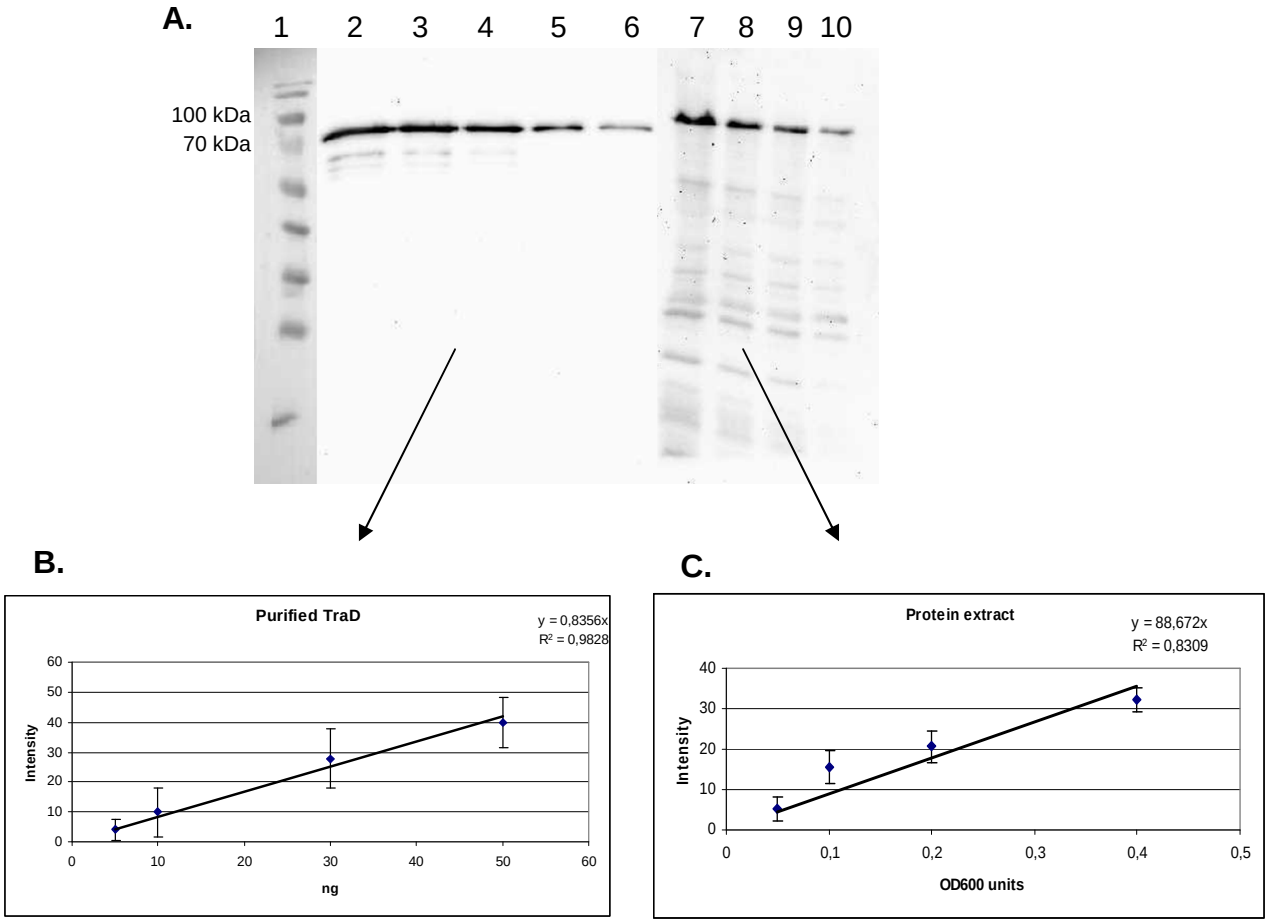

Figure 3.23: **Quantitative western blot TraD**. (A.) 1) Page Ruler^{TM} Prestained Protein Ladder, 2 – 6) 70, 50, 30, 20, 10 and 5 ng purified TraD, 7 – 10) MS614 [R1-16] protein extract OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1 ml. To quantify the amount of TraD per cell the intensity of the bands were measured. A linear regression was made for (B.) the purified TraD and (C.) the protein extract. The trend lines are indicated in black. The linear equations and the coefficients of determination are shown.

