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Expression and Interaction Studies of the Tra-Proteins from the Gram-Positive Conjugative Model Plasmid pIP501

MASTERARBEIT

zur Erlangung des akademischen Grades

Master of Science

Masterstudium Biochemie und Molekulare Biomedizin

eingereicht an der

Technischen Universität Graz

Betreuer:

Ao.Univ.-Prof. Dr. Walter Keller

Institut für Molekulare Biowissenschaften

Graz, Juli 2017

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Acknowledgement

I would first like to thank my thesis advisor Ao. Univ.-Prof. Dr. Walter Keller of the Structural Biology at the Institute of Molecular Biosciences at the University Graz. He gave me the opportunity to work on this interesting topic and his good guidance.

Besides my advisor I would like to thank MSc Verena Kohler, who was introducing me in this theme and work. Her guidance helped me in all the time of research and writing of this thesis.
I thank my labmate Kristin Hunger, for the stimulating discussion and for her help and also all the other labmates from the Structural Biology department.

Further I wanted to thank PD Dr. Elisabteh Grohmann for providing *Enterococcous feacalis* strains and the plasmid pIP501.

Finally, I must express my very profound gratitude to my parents and to my boyfriend for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

This work was sponsored by the FWF.

1 Index

2 Zusammenfassung

Konjugation ist eines der wichtigsten Mechanismen für Horizontalen Gentransfer. Dieser Mechanismus ist verantwortlich für die Weitergabe von Plasmiden, die wiederum Antibiotikaresistenzen oder Virulenzfaktoren tragen. Diese konjugativen Plasmide enthalten auch alle Informationen die benötigt werden um diese von einer Donor-Zelle auf eine Rezipienten- Zelle zu übertragen. In Gram-positiven Bakterien sind bisher zwei Mechanismen bekannt, wobei der vorherrschende das Typ IV Sekretionssytem ist. Trotzdem ist nur sehr wenig über das Typ IV Sekretionssytem in Gram-positiven Bakterien bekannt, dass nicht nur Antibiotikaresistenzen überträgt, sondern auch Pathogenitätsfaktoren, die wiederrum zu schwerbehandelbaren infektiösen Krankheiten führen können.

Darum studieren wir das Gram-positive Konjugative Model Plasmid pIP501. Es besteht aus 15 verschieden Transfer-Proteinen, die aus einem einzigen Operon bestehen. Bisher konnten erst sechs interagierende Proteine identifiziert werden. Diese sind wahrscheinlich auch beteiligt an der Formation des sogenannten Core complexes. Diese Interaktionen wurden alle mit Hilfe des Bacterial Two Hybrid Assays identifiziert. Unter zu Hilfe nahme dieser Methode haben wir nun weitere bisher noch unbekannte Interaktionspartner identifiziert. Wir erhielten auch Informationen darüber welche Domänen, der Proteine, mit den ganzen Proteinen interagieren. So fanden wir zum Beispiel, das TraM mit allen drei Domänen von TraG interagiert. Und umgekehrt interagiert auch TraG mit allen drei Domänen von TraM. Dies könnte wiederrum auf die Wichtigkeit der beiden Proteine während der Formation des Core complexes hinweisen. Weiters fanden wir noch Interaktionspartner von TraL, einem wahrscheinlich wichtigen Protein des core cpmplexes. Dabei fanden wir Interaktionen mit TraM, TraG und TraK. Diese Interaktionen waren zuvor noch nicht bekannt und bestärken nun unsere Hypothesen über die Formation des Core Complexes.

Mit der in vivo Crosslinking Technik fanden wir wahrscheinlich Interaktionen zwischen TraM und TraK direkt in *Enterococcus feacalis*, einem natürlichen Wirt des Plasmides pIP501. Als auch zwischen TraK und TraN, eine Interaktion die davor schon gezeigt werden konnte.

Zusätzlich haben wir noch die verschiedenen Expressionslevels der mRNA der Tra-proteine untersucht. Dafür haben wir real time PCR verwendet. Dabei fanden wir, das es zur Überexpression von allen Tra-proteinen kommt, wenn TraN ausgeknockt wird.

3 Abstract

Conjugation is one of the most important mechanisms of horizontal gene transfer to shuttle plasmids with genes encoding antibiotic resistance or virulence factors among bacteria. Conjugative plasmids inherently contain the genetic information that is needed to mobilize them from a donor into a recipient cell. In Gram-positive bacteria, two methods are known for horizontal plasmid transfer of single stranded DNA, the more prevalent of which being the Type IV Secretion System. Nevertheless, we know very little about secretion systems in Gram-positive bacteria, which are responsible for the spread of antibiotic resistance and pathogenicity plasmids, making simple infectious diseases difficult to treat.

Therefore, we study the Gram-positive conjugative model plasmid pIP501. The *tra*-Region of pIP501 has been identified as a single operon, which encodes fifteen different transfer proteins.

In previous experiments we identified six different interacting key players in the membrane integrated core complex of this secretion system. These transfer proteins were identified via Bacterial two hybrid experiments. Through this method we identified previously unknown interaction partners and gained more detailed information about which domains are involved in these interactions. We were able to show that all of the TraM domains interact with the complete TraG protein and vice versa, which could indicate that both proteins are involved in the formation of the core complex. We also found interactions between TraL, a postulated part of the core complex of the pIP501 encoded Type IV Secretion System, and TraK, TraM and TraG. Those are completely novel and further strengthen as well as expand our working hypothesis.

With the in vivo Crosslinking approach we found a likely interaction between TraM and TraK as well as between TraN and TraJ, which was shown before with Yeast two hybrid, but could never be demonstrated in a natural host of the pIP501 before.

We also performed real time quantitative PCR to test the different mRNA levels of the transfer proteins, furthermore to quantify the expression of the *tra* genes. Herein we found that a TraN knock out strain of the pIP501 displays overexpression of the other Tra-proteins. This could prove the thesis that TraN is a negative regulator of the *tra*-operon.

4 Introduction

Infectious diseases with antibiotic resistant bacteria became a big issue in the last decades, especially in the health care system, but also in the community. This gave rise to untreatable infectious diseases and with no alternative therapy. The biggest threats nowadays are methicillin resistant *Staphyloccocus aureus* and vancomycin resistant *Enterococcus sp*. Unfortunately, resistance against nearly all antibiotics has been observed.(Gastmeier, Sohr et al. 2002)

There are many different reasons why the spread of antibiotic resistance takes place so fast; the biggest of them is overuse of antibiotics. They are inappropriately prescribed, which not only promotes the development of resistance in bacteria, but it is also a questionable treatment, with potential complications for the patients. It is further used extensively in agriculture, for example as growth supplement in livestock feed. In the US 80% of the total sold antibiotic is used for animals to promote growth and prevent infections. Antibacterial products, as used for hygienic or cleaning purposes, may also contribute this problem.(Ventola 2015)

The problem of antibiotic resistance is not recent – in the 1950s, only 10 years after the discovery of penicillin, resistance against it became a big threat. In 1962 the first case of methicillin resistant *Staphylococcus aureus* appeared in the United Kingdom. As an alternative to methicillin, vancomycin was introduced in 1972. Only 7 years later there were cases of resistance reported in coagulase negative *Staphylococci*. In the next decades a lot of new antibiotics were introduced by the pharmaceutical industry to combat the resistances. Nowadays only 3 of the 18 biggest pharmaceutical companies are still researching in the antibiotic field because they are economically non profitable. This lack of improvement could lead to a future lack of treatment options.(Ventola 2015) Therefore, the research of the mechanism of horizontal gene transfer, which is the predominant form of the spread of antibiotic resistance among bacteria, is the first step towards finding alternate solutions to interfere with that way of transport among bacterial communities. (von Wintersdorff, Penders et al. 2016)

4.1 Horizontal Gene Transfer

It is a transfer of genetic information, typically external genetic material that bacteria acquire or transfer from a mother to a daughter cell. It is an important mechanism for genetic variation and evolution and enables bacteria to adapt to environmental variations. Prokaryotes have three main strategies to obtain new DNA sequences: transformation, transduction and conjugation.(von Wintersdorff, Penders et al. 2016)

4.1.1 Transformation

During transformation the bacteria incorporate naked DNA. This strategy is the simplest type of horizontal gene transfer and is also relevant for spreading pathogenicity factors as Griffith demonstrated in *Staphylococcus pneumonia* 1928. (Griffith 1928) Most of the time it serves as substrate source for DNA repair or as source for genetic innovation.

4.1.2 Conjugative Transfer

This is a very efficient way of gene transfer. It requires tight contact between the donor cell and the recipient cell as well as the so-called F-factor (fertility factor) from the donor cell, which is named the F+ cell and the recipient is the F- cell. This is a very small and autonomous DNA molecule.(Baron, Carey et al. 1959) Lederberg and Tatum first demonstrated the genetic recombination of bacterial cells 1946 in their famous experiment where they mixed an F- strain and F+ strain and plated them on minimal medium. On minimal medium only recombinates could grow. (Lederberg and Tatum 1946) We now know that these are mostly relying on a plasmid or satellite DNA. Later a strain was found, which was able to more efficiently recombine than the F+, those are called hfr+ (high frequency of recombination). DNA transfer takes place from the hfr+ to the hfr- and is unidirectional. In hfr+ strains the F-factor is integrated in the host chromosome. During conjugation, parts of the host chromosome get transferred over, which is called the real bacterial conjugative transfer.(Lederberg, Cavalli et al. 1952) Either dsDNA or ssDNA are transported from the donor to the recipient. The transfer of ds DNA depends on one single protein molecule, an FtsK like ATPase and is found only in *Actinobacteria*,(Vogelmann, Ammelburg et al. 2011) whereas ssDNA transfer is ubiquitous in bacteria and archaea. It relies on a dedicated, cell envelope spanning DNA transfer machinery ancestral to the Type IV secretion system. This mechanism is relevant for conjugative plasmids and integrative conjugative elements (ICE). (Koraimann and Wagner 2014)

4.1.3 Transduction

In this case bacterial viruses such as bacteriophages serve as natural vectors for genes between bacteria. The bacterial/host DNA is mistakenly packaged into the head of the empty bacteriophage when the phage particle is produced. Furthermore it is transferred to another bacteria cell.(Zinder and Lederberg 1952) Nowadays we know that not only chromosomal DNA can be transferred with bacteriophages, but also different mobile genetic elements such as transposons or plasmids.(von Wintersdorff, Penders et al. 2016)

4.2 Mobile Genetic Elements

Those are segments of DNA encoding proteins, which are important for the mediated movement of DNA between genomes. They play an integral role in the evolution of the bacterial genome and they are the backbone of horizontal gene transfer. Mobile Genetic Elements like transposons can change their genetic location. Integrons, which are a natural cloning and expression mechanism using site-specific homologue recombination, have insertion sequences that are widely distributed among bacteria and can be found on either plasmids or chromosomes, bacteriophages and genomic islands, which may contain pathogenicity factors. Plasmids are also regarded as mobile genetic elements; they will be the focus of this work.(Bennett 2008)

4.3 Plasmids

The term plasmid was first invented 1952 by J. Lederberg. (Lederberg 1952) He divided plasmids into infectious or non-infectious categories. The infectious ones were able to be transported from one cell to another via conjugational transfer. To promote this transfer, they have sex factors. Non-infectious plasmids do not have these sex factors, which means they need another sex factor containing plasmid in the same cell for conjugation or they may be transferred by transducing phages. (Clowes 1972) It was discovered that plasmids are responsible for the spreading of antibiotic resistance, for example against penicillin, in *Staphylococcus aureus*.(Lacey 1975) Nowadays plasmids are very powerful tools in molecular biology.

Plasmids are circular DNA molecules, which exist usually as extra-chromosomal replicons in a cell. Plasmids are double stranded, self-replicating DNA molecules. They range in size between 2-3 kb (2-3genes) up to 400kb (400 genes).

As mentioned before, they are self-replicating with a characteristic copy number within the host. There are three known replication types for circular plasmids: the theta type, strand displacement and the rolling circle (RC).

DNA replication through the theta type has been mostly studied in Gram-negative bacteria. The initiation needs a specific plasmid-encoded Rep initiator protein. Additional features are an AT-rich region, where opening of the parental strand and binding of the host initiation factors occur, including binding sites for the host DnaA factor. For elongation, a host encoded DNA-Polymerase is needed and for termination the host system is needed as well.(Meijer, de Boer et al. 1995)

The IncQ-family of plasmids replicate through a mechanism called strand displacement. They require three plasmid-encoded proteins for the initiation of replication. It is independent from host encoded replication proteins. The initiation takes place at a complex single stranded origin region (ssiA and ssiB) positioned on both strands. The synthesis of both strands occurs continuously and results in the displacement of the complementary strand.(Scherzinger, Haring et al. 1991)

Another mechanism is the rolling circle, where the replication has to be unidirectional and asymmetric, because the syntheses of leading and lagging strand are uncoupled. During initiation of the replication a plasmid-encoded Rep-protein introduces a site-specific nick in a region called double-stranded origin (dso) on the plus strand. In this way a 3´OH end is introduced, which is used as the primer for the leading strand synthesis. The elongation from the 3´OH-end starts with the displacement of the parental plus strand and continues until the replisome reaches the dso. During termination of the leading strand replication a DNA strand transfer reaction takes place. The end products are a dsDNA consisting of the parental minus strand and the newly synthesized plus strand and the ssDNA molecule, which is the parental plus strand. This single stranded plus strand is then converted in a dsDNA strand by host proteins. The initiation takes place at the single-strand origin (sso), which is physically distant from the dso. During last step the DNA-Gyrase of the host supercoils the products of replication.(Khan 1997, del Solar, Giraldo et al. 1998)

After replication, plasmid segregation takes place during cell division. As such, it has to be ensured that the daughter cells receive at least one copy of the plasmid. For this, different

strategies are available. One of those strategies is the multimer resolution system (mrs). During this process naturally formed plasmid oligomers from replication and recombination have to be resolved into monomers, which increases the number of molecules that are accessible. Therefore, a site-specific recombinase, so called resolvase, is needed. These resolvases could be host- or plasmid-encoded. (Zielenkiewicz and Ceglowski 2001)

Another strategy is active partitioning, which is an active process and often used by low copy plasmids. Partitioning loci consist of three essential components: two genes encoding trans acting proteins (parA/sopA and parB/sopB) and cis acting centromere like sites (for example the par-system of P1-plasmid and the stop system from F-plasmid). The B-proteins bind to the cis acting centromere site and the A-proteins are Walker motif-type ATPases. Those proteins make contact with the partitioning complex, which also stimulates their ATPase function.(Watanabe, Inamoto et al. 1989, Bouet and Funnell 1999) The partition operon is negatively auto-regulated by protein A. It could be analog to chromosome segregation, which also includes pairing of the plasmid molecules and separation of single plasmid copies to the poles of the cell. (Moller-Jensen, Jensen et al. 2000)

A further strategy is the post-segregational killing or plasmid addiction, where plasmid-free cells are selectively killed. Those systems code for a stable toxin and an antidote. The antidote protects the cell from the lethal toxin by forming tight complexes with the toxic molecules. There are two different groups of antidotes; they could be either proteins or antisense RNAs. With the antisense RNA, the mRNA of the toxin is regulated posttranscriptionally, whereas the protein based antidote directly interacts with the toxin. (Zielenkiewicz and Ceglowski 2001) Protein-based antidote is degraded by cellular proteases. (Jensen and Gerdes 1995)

Not all plasmids spread through cell division; many are distributed through conjugative transfer.

4.4 Type IV Secretion System

Gram-negative and positive bacteria employ Type IV Secretion Systems (T4SS) to translocate DNA and protein substrates, generally through contact-dependent mechanisms to other cells. The T4SS are functionally grouped into conjugation systems, effector translocator and contact independent DNA/protein exchange systems. (Cascales and Christie 2003) The function of this effector translocator has been shown only in Gram-negative pathogens and symbionts so far. They deliver effector proteins to eukaryotic cells and the translocated substrates disrupt host

cells' physiological processes, enabling bacterial colonization and spread.(Asrat, Davis et al. 2015, Kubori and Nagai 2016) Of the family of contact independent exchange systems only of a few examples are known; their function lies in the release of DNA and protein substrates to the milieu or alternatively the uptake of exogenous DNA. (Ramsey, Woodhams et al. 2011) The conjugation systems are the largest subfamily present in nearly all bacteria (Gram-negative and positive) and also in archaea, (Christie 2016), which this work will also focus on.

The conjugation system mediates DNA transfer within and between phylogenetically diverse species and some systems also deliver DNA to fungi, plants and human cells. Conjugative plasmids of Gram-negatives are the best-studied paradigms. (Fig.1) (Christie and Cascales 2005)