The concentration of the protein extract containing TraD in ng per OD_{600} unit could be calculated with linear regression analyses. From this amount the TraD molecules per OD_{600} unit could be estimated with 82 kDa = $1.36x10^{-10}$ ng and so 1 ng = $7.35x10^{9}$ TraD molecules. The TraD amount was normalized to an OD_{600} 0.1 to get the mean value of $9.78x10^{10} \pm 2.78x10^{10}$ TraD molecules per OD_{600} 0.1.

To determine the living cells per OD_{600} unit the same volumes of the MS614[R1-16] culture as for protein extract preparation (OD_{600} 0.4, 0.2, 0.1 and 0.05 in 1 ml) were harvested and diluted for plating on Km agar plates (40 µg/ml). The plates were incubated over night at 37℃ and the colonies were counted the next day (Figure 3.24).

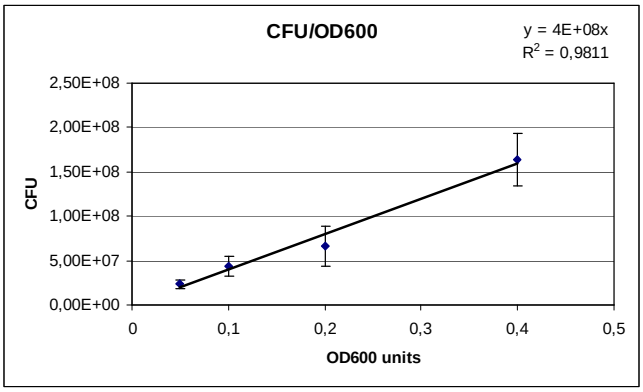

Figure 3.24: **Viable cells per OD_{600} unite**. The figure shows the mean value and standard deviation out of two counting. The trend line is indicated in black. The linear equation and the coefficient of determination are shown.

The calculated TraD molecules per OD_{600} unit were divided by the number of viable cells per OD_{600} unit to get the TraD molecules per cell. I detected 2382 ± 641 TraD molecules per cell.

4 Discussion and Outlook

4.1 Variant amino acid 626 in TS A determines specificity and a conserved cluster is relevant for efficient TS translocation

4.1.1 Molecular specificity determination in TS A

Two minimal TS in F and R1 TraI were mapped by Silvia Lang. Both TS are translocated independently from each other and are specific for their cognate T4SS. This leads to the assumption that they must have something in common because of the independent translocation and that they must have differences that determine the specificity for the T4SS. A predicted structure, based on the already known RecD2 structure, was found for both TS in both systems as common feature.

Comparing the amino acid sequences of F and R1 TSs I found amino acid variations in TS A and TS B. The resulting question was whether these variations are responsible for the specificity of the TS.
Based on the location of the variant amino acids on the predicted model for the TS I exchanged amino acid 626 and 757 in TS A and 1382 in TS B from F to R1.
Amino acid 626 is a single positioned amino acid of TS A with no matching variation of TS B on the same position. For this mutation I predicted that it is a promising candidate for a specificity determinant.
The hybrid TS was no longer transferred by the cognate F system and a 12-fold gain of function appears in the heterologous R1 system. The exchange leads to a fidelity switch.
This result confirms my prediction that amino acid 626 of TS A fulfils a key function in the specific recognition by the T4SS.

Amino acids 757 of TS A and 1382 of TS B are co-located on the same position on the predicted model. Due to the observation that natural amino acid variations exist on this position, I assumed that another mutation should not have a dramatic influence on the specificity of the TS.

The exchange was tolerated in TS A and leads to a reduction of the protein translocation of TS B. A transfer in the heterologous system was not observed for both hybrids. My results confirmed the prediction that this position is not essential for the specificity discrimination.

4.1.2 Exchange of a variant amino acid outside of the predicted model shows no change in specificity

As a proof for the predicted TS model as a recognition motif I exchanged variant amino acid 1283 in TS B that does not lie on the overlapping structure with RecD2. The exchange was made in TS B of both systems. The CRAfT shows no difference in protein translocation compared to the positive control in R1. In F the transfer was reduced 10-times.

The fact that the exchange in R1 had no influence on protein translocation confirms our assumption that this variant amino acid 1283 outside of structure is not essential for the specific recognition of the signal.

Out of my results we can not say that all variant amino acids of TS B not positioned on the model are irrelevant for TS translocation. The structures of the TS and RecD2 show only 20 % similarity compared to each other and 5 not tested non identical residues out of 9 are positioned on the residual 80 % non overlapping part. It is very unlikely that the rest of the structure of the TS is unimportant for its function.