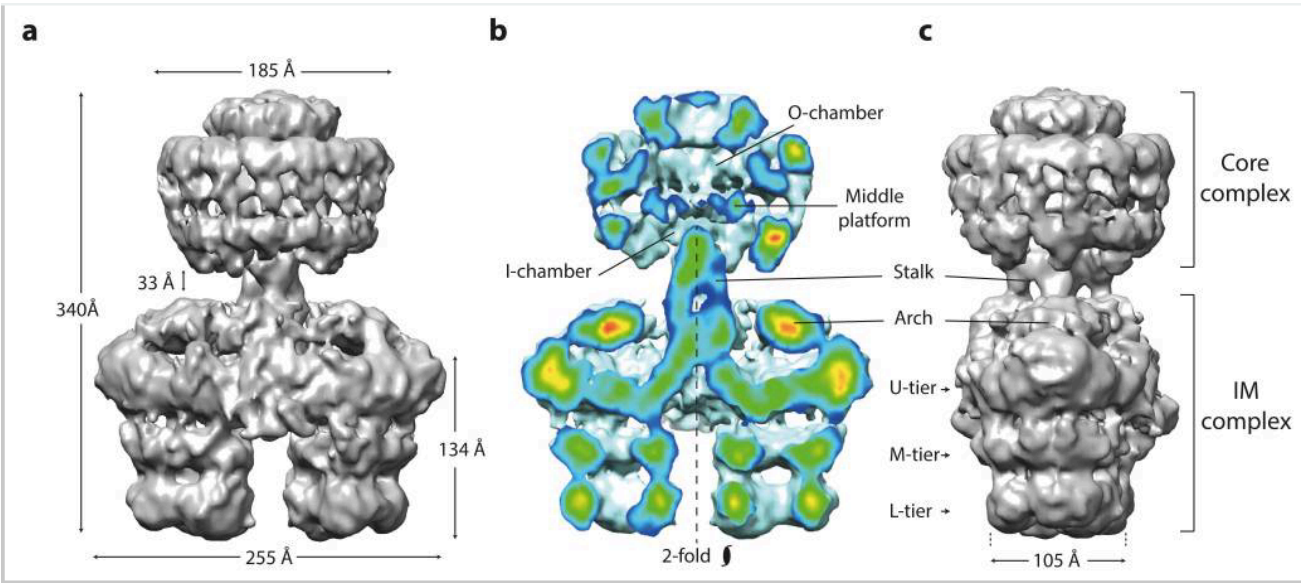

Figure.1: Structure of the Gram-negative T4SS. The data was gained with electron microscopy and negative staining a) Front view of the T4SS model. Merging independently processed core complex and IMC data generated the map. They both are connected through a stalk b) Cut of front view with electron density map ranging from red to blue, which indicates regions from strong to weak density. c) Side view of the core complex and the IMC. (Low, Gubellini et al. 2014)

The VirB/VirD system of *Agrobacterium tumefaciens* is one of the most extensively studied and the prototype for all T4 conjugation systems. Closely related homologues exist in other organisms for most of the components. (Zechner, Lang et al. 2012) The T4SS in Gram-negative bacteria consists of four distinct proteins or subassemblies – the VirD4 coupling protein (CP); the inner membrane complex (IMC), consisting of VirB4 ATPase and VirB11 ATPase, polytopic VirB6 and bitopic VirB8; the outer membrane complex (OMC), composed of outer membrane associated VirB7 and VirB9 and a cell envelope subunit VirB10; and the conjugative pilus

which insists of VirB2 pilin, a proteolytic fragment of VirB1 transglycosylase and the pilus tip protein VirB5. The proteins VirB1-VirB11 are also called the mating-pair-formation (mpf). Related P-type systems of the T4SS are the simplest functioning examples in Gram-negative bacteria. Other P-type systems as well as F-type and I-type systems encode homologues or orthologues to the VirD/VirB subunits but also possess additional domains, protein-complexes and subunits. (Fig.3) (Christie 2016, Christie, Atmakuri et al. 2005)

The general mechanism of the conjugative transfer starts with a multiple protein assembly at the origin of transfer (oriT) to form the relaxosome. This stable complex prepares the single strand of plasmid DNA designated for transfer via a nicking activity of a relaxase enzyme. At the nic position of the oriT the phosphodiesterbond is cleaved for initiation of the transfer. This is mediated by a tyrosin residue of the relaxase, so a tyrosinyl-DNA adduct is formed. This is specifically recognized by the plasmid-encoded Type IV coupling proteins (T4CP). In response to contact-dependent initiation signals, relaxase-linked transfer DNA is probably actively pumped through the mpf complex. In the donor, following termination of transfer, the nicking reaction reverses, yielding the original plasmid DNA strands. (Fig.2) (Zechner, Lang et al. 2012)

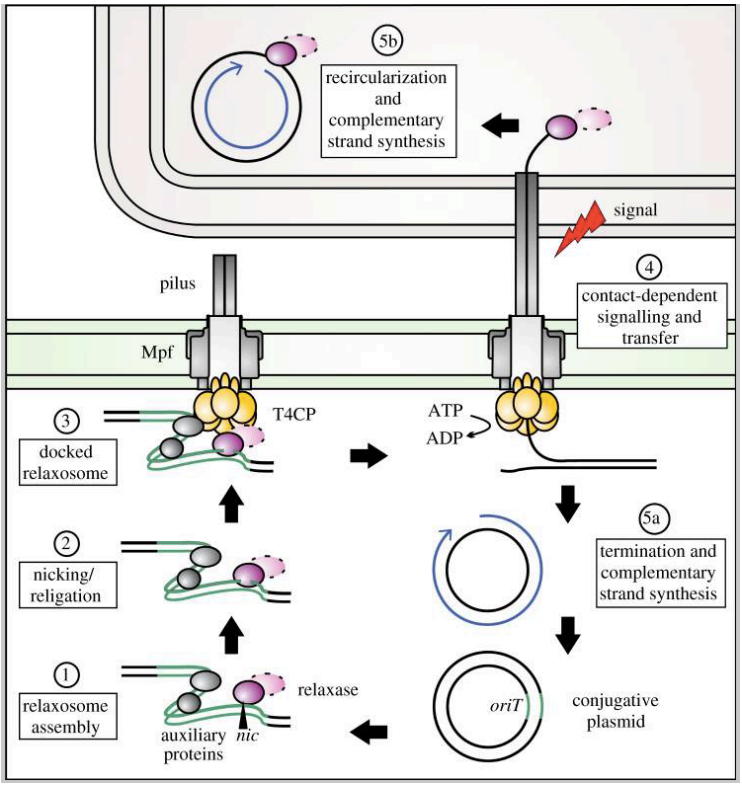

Figure 2: General mechanism of plasmid transfer. 1) Conjugative plasmids, which carry an *oriT* start to form the relaxosome. 2) Now the DNA gets nicked for initiation of transfer. 3) The relaxosome gets recognized by the T4CP (yellow) 4) and gets pumped through the envelope spanning channel (mpf). 5a) In the donor cell, following termination and complementary strand synthesis. 5b) In the recipient

recircularization of the plasmid DNA and complementary strand synthesis start out. For simplicity, plasmid DNA processing of stages 1-4 are illustrated with linear DNA fragments.(Zechner, Lang et al. 2012)

Determination of the nucleotide sequence of their putative transfer (tra) regions or whole gnomes of Gram-positive bacteria have revealed homologies to proteins that belong to the TraG/TrwB/VirB family of coupling proteins, the conjugative transfer ATPase VirB4 and to the VirB1 family of transglycosylases. (Fig.3)

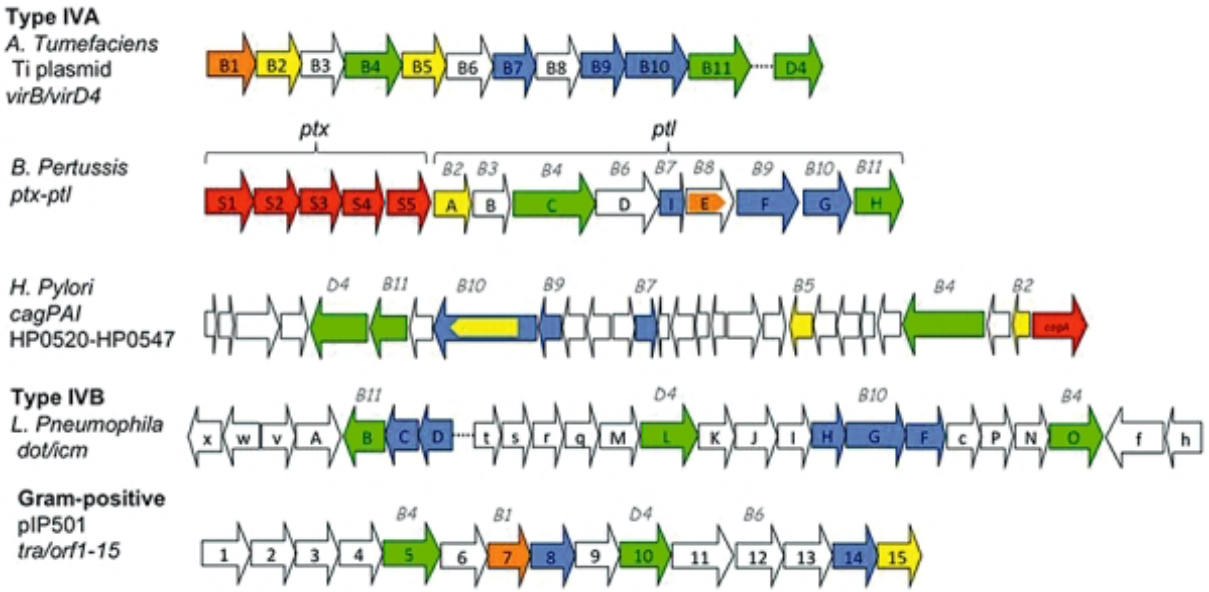

Figure 3: Genetic organization of T4SSs. Blue genes corresponds to core components or plausible ones. Genes in green are ATPases and in yellow surface components. Orange genes are lytic transglycosylases and effector proteins are red. Homology to the *VirB/D4* system is displayed in grey above the schemes. For example a *B4* above a gene denotes that this gene is homologous to *virB4*. (Wallden, Rivera-Calzada et al. 2010)

We still know little about the T4SS of Gram-positive bacteria; we do not know how they achieve cell-cell contact. Whereas Gram-negative bacteria establish the physical contact by a complex extracellular filaments, designated sex pili, for the majority of Gram-positives this have not been identified yet. (Grohmann, Muth et al. 2003) Various model systems are used for these T4SS, such as the broad host range models of the IncQ18 family, to which the pIP501 belongs – which will be described below – and the *Enterococcus* sex-pheromone responsive plasmids.

For the broad host range models all important protein families encountered in Gram-negative T4SS have also been detected in Gram-positive bacteria. One of those is the motor protein family, which has ATPase-activity; the peptidoglycan hydrolase family, which is acting as a

transglycosylase, as well as the T4SS channel/putative core component family and surface factor/adhesins family. (Goessweiner-Mohr, Arends et al. 2014)

For the pheromone responsive DNA-transfer systems, the donor recipient contact is mediated via a tightly controlled system based on small peptides, the so called sex-pheromones, which are secreted by potential plasmid recipients, for example pAD1 in *Enterococcus faecalis*. (Dunny 2007)

Generally, the conjugative T4SS of Gram-positive bacteria implicate similar steps as their counterparts the Gram-negative bacteria; the first step is processing the plasmid DNA to be transferred with the relaxase, covalently attached to its 5´-end. However, the actual DNA translocation process including cell-cell contact from donor to recipient and also passing through the cell envelope differs between Gram-positive and Gram-negative bacteria. This might be due to the differences in the cell membrane. In Gram-positive bacteria the cytoplasmatic membrane is followed by a thick multilayer peptidoglycan. (Goessweiner-Mohr, Arends et al. 2014) Gram-positives also lack genes for subunits comprising the "core complex", which serves as a structural scaffold for the translocation channel and also to couple energy derived from ATP hydrolysis with the outer membrane gating. The channel architecture and the dynamics of channel gating must therefore be different.(Bhatty, Laverde Gomez et al. 2013)

4.5 Plasmid pIP501 and the DNA transfer

pIP501 is a Gram-positive conjugative model plasmid with a broad host range and was isolated for the first time from *Streptococcus agalactiae*. (Horodniceanu, Bouanchaud et al. 1976) It is a multiple antibiotic resistance plasmid frequently detected in clinical *Enterococcus faecalis* and *Enterococcus faecis* strains, but it has also been found in *Lactococcus*, *Bacillus*, *Listeria*, *Staphylococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc sp*. It belongs to the Inc18-plasmid family together with pAMβ1 and pSM19035 and the replication proceeds unidirectionally by a theta mechanism. pIP501 encodes a Gram-positive T4SS in the form of the *tra* operon, coding for 15 putative transfer (tra) proteins. Seven of these 15 proteins show homology to the Vir-proteins of the Gram-negative T4SS of *Agrobacterium tumefaciens*. (Fig.4)

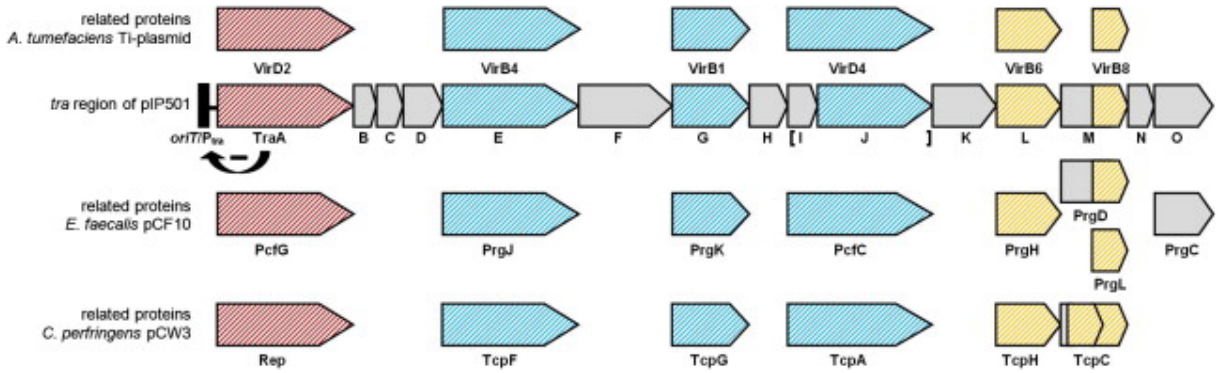

Figure 4: Genetic organization of the pIP501 *tra* operon. Comparison of sequence similarities and related proteins to the *A. tumefaciens* Ti-plasmid VirB/D4, *E. faecalis* pCF10 and *C. perfringens* pCW3 T4SS proteins. Proteins, which are colored in blue, are coupling proteins. Relations based on structure and/or domain prediction based similarities are highlighted in yellow. The gene encoding the putative relaxase is colored in red. The TraA-mediated regulation of the expression is indicated with the bent arrow. (Goessweiner-Mohr, Arends et al. 2013)

TraA has been biochemically characterized as a relaxase and shows similarity to VirD2. In addition to its site-specific DNA-nicking activity it also negatively regulates the expression of all T4SS proteins encoded by the *tra* operon. So far, no auxillary factors have been identified for the TraA mediated oriT cleavage.(Goessweiner-Mohr, Arends et al. 2014)

The pIP501 also codes for two putative ATPases, TraE and TraJ. Both contain the typical NTP-binding motif necessary for ATPase activity. TraE further shows a relation to the VirB4 protein from Ti-Plasmid. TraJ is postulated as a coupling protein together with TraI. Another encoded protein is TraG, which has a peptidoglycan cleavage site. It appears to be essential for the transfer of pIP501 and it might act as a lytic transglycosylase. (Atmakuri, Cascales and Christie 2004)

Two putative channel components of the pIP501 are TraM, a VirB8-like protein and TraL, a VirB6 homolog. A possible scaffolding role for TraM based upon the structure similarity to VirB6 has been suggested, but it could also have a divergent role because of its largely different domain composition. The role of the TraL is not clear so far, but because of its homology to the VirB6 it is assumed that it could play a role as a scaffolding or inner membrane secretion channel component, as suggested for the Gram-negative transfer system. The only protein that could act as a surface adhesin is TraO. A cell wall motif was discovered at its carboxy-terminal end. Moreover, TraO is related to the putative cell wall anchored surface protein PrgC, which is encoded by the sex pheromone responsive plasmid pCF10, further reinforcing this hypothesis. (Fig.4) (Goessweiner-Mohr, Arends et al. 2013, Atmakuri, Cascales and Christie 2004)