4.1.3 A conserved cluster is important for efficient TS transfer

A conserved consensus sequence was found in both TS from F and R1. This cluster is located in a ß-sheet within the predicted structure near the position of residue 757 of TS A and 1382 of TS B. Because of the conservation and the position on the model I predicted that this cluster could be important for the function of the recognition motive and it could act as a structure giving feature.

The conserved amino acid 1344 in the cluster was mutated R to Q in F and R1 TS B. The exchange leads to a reduced protein translocation in both systems. In R1 the translocation of the mutated TS B was only 0.8 % compared to the wild type TS B.

A mutation in this conserved motif could affect protein folding and this would explain the reduced affinity of the T4SS for the TS.

The conserved cluster was also found in $MobA_{R1162}$, the only other relaxase where TS have been mapped and the motive was found in both. An insertion of three amino acids (LDR) near this conserved consensus in the N-terminal TS leads to a disruption of the protein translocation (Parker & Meyer, 2007).
This circumstance underlines the importance of the cluster for the function of the TS as a recognition motive.

4.1.4 Outlook

Further experiments should be made to exchange the remaining seven variable amino acids in TS B. The new hybrids can be tested in the CRAfT in the F and R1 background. Maybe one of the seven amino acids works also as a key residue for the specificity of TS B. An exchange could lead to a fidelity switch from F to R1 like the exchange of amino acid 626 in TS A does. If this is not the case, we know that the specificity determination of TS B is more complex than in TS A. Maybe more than one variant amino acid is responsible for the stringent translocation by the cognate T4SS.

The TS are conserved in MOB_Q and MOB_F relaxase families. Within the MOB_F family a duplication event of the TS took place. The MOB_{F12} relaxases harbour TS A and TS B (Lang et al., 2010). It would be interesting to determine for example the TS from the closely related and 95% identical TraI from plasmid R100 (Byrd et al., 2002). Maybe it is common for the TS of closely related MOB_F relaxases that they have a conserved structure and variant key-amino acids alter their specificity for the recognition of the T4CP.

The CRAfT showed that TS A and TS B are transferred specifically because we see a phenotype switch in the recipient strain. This method does not tell anything about the specific TS recognition within the relaxosome or at the conjugative pore in the donor. Our group already started with experiments to find out more about these processes in the donor strain. Paul Kirchberger started to test protein interactions between TS and relaxosome components in two hybrid assays (unpublished data) and I made experiments to find the specificity determinants of TraD for TraI during this study (described in section 3.2 and discussed in section 4.2).

It would also be great to solve the crystal structure of the TS. Given that it is possible, the TS could be co-crystallized with proteins form the relaxosome e.g. TraM or the CP TraD to find interaction partners.
Further experiments should be made to find out more about the mechanism of specific substrate recognition and selection for conjugation within the donor cell in the future.

4.2 Molecular specificity determinants of TraD for TraI recognition

4.2.1 Specific TraI recognition requires not only TraD

TraD, the coupling protein of IncF systems, connects the relaxosome with the transport machinery of the T4SS. The CP is responsible for the substrate selection and recruitment to the secretion apparatus (Cascales & Christie, 2004).
A sequence alignment of F and R1 TraD was made and a sequence of PQQ repeats in R1 and a longer C-terminal tail of F was found. TraD of R100 shows also PQQ repeats and has a shorter C-terminus compared to F TraD. I asked if these differences are responsible for the specific recognition of TraI.
TraI from F and R1 were tested in the CRAft for the protein translocation in the R100 background. Translocation of both TraI proteins failed in the R100 system. This means that other molecular differences of TraD or proteins of the relaxosome complex or the T4S channel could be involved in the specific recognition of TraI.

This assumption was confirmed by a TraD swap experiment performed by Silvia Lang. She complemented the R1-16Δ*traD* strain with homologous R1 TraD or the heterologous F TraD. The protein translocation of the full length TraI from F and R1 was detected. The T4CP did not discriminate between the TraI from F or R1 (Lang et al., 2010)

Promising candidates within the relaxosome complex as TraI recognition receptor are TraM and TraY. Experiments with TraM and TraY from F, R1 and R100 showed that these proteins are specific for their *oriT* binding region on the plasmid (Everett &

Willetts, 1980, Willetts & Maule, 1986, Fekete & Frost, 2000). Moreover TraM interacts with TraI (Ragonese et al., 2007) as well as with the C-terminus of TraD (Disqué-Kochem & Dreiseikelmann, 1997; Beranek et al., 2004; Lu et al., 2008). That suggests that TraM could recognise TraI as a substrate for translocation and deliver it to TraD for transfer.