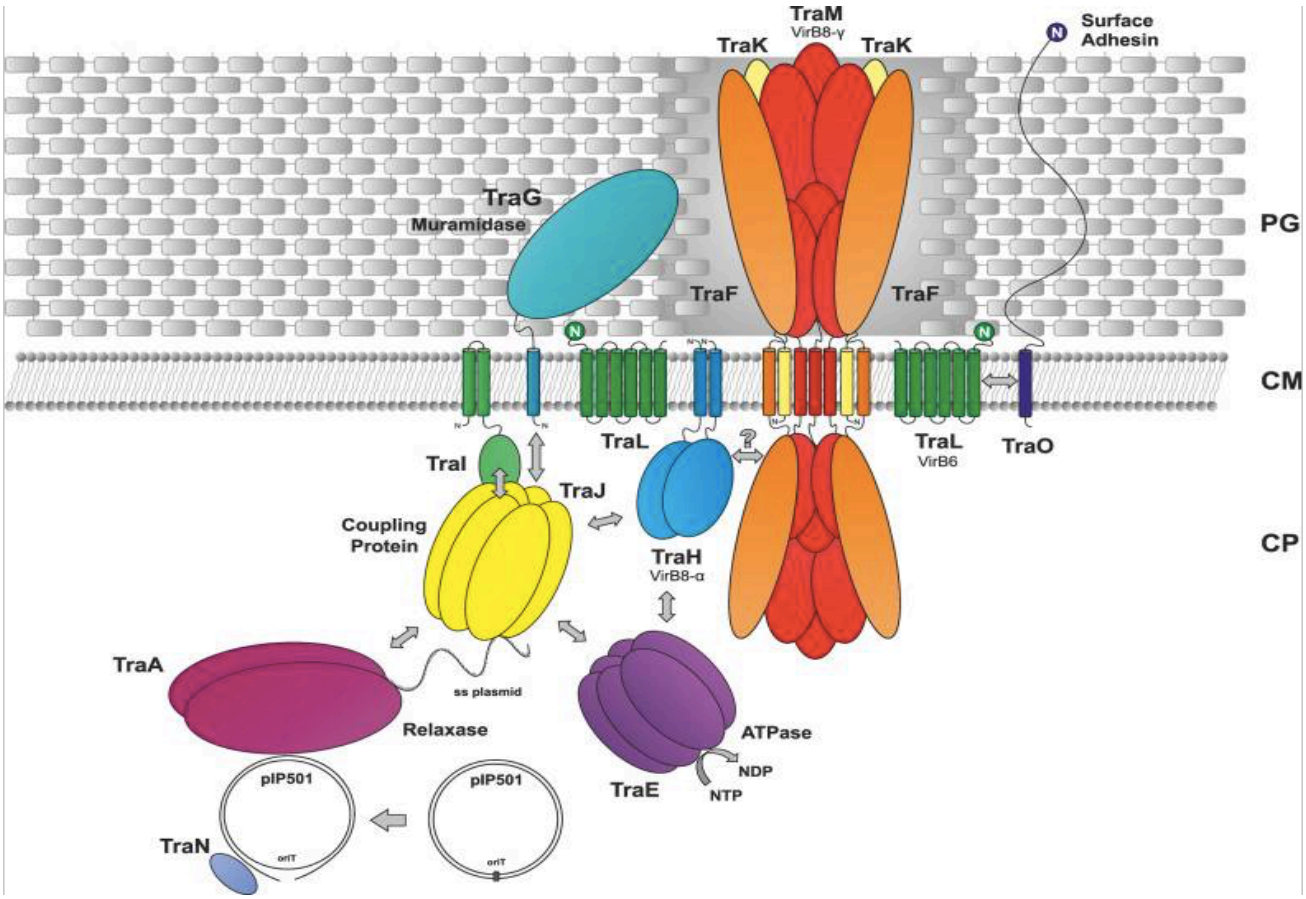

Figure 4: Model of the possible pIP501 DNA transfer pathway. It starts with the relaxase TraA binding to the oriT. After nicking, the single-stranded plasmid is recruited to the putative transfer channel. The putative coupling protein TraJ mediates the recruitment. TraG could mediate local opening of PG.. The putative function of other key members of the pIP501 *tra* operon during the DNA secretion process is indicated. Using in silico approaches the localization and orientation of the T4SS proteins is predicted. Additionally localizations studies were consulted. PG, peptidoglycan; CM, cytoplasmic membrane; CP, cytoplasm (Fercher, Probst et al. 2016)

4.6 Bacterial Two Hybrid

It is based on the reconstitution of an artificial cAMP signal transduction pathway in an adenylate cyclase deficient *E. coli* strain and was first set up by Karimova in 1998. Its advantage is the modular structure of the two catalytic domains of *Bordetella pertussis* adenylate cyclase, which consist of two complementary fragments, T25 and T18.(Dautin, Karimova et al. 2002) They are inactive when they are expressed separately in *E. coli*. They can become active again when two polypeptides fused to the fragments interact with each other. In E.coli, cAMP is a key-signaling molecule and binds to a transcriptional activator, CAP (catabolite activator protein). When cAMP accumulates, it binds to CAP, enabling it to activate a large number of genes, such as the lactose or maltose metabolizing family. Adenylate cyclase

deficient *E. coli* strains are unable to ferment lactose or maltose, so the cells can be discriminated easily on indicator media or selective media. (Karimova, Pidoux et al. 1998)

The genetic screening of protein-protein interactions requires coexpression of two hybrid proteins in the same adenylate cyclase deficient strain. For this purpose, the two fragments are cloned into two compatible vectors to fuse them with the adenylate cyclase subunits:

- pKT25 encodes the T25 fragment that is expressed under the control of a lacZ promotor. It includes a kanamycin resistance selectable marker and a multiple cloning site (MCS). The MCS is at the 3´end of T25, which allows in-frame fusion at the C-terminal end of T25. pKNT25, which also contains the T25 polypeptide, has its MCS fused in-frame upstream, which allows in-frame fusions at the N-terminal end of T25.
- pUT18 it encodes the T18 fragment which is also under the control of a lac-promotor. It contains an ampicillin resistance selectable marker. The T18 lies downstream of the MCS, this allows fusion to the N-terminal side of T18. pUT18C has the MCS at 3´end of T18, so a heterologous protein fusion on the C-terminal side of T18 is possible.

Now an adenylate cyclase deficient *E.coli* strain is co-transformed with the two vectors and the interaction of two proteins brings together the two fragments and restores the enzymatic activity of the adenylate cyclase. cAMP then induces the expression of lactose or maltose metabolizing genes. This way they may be screened on selective media or indicator media for possible interactions and additionally a β-galactosidase assay can be performed. (Fig.6) (Karimova, Ladant et al. 2002, Battesti and Bouveret 2012)

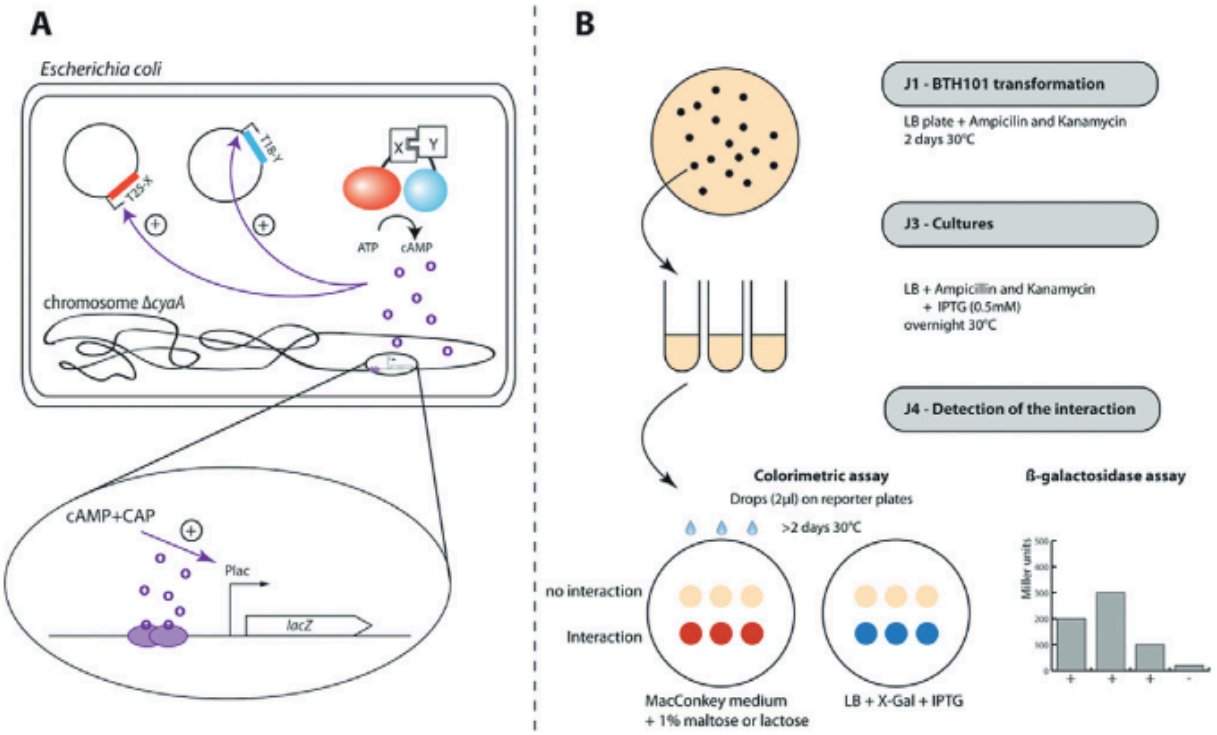

Figure 5: B2H scheme: A: An *E. coli* Δ*cya* strain gets transformed with the two compatible plasmids carrying the fusion proteins with T25 and T18 domains. The interaction of proteins X and Y brings back together the domains of T25 and T18 and reconstitute the adenylate cyclase activity. Diffusible cAMP then accumulates and together with CAP activates the expression of lactose and maltose operons. A positive feedback mechanism promotes the expression of the hybrid genes. B: Workflow as it is practiced in our own lab. (Battesti and Bouveret 2012)

5 Aim of the Thesis

The pIP501 is a conjugative model plasmid, which we use to study the Gram-positive T4SS. The goal of this thesis was to find out more about the interactions of the different Tra-proteins and their expression patterns. The different fields of this work are:

1. In our current study we are focusing on reproducing the results of the bacterial two hybrid and identifying the domains of the core key players that interact during conjugation. If we know more about the interacting domains of this complex, it might help with the general understanding of the assembly of the core complex of the T4SS.

2. We are further focusing on in vivo crosslinking in different growth phases of *Enterococcus faecalis*, to verify interaction partners found via bacterial two hybrid experiments. This is a protein-protein interaction study in a Gram-positive bacterium. The reason this is particularly interesting is because *Enterococcus faecalis* sustains the pIP501 naturally. We further attempted to analyze the crosslinked complexes with mass spectrometry.

3. To get more detailed information, we wanted to know more about the expression levels of the different tra proteins. Therefore, we performed real time quantitative PCR to test the different mRNA levels of the Tra–proteins and to quantify the expression of the *tra* genes. We suspect that TraN functions as a repressor, however putative interaction partners are thus far unknown. We further want to investigate if knockout of TraN influences expression levels of other Tra-proteins.

6 Materials and Methods

6.1 Materials

6.1.1 Chemicals and Kits

6x Loading Dye	NEB
Acrylamid/Bisacrylamid	Roth
GeneJET Plasmid Miniprep Kit	Thermo Fischer Scientific
GeneJET PCR Purification Kit	Thermo Fischer Scientific
GeneJET Gelextraction Kit	Thermo Fischer Scientific
IPTG (Isopropyl-β-D-thiogalactopyranosid)	Roth
ONPG (o-Nitrophenyl-β-D-galactopyranosid)	Roth
TRIzol-Reagent	Thermo Fischer Scientific
X-Gal (5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid)	Roth
Para-formaldehyde	Roth

6.1.2 Enzymes, Standards and Antibiotics

HotFIRE POL	SOLIS BioDyne
T4-DNA Ligase	NEB
Restriction enzymes	NEB
M-MLV Reverse Transcriptase RNase H	SOLIS BioDyne
5x Hot FIREPol EvaGreen qPCR Supermix	SOLIS BioDyne
Low Range Standard	Promega
100bp DNA Ladder	Promega
Mid Range molecular prestained Standard	Promega

The size range and figures of the standards are in the attachment.

6.1.3 Microorganisms

Species and Strain	Genotype	Reference
Escherichia coli		
BL21		
BTH101	F-,cya-99, araD139, galE15, galK16, rpsL1(Strr), hsdR2, mcrA1, mcrB1	EUB001
Enterococcus faecalis		
OG1RF		from Elisabeth Grohmann
JH2-2		from Elisabeth Grohmann

6.1.4 Plasmids

Name	Size (kb)	Reference	Resistance
pKT25	3.4	EUP-25C	Kanamycin
pKNT25	3.4	EUP-25N	Kanamycin
pUT18	3.0	EUP-18N	Ampicillin
pUT18C	3.0	EUP-18C	Ampicillin
pUT18C-zip	3.0	EUP-18Z	Ampicillin
pKT25-zip	3.4	EUP-25Z	Kanamycin
pIP501	30.6		

6.1.5 Media

- Luria-Bertani (LB)-Medium for the cultivation of *E. coli* from Roth
- Brain Heart Infusion from Roth for *E. faecalis*
- Todd Hewitt Broth from for *E. faecalis*
- LB-Agar from Roth for the plates of *E. coli*

All media were prepared with deionized water.

6.1.6 Synthetic Oligonucleotides

The upper case letters in the non-binding sequence are recognition sites for the restriction enzyme digestion.

Name	SEQUENCE (5´-3´) Binding + non binding
For Bacterial Two Hybrid	
TraF (pUT18/pKNT25)	
N-terminal fw	atatCTGCAa ATGAAATACAAAATCTTGAAAAAT
N-terminal rev	TAATTCAGCAGAAAAGAAATTCaattGGATCCaa
membrane fw	atatCTGCAaCAGGACAAGAAAGCAG
membrane rev	CTCATTTACTTTCATAAGTAAAACaattGGATCCaa
C-terminal fw	atatCTGCAaAAGAAACAAGTTACGGC
C-terminal rev	ATCTTTTTCAGCGTTTGTaattGGATCCaa
TraG (pUT18/pKNT25)	
Membrane fw	atatCTGCAaATGGGAGCAACAGC
Membrane rev	GTTTTCTGTTGCTAGTCCaattGGATCCaa
SLT+linker fw	atatCTGCAaAAAAATTTAAGTGAATCTGTTTTG
SLT+linker rev	ATTGTTTTGAACGCCCaattGGATCCaa
Chap+linker fw	atatCTGCAaGAAAGCCAGGCAAG
Chap+linker rev	TTCAACATAACTTGCAATATTTGaattGGATCCaa
TraI (pUT18/pKNT25)	
Sol-TM1 fw	atatCTGCAaATGGCGAAGAAGAAGC
Sol+TM1 rev	AAAAGGTAATAATTTAAACACAATCaattGGATCCaa
TM2 fw	atatCTGCAaATCATTGCGAATGATACA
TM2 rev	TAGAAAGACAAATAAAATAATTAAACaattGGATCCaa
Soluble fw	atatCTGCAaCACCCTACCAAACG
Soluble rev	GTCATTTTTCCCCCTCaattGGATCCaa
TraK (pUT18/pKNT25)	
Cytmem fw	atatCTGCAaATGAATGGACTGAAAGAA
Cytmem rev	ATTGTTAAAATAAGAAATGCCCaattGGATCCaa
Extracellular fw	atatCTGCAaAAGAATCAACCACCAG
Extracellular rev	GTAAACACCTCCAACATTaattGGATCCaa
TraM (pUT18/pKNT25)	

N-terminal fw	atatCTGCAaATGTCTTATTATTTTGAAATACGT
N-terminal rev	TTGGCGTTCATTACTCaattGGATCCaa
Membrane fw	atatCTGCAaGAAATACCGCAGACAG
Membrane rev	AGAAATATCCGTTTCTTTGaattGGATCCaa
C-terminal fw	atatCTGCAaCAATCGAAAATAGATACTTTTG
C-terminal rev	GTTAAAAGAGAAGTCTGTAAGaattGGATCCaa
TraF (pKT25)	
N-terminal fw	atatCTGCAGaaATGAAATACAAAATCTTGAAAAAT
N-terminal rev	TAATTCAGCAGAAAAGAAATTCaattGGATCCTCA
membrane fw	atatCTGCAGaaCAGGACAAGAA AGCAG
membrane rev	CTCATTTACTTTC ATAAGTAAAACaattGGATCCTCA
C-terminal fw	atatCTGCAGaaAAGAAACAAGTTACGGC
C-terminal rev	ATCTTTTTCAGCGTTTGTaattGGATCCTCA
TraG (pKT25)	
Membrane fw	atatCTGCAGaaATGGGAGCAACAGC
Membrane rev	GTTTTCTGTTGCTAGTCCaattGGATCCTCA
SLT+linker fw	atatCTGCAGaaAAAAATTTAAGTGAATCTGTTTTG
SLT+linker rev	ATTGTTTTGAACGCCCaattGGATCCTCA
Chap+linker fw	atatCTGCAGaaGAAAGCCAGGCAAG
Chap+linker rev	TTCAACATAACTTGCAATATTTGaattGGATCCTCA
TraI (pKT25)	
Sol-TM1 fw	atatCTGCAGaaATGGCGAAGAAGAAGC
Sol+TM1 rev	AAAAGGTAATAATTTAAACACAATCaattGGATCCTCA
TM2 fw	atatCTGCAGaaATCATTGCGAATGATACA
TM2 rev	TAGAAAGACAAATAAAATAATTAAAACaattGGATCCTCA
Soluble fw	atatCTGCAGaaCACCCTACCAAACG
Soluble rev	GTCATTTTTCCCCCTCaattGGATCCTCA
TraK (pKT25)	
Cytmem fw	atatCTGCAGaaATGAATGGACTGAAAGAA
Cytmem rev	ATTGTTAAAATAAGAAATGCCCaattGGATCCTCA
Extracellular fw	atatCTGCAGaaAAGAATCAACCACCAG
Extracellular rev	GTAAACACCTCCAACATTaattGGATCCTCA
TraM (pKT25)	

N-terminal fw	atatCTGCAGaaATGTCTTATTATTT TGAAATACGT
N-terminal rev	TTGGCGTTCATT ACTCaattGGATCCTCA
Membrane fw	atatCTGCAGaaGAAATACCGCAGACAG
Membrane rev	AGAAATATCCGTTTCTTTGaattGGATCCTCA
C-terminal fw	atatCTGCAGaaCAATCGAAAATAGATACTTTTG
C-terminal rev	GTTAAAAGAGAAGTCTGTAAGaattGGATCCTCA
TraF (pUT18C)	
N-terminal fw	atatCTGCAGaATGAAATACAAAATCTTGAAAAAT
N-terminal rev	TAATTCAGCAGAAAAGAAATTCaattGGATCCTCA
membrane fw	atatCTGCAGaCAGGACAAGAAAGCAG
membrane rev	CTCATTTACTTTCATAAGTAAAACaattGGATCCTCA
C-terminal fw	atatCTGCAGaAAGAAACAAGTTACGGC
C-terminal rev	ATCTTTTTCAGCGTTTGTaattGGATCCTCA
TraG (pUT18C)	
Membrane fw	atatCTGCAGaATGGGAGCAACAGC
Membrane rev	GTTTTCTGTTGCTAGTCCaattGGATCCTCA
SLT+linker fw	atatCTGCAGaAAAAATTTAAGTGAATCTGTTTTG
SLT+linker rev	ATTGTTTTGAACGCCCaattGGATCCTCA
Chap+linker fw	atatCTGCAGaGAAAGCCAGGCAAG
Chap+linker rev	TTCAACATAACTTGCAATATTTGaattGGATCCTCA
TraI (pUT18C)	
Sol-TM1 fw	atatCTGCAGaATGGCGAAGAAGAAGC
Sol+TM1 rev	AAAAGGTAATAATTTAAACACAATCaattGGATCCTCA
TM2 fw	atatCTGCAGaATCATTGCGAATGATACA
TM2 rev	TAGAAAGACAAATAAAATAATTAAACaattGGATCCTCA
Soluble fw	atatCTGCAGaCACCCTACCAAACG
Soluble rev	GTCATTTTTCCCCCTCaattGGATCCTCA
TraK (pUT18C)	
Cytmem fw	atatCTGCAGaATGAATGGACTGAAAGAA
Cytmem rev	ATTGTTAAAATAAGAAATGCCCaattGGATCCTCA
Extracellular fw	atatCTGCAGaAAGAATCAACCACCAG
Extracellular rev	GTAAACACCTCCAACATTaattGGATCCTCA
TraM (pUT18C)	