4.2.2 Outlook

To confirm my result a TraD swap experiment could be made, where R1-16Δ*traD* is complemented with R100 TraD. If it is true that TraD is not responsible for the discrimination of TraI, than there should be no difference in TraI protein translocation.

4.3 Conjugative transfer of ParM by the R1 T4SS

The parMRC plasmid partitioning complex of plasmid R1 is necessary and sufficient for the stable and correct distribution of the plasmid copies during cell division. Previous experiments from our group showed that especially ParM interacts with parts of the R1 T4SS. I showed that ParM is translocated during R1 conjugation and that ParR could also be involved in this machinery.

4.3.1 The absence of ParM does not effect protein translocation and conjugation

It was known that the disruption of *parM* in R1-16miniTn5CmE5 has no influence on the conjugation frequency but if it also influences the protein translocation was not know. I created a R1-16Δ*parM* strain and tested it in the CRAfT for conjugation and the transfer of the full length TraI.

My results showed that the disruption of *parM* in the R1-16 plasmid had no influence on conjugation and the protein translocation of the full length TraI under my conditions.

4.3.2 ParR is necessary for R17 infection

The R1-16Δ*parM* strain was also tested for R17 phage infection by Sandra Raffl. The strain showed the same infection level as wild type R1-16. This result differs from the result of R1-16miniTn5CmE5 which shows immunity to R17 infection.
Recent experiments discovered why these two *parM* knock out strains respond differently to the R17 phage. Paul Kirchberger tested the R1-16miniTn5CmE5 once again for phage infection and complemented it with *parR*. With ParR the strain was sensitive for phage infection. This shows that ParR is necessary for R17 phage infection and explains why R1-16Δ*parM* was fully infected.

4.3.3 ParM is translocated in the R1 system

In a CRAfT it was tested if ParM is translocated in the R1 system to the recipient and it was transferred.

The next step was to find out how the ParM is translocated and I made three hypotheses: (i) as free protein, (ii) bound to TraI or DNA, (iii) bound to the DNA via ParR all three with the possibility as single protein or as filament.

To find out more about how the protein is translocated three ParM mutants were also tested in the CRAfT in the R1-16 background. ParMK123A is not able to bind to ParR and ParMD170E does not form correct filaments. Both were transferred at a reduced frequency. From the result in the R1-16 background I conclude that ParR binding and a correct filament formation is required for efficient ParM translocation.

To get more information the same ParM mutants were tested in the R1-16Δ*parM* background. Especially ParMK123A should bring clarity whether ParR binding and filament formation is necessary for translocation. The detection of the wild type ParM and mutated ParM protein translocation failed. It seems that the N-terminal Cre fusion interferes with correct ParR binding, filament formation or interaction with the T4SS proteins without the help of natural ParM proteins.

With these two experiments the question how the ParM is transported could not be answered but we got some hints.

(i) As free protein

Until now we have no evidence that ParM is translocated as free protein or free filament. The CRAfT with ParMK123A in the R1-16Δ*parM* background should bring clarity because the mutated protein is not able to bind to ParR and make filaments. In the experiment ParMK123A showed no protein translocation.

(ii) Bound to TraI or DNA

Experiments performed by Christian Gruber from our lab gave evidences for this possibility. In relaxase assays, where the nicking activity from the full length TraI was observed, the presence of ParM showed a 2-times stimulating effect. In this assay no *parC* and ParR are present and so ParM can not start to form filaments. That means that ParM stimulates TraI as single protein or it uses another way to start filamentation. If the stimulation comes from protein-DNA or protein-protein interaction of ParM remains unclear until now.

(iii) Bound to the DNA via ParR

Several findings confirm this hypothesis.

The result of my CRAfT in the R1 background shows that ParM is only translocated at wild type frequency when it is able to bind to ParR and to form correct filaments.

ParR is also needed for R17 phage infection and that indicates a connection with the relaxosome.

The working group of P. Chirstie showed that the *A. tumefaciens* T-DNA delivery system to the T4SS influences the relaxosome assembly at the T-strand as well. Responsible therefore is an ATPase of the ParA/MinD-like family called VirC1 and its partner protein VirC2. They predicted that the VirC1/VirC2/T-DNA complex is equivalent to a ParA/ParB/*parC* partitioning system that has been converted to a DNA processing machinery for conjugation (Atmakuri et al., 2007).

4.3.4 ParR translocation

For the case that ParM is translocated because it is bound to ParR, this protein should also be transferred together with ParM. Therefore ParR was tested in the CRAfT for its transfer and it did not show protein translocation. The absence of the transfer could have two possible explanations. The first explanation is that it is really not translocated. The second is that the Cre fusion disrupts its DNA binding activity.

The Cre recombinase was fused to the *parC* binding N-terminus of ParR (Salje & Löwe, 2008) and could avoit protein-DNA interaction.

4.3.5 Hypothetical role of the Par proteins within the conjugative machinery

The resulting question out of the previous data and my results is: What function fulfil the proteins of the R1 partitioning system during the R1 conjugation?

Out of my data, under my conditions, I can say that the disruption of *parM* does not influence the plasmid delivery to the T4SS, because of the normal conjugation frequency. Maybe this is just an effect of the long conjugation time in my experiments. During two hours and 30 minutes the plasmid has enough time to find the T4SS. Further experiments should be made to see, whether a shorter conjugation time has an effect on conjugation or not.

As I saw that ParM is transported to the recipient cell my first idea was that the growing ParM filament pushes the plasmid through the T4S channel to the recipient cell. But this assumption did not fit to the normal conjugation frequencies of both *parM* knock out strains, showing that the plasmid is transported without ParM in the cell. The movement of the processed plasmid through the T4 channel could also not be conjugation time dependent, like the delivery of the plasmid. The transport could not be possible without the provided transport system, no matter how long the conjugation time is.

For me it is more likely that the Par proteins are somehow involved in substrate processing and relaxosome docking. As mentioned before, ParM stimulates the nicking activity of TraI (Christian Gruber) and it interacts with TraD (Barbara Klug). Furthermore, the R17 phage needs a docked and functional relaxosome complex for infection and without ParR this condition seems to be not met. At this point I would say that the proteins from the *par* locus are not quite necessary for processing and docking but maybe they fulfil a “fine tuning” function not defined yet.

4.3.5 Outlook

Several approaches should be made to find out more about the connection between the R1 plasmid partitioning system and the conjugation machinery.

Further CRAfTs with the *parM* knock out strains should be made with shorter conjugation time to test, if it has an influence on the conjugation frequency. For the

case that the conjugation frequency goes down depending on the shorter conjugation time it could be a hind for a plasmid delivery problem.
It should be proved if the ATPases of the partitioning system are possible substrates for conjugative T4SS or if this is unique for the R1 system. For example it could be tested if SopA from F (Type I) or ParM from R100 (Type II) is transferred.
Further experiments should also be made to find out how ParM is translocated in R1. A CRAfT in the R1-16Δ*parR* background should bring clarity if ParM needs ParR interaction to be transferred. Furthermore a C-terminal Cre fusion to ParR would answer the question if ParR is not translocated or if the N-terminal fusion disrupts its interaction with the DNA.
It should also be tested if ParM binds to DNA (*oriT*) or proteins of the relaxosome complex or the CP, e.g. using mobility shift assays or coimmunoprecipitation.
It would also be interesting to perform fluorescence microscopy with tagged ParM, ParR and the conjugative plasmid to visualize these components and its movements during conjugation. Similar experiments were made to observe ParM filaments during partitioning in fixed cells and *in vivo* (Møller-Jensen et al., 2002; Campbell and Mullins, 2007; Garner et al., 2004; Salje et al., 2009)

4.4 Western blot analysis

A part of my work was to test three antisera for their ability to detect protein in purified form and within protein extract to perform a quantitative western blot.

4.4.1 TraI relaxase antiserum

The antiserum can be used for assays with purified TraI full length protein and with the TraI relaxase domain.
I would propose an antiserum dilution of 1:1000 for the work with full length TraI and 1:10000 anti serum dilution for the work with the relaxase domain of TraI.
For me it was not possible with this TraI antiserum to perform a quantitative western blot, because the signal of TraI within protein extract was too weak for quantification.

4.4.2 TraY/lysoszyme antiserum

I would not advise to use this antiserum for any assay with TraY because of the TraY/lysozyme antibody mix and the small share of TraY antibodies in it.

4.4.3 TraD antiserum

The results showed that the antiserum against TraD is very specific and competent. It is possible to detect TraD as purified protein and within a protein extract. As antiserum dilution I would suggest 1:40000.

With this antiserum it was possible to perform a quantitative western blot. The amount of TraD molecules per cell was detected. I calculated a number of 2382 ± 641 TraD molecules per cell. As its function as T4CP, TraD forms a hexameric ring. With the calculated number of TraD it would be possible to form more than 300 hexamers per cell.

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