N-terminal fw	atatCTGCAGaATGTCTTATTATTTTGAAATACGT
N-terminal rev	TTGGCGTTCATT ACTCaattGGATCCTCA
Membrane fw	atatCTGCAGaGAAATACCGCAGACAG
Membrane rev	AGAAATATCCGTTTCTTTGaattGGATCCTCA
C-terminal fw	atatCTGCAGaCAATCGAAAATAGATACTTTTG
C-terminal rev	GTTAAAAGAGAAGTCTGTAAGaattGGATCCTCA
For Colony PCR	
pKNT25 fw	TTGTGTGGAATTGTGAGCGG
pKNT25 rev	CCAGCCTGATGCGATTGCTG
pKT25 fw	TCGGTGACCAGCGGCGATTC
pKT25 rev	GGGTAACGCCAGGGTTTTCC
pUT18 fw	GGAATTGTGAGCGGATAAC
pUT18rev	CCATGGCCTCGCTGGCGGCT
pUT18C fw	CTGGAAACGGTGCCGGCGTC
pUT18C rev	ATTGTACTGAGAGTGCACCA
For quantitative real time PCR	
TraB fw	AACAGCCTTGCTTGTTACGG
TraB rev	AAGTAAGCCATACGCCCAAG
TraG fw	GGACTTGATGAAACGGAAGC
TraG rev	CTTCCCCTTGACGTTTTGAG
TraJ fw	TAGTGAAGAATGGCGGAACG
TraJ rev	GCGGTAATTGTAGCCCATTG
TraK fw	TCATGGATCGGCTTATTGG
TraK rev	AGTCATTTTTCCCCCTCCTC
TraM fw	ATGAACCAACAGAACCAACG
TraM rev	CTGTCTGCGGTATTTCTTGG
TraO fw	GTTAGTTTTGCGGACGAAGC
TraO rev	TGGAGTAGTTGGATCGGTTG
Houskeeping Genes	
proC fw	GTCTTCAGGGGATGCAAAAG
proC rev	TCGTCTTGTTCGTGTGATGC
16sRNA fw	AGCAACGCGAAGAACCTTAC

16sRNA rev	ATGCACCACCTGTCACTTTG
GAPDH fw	ATTAGATGGCCCTGTTCGTG
GAPDH rev	TGCTTTAGCAGCACCAGTTG

6.1.7 PCR-Programs

B2H-PCR								
	Tm	t (m:s)	Tm	t (m:s)	Tm	t (m:s)	Tm	t (m:s)
Denaturing	95° C	13:00	95°C	00:20	95°C	00:20		
Annealing			54°C	00:30	66°C	00:30		
Elongation			72°C	1:00	72°C	1:00	72°	10:00
Cycles	1		10		20		1	

Colony PCR						
	Tm	t (m:s)	Tm	t(m:s)	Tm	t (m:s)
Denaturing	95°C	15:00	95°C	00:30	95°C	00:30
Annealing			54°C	00:35	54°C	00:35
Elongation			72°C	00:30	72°C	10:00
Cycles	1		25		1	

Quantitative real time PCR						
	Tm	t (m:s)	Tm	t(m:s)	Tm	t (m:s)
Denaturing	95°C	15:00	95°C	00:15	95°C	00:15
Annealing			60°C	00:20	60°C	00:20
Elongation			72°C	00:20	72°C	10:00
Cycles	1		40		1	

6.1.8 Buffers and Solutions

All buffers and solutions were prepared with deionized water or bi-distilled water.

6.1.8.1 Bacterial Two Hybrid Assay

Subsequently listed are all buffers and solutions that were used during cloning for the B2H-Assay and during the assay.

TAE buffer for agarose gel electrophoresis 50x

242 g Tris free base

18.61 g Disodium EDTA

57.1 mL Glacial Acetic Acid

ddH_2O to 1 liter

TAE buffer for agarose gel electrophoresis 1x

20mL 50x Stock

980mL H_2O

TB buffer (1L) for chemically competent cells

10mM HEPES pH 6,7 (2,38g)

15mM $CaCl_2$ (2,13g)

55mM $MnCl_2$ (10,89g)

250mM KCl (10,87g)

Z buffer for β-galactosidase assay

60mM Na_2HPO_4

40mM NaH_2PO_4

10mM KCl

1mM $MgSO_4$

50mM β-mercaptoethanol

ONPG	4mg/mL
SDS	0.1%
IPTG	1M
Na_2CO_3	1M

6.1.8.2 In vivo Crosslinking

Listed below are all buffers and solutions that were used during protein analyses and in vivo crosslinking as well as for SDS-PAGE and immunoblotting.

1M-Sodiumphosphate buffer for in vivo crosslinking

Solution A: 138.0 g NaH_2PO_4–H_2O in 1 liter dH_2O (pH 7.0).
Solution B: 142.0 g Na_2HPO_4 in 1 liter dH_2O (pH 7.0).
Mix 423 ml Solution A with 577 ml Solution B.

.

4% para-formaldehyde solution for in vivo crosslinking

2.0 g para-formaldehyde

50ml 1M-Sorbitol-solution

Laemmli buffer for SDS-PAGE

SDS 10%

Glycerol 50%

Bromophenol blue 0,01%

Tris-HCl pH 6,8 1,5M

Transfer buffer (after Towbin) 10x 1L for Western Blot

30,3 g Tris

144,1g Glycin

5mL SDS (10%)

Transfer buffer 1x 1L (usage: 4 times) for Western Blot

100mL 10x Stock

700mL H_2O

200mL MetOH

TBS 10x 1L pH 7,4 for Western Blot

500mM Tris (60,6g)

1.5M NaCl (87,65g)

TBST 10x 1L pH 7,4 for Western Blot

500mM Tris (60,6g)

1.5M NaCl (87,65g)

10mL TritonX (20% Stock)

Stripping buffer

20 mL SDS 10%

12.5 mL Tris HCl pH 6.8 0.5M

67.5 mL ultra pure water

0.8 mL ß-mercaptoethanol

6.1.8.3 RNA-Isolation

RNA-Gel-Buffer

TAE buffer 1x

0.3% sodium hypochlorite

6.2 Methods

All methods that are not explicitly described, were done according to Sambrook et al. (1989)

6.2.1 Bacterial Two Hybrid Assay

For the B2H Assay, preparative cloning of the domains had to be done. Therefore, we used the following microbiology methods.

6.2.1.1 PCR for Amplification of the Domains

For amplification of our fragments we used specific primers for every fragment in a total volume of 40µL. We used 1µL of each primer, 4µL of the template 8µL PCR-Mix and the rest we filled up with Fresenius water.

For identification of the fragments we used a 1% agarose-gel onto which 3µL of the reactions were loaded. The rest of the PCR-volume was used for purification.

6.2.1.1 Extraction of DNA from Agarose Gels

All extractions were done with the GeneJET Gel Purification Kit. The eluation of the DNA was done with Fresenius water in 50µL.

6.2.1.2 Plasmid Isolation from E. coli

All plasmid isolations were done with the GeneJET Purification Kit. Plasmids were eluated in 50µL Fresenius water.

6.2.1.3 Restriction Enzymes and Ligation

All DNA-Fragments were cut with restriction endonucleases over night at 37°C.
For ligation we used a molar ratio of 1:3 of vector-to-insert in a volume of 10µL followed by overnight incubation at 16°C.

6.2.1.4 Competent Cells and Transformation

For the preparation and transformation of competent cells we followed the protocol of Sambrook et al (1989). 100µL competent cells were incubated with 5µL of ligation product or 1µL of an isolated plasmid on ice for 30 min. The duration of the heat shock was 1 min at 42°C and the regeneration time was 2 min on ice followed by 1 hour (*E.coli* BL21) or 90min (*E.coli* BTH101) at 37°C in SOC-media. (Sun, Ding et. al 2009)

6.2.1.5 Determination of Concentration of the DNA

It was done photometrically with the NanoDrop.

6.2.1.6 Identification of Transformants

We used colony PCR. For this procedure, one colony was suspended in 50µL Fresenius Water and lysed by heating it for 10 minutes in 95°C and afterwards placed on ice for 10 minutes. After centrifugation (for 10 minutes at 13200rpm) we used 1µL in a volume of template for 20µL for PCR. We used the primers for colony PCR.
Alternatively we used the isolated plasmids (1µL) in a volume of 40 µL; here, the same primers and the same conditions as for the amplification were applied.

6.2.2 Bacterial-Two-Hybrid

For our research we used the BACTH system of EUROMEDEX and carried out all experiments according to their protocol. To begin, the amplified PCR fragments were ligated with the four different plasmids.

6.2.2.1 Transformation

We co-transformed *E. coli* BTH101 with two complementing plasmids with fused proteins. We used approximately 1µL of each plasmid per transformation. Followed by 30 min chilling on ice and a heatshock for 1 min at 42°C, then 2 minutes on ice. For regeneration we used 700µL of SOC-media and incubated the cells at 37°C for 90 min. (Sun, Ding et. al 2009)
Afterwards we plated them on X-Gal agar plates containing ampicillin, kanamycin and IPTG. (Battesti and Bouveret 2012)

6.2.2.2 β-Galactosidase Assay

For the β-galactosidase Assay we picked 4 different transformants per plate to take measurements of 4 replicates of each transformant per experiment.
For the overnight culture we used 96-deepwell plates with 500µl LB-media in each well. On each plate we had 3 positive controls, 2 negative controls and 4 different variants of clones. On the next day, 20µL of each culture were transferred into a second deepwell plate, also containing 500µL LB-media plus kanamycin and ampicillin per well, each strain in 4 wells. Incubation proceeded for 90 min at 30°C and 200 rpm, followed by induction with 25µL IPTG (25mmol) and additional incubation of 2 hours.
Afterwards, 100µL samples were taken for OD measurement at 600nm and 100µL were transferred into another 96-deepwell plate containing 1ml Z-Buffer, 20µL 0.1% SDS and 40µL chloroform per well. The cells were pipetted up and down 5-10 times to mix the reagents. 50µL ONPG (4mg/mL) were then added and after 15 min the reaction was terminated with 100µL Na_2CO_3 (1M). For OD measurement at 402nm we took one 100µL aliquot from each well.(Griffith and Wolf 2002)

6.2.3 In vivo Crosslinking Technique

6.2.3.1 Harvesting of E. faecalis

The main culture was inoculated with ONC to an OD of 0,05, grown for two hours at 37°C and harvested in aliquots of an OD of 5 for in vivo crosslinking. For in vivo crosslinking we grew them another 2 hours, also harvesting an OD of 5. Aliquots were centrifuged at 4000rpm for 10min. After discarding the medium, the pellets were frozen in liquid nitrogen and stored at -20°C.

6.2.3.2 In vivo crosslinking

The pellet was resuspended in 5mL sodium phosphate buffer and aliquoted at a volume of 1mL. After centrifugation for 30 seconds the supernatant was discarded. Pellets were resuspended in 1 mL sodium phosphate buffer for the control, 0,5% paraformaldehyde sodium phosphate buffer, 1,0%, 1,5%, 2% and 2.5% paraformaldehyde sodium phosphate buffer. Reactions were incubated for 7 min at 42°C with gentle shaking and then centrifuged at 13200rpm for 1min. The supernatant was discarded. The pellet was resuspended in 1mL 1,25M Glycine in sodium phosphate buffer and incubated for 3min at room temperature., followed by centrifugation for 1 min at 13200 rpm and discarding of the supernatant. Pellets were washed with 1mL sodium phosphate buffer and centrifuged. After discarding the supernatant, pellets were stored at -20°C for Western Blotting. (Klockenbusch and Kast 2010)

6.2.3.3 Lysis and SDS-Page

The *E. faecalis* pellet for in vivo crosslinking was resuspended in 200µL NaOH (1M) and incubated for 10min and 13450 rpm at 42°C. Afterwards it was spun down and the pellet resuspended in 200µL 5x Laemmli buffer without β-mercaptoethanol and incubated for 10min and 13450rpm at 65°C. 20µl of this suspension was prepped on a 10-15% acrylamide SDS-gel. The bands were separated in the gel at 180V for approximately 50min.

6.2.3.4 Western Blot

For Western Blotting we used a PVDF membrane, which were equilibrated 10 sec in methanol and 5 min in Transfer buffer. Proteins were blotted for 35 min at 220mA.

6.2.3.5 Detection

After blotting, the membrane was incubated for 1 hour for blocking with 5% milk powder or 3% BSA. Incubation with the first antibody was done overnight followed by 4 washing steps with TBS buffer. The membrane was incubated for 1h with the secondary antibody and then rinsed 4 times with TBS buffer. For detection we used ECL as described by the manufacturer and detected with the ChemiDoc.

6.2.3.6 Stripping

The stripping buffer was warmed to 50°C and added to a small plastic box with a tight lid to a volume that would cover the membrane. The submerged membrane was incubated at 50°C for up to 45 minutes with some agitation and then washed multiple times with TBS until no β-mercaptoethanol could be detected via smell-test. The membrane was then incubated with blocking solution before probing it with the next antibody.

6.2.4 RNA-Analysis

6.2.4.1 Harvesting of E. faecalis

The main culture was inoculated with ONC to an OD of 0,05 and grown for two hours at 37°C and harvested in aliquots of an OD of 1 for RNA isolation. Aliquots were centrifuged at 4000rpm for 10min. After discarding the medium, the pellets were frozen in liquid nitrogen and stored at -20°C.

6.2.4.2 RNA-Isolation

The pellet normalized to an OD of 1 was supplied with 200µL glass beads and 500µL TRIzol-Reagent (Thermo Fischer Scientific). The cells were cracked in the PowerLyzer for 1min at 3000rpm; this was done three times with a 1 min break on ice each time. To get rid of the glass beads, the suspension was centrifuged and transferred into a fresh Eppendorf tube. It was incubated for 5 min at room temperature and after adding 100µL BCP (1-bromo-3-chloropropane) was shaken for 15 seconds. After 3 min of incubation at room temperature the suspension was centrifuged at 4°C at 12000rpm for 15min. The upper colorless phase was transferred into a fresh tube. For isolation, the RNA was incubated with 250µl 100% isopropanol for 10min at room temperature, followed by centrifugation at 4° and 12000rpm

10min. The supernatant was removed and the pellet washed with 500µL 75% Ethanol and vortexed briefly, followed by centrifugation of the RNA at 4° and 7500rpm for 5min. (Chomczynski and Sacchi 1987) The supernatant was removed and the pellet dried in the SpeedVac for 5min. Afterwards the pellet was resuspended in RNAse-free water and incubated at 55°C for 10min to dissolve the pellet. The RNA concentration was determined via NanoDrop and also on a control agarose gel with a TAE buffer containing 0.3% sodium hypochlorite. (Aranda, LaJoie and Jorcyk 2012)

6.2.4.3 DNA Removal and Reverse Transcription

We used the Thermo-Scientific DNA Removal Kit and followed the procedure for routine DNase treatment. After DNA removal we transcribed our RNA into cDNA using the M-MLV-Reverse Transcriptase RNase H from SOLIS BioDyne, following the manufacturer's protocol.

6.2.4.4 Quantitative Real Time PCR

For expression analysis we used quantitative real time PCR. We used the 5x Hot FIREPol EvaGreen qPCR Supermix from SOLIS BioDyne, sticking to the recommended protocol. For each reaction we used 100ρmol of every primer and 4µl of cDNA.

7 Results

7.1 Interactions of the Tra-proteins using Bacterial two hybrid

We do not know much about the protein-protein interactions of the different Tra-proteins. However, new insight may offer clues towards the composition of the core complex and function of the different proteins. Therefore, we wanted to find out which of them were able to interact using Bacterial two hybrid assay. The biggest advantages of the B2H are quantitative analysis additional to the qualitative approach, so there are less false positive results.

First, we started to test the full Tra-proteins against other full Tra-proteins. Previous studies uncovered interactions (Kohler et. al unpublished data), which we wanted to reproduce. Further we wanted to find interactions between the full proteins and different domains of the other interacting protein. Hence we divided the following tra proteins in the following domains:

Tra-Protein	domains	length (bp)
TraF		1353
	- N-terminal	600
	- membrane	117
	- C-terminal	211
TraG		1110
	- membrane	150
	- SLT_link	518
	- CHAP_link	466
TraI		432
	– sol_TM1	102
	– TM2	155
	– soluble	174
TraK		924
	– cytmem	198
	– extracellular	725
TraM		969
	– N-terminal	117
	– membrane	93
	– C-terminal	108

We cloned them into the 4 provided vectors of the Bacterial two hybrid and tested all of them as both N-terminal and C-terminal fusions and both as bait and prey. All Tra-protein-domains plus positive (Leucine-zipper fused to adenylate cyclase fragments) and negative (fragments of

adenylate cyclase without fusion-protein) control was co-transformed into bacteria lacking endogenous adenylate cyclase activity. In the qualitative approach, we could differentiate the strength of interaction by the different shades of blue. To exclude false positive results, we did not only divide between blue and white colonies. Additionally, we carried out a β-galactosidase assay. After performing the LacZ assay, we calculated the Miller Units (per OD_{600} as well as per milligram cell dry weight). To simplify matters, we normalized all measurements to the positive control. Only those Tra-protein combinations that showed both blue colonies on X-gal agar plates and significant higher β-galactosidase levels than the negative control were considered.

First, we started testing the full-length proteins against other full-length proteins to find out in which position the interaction takes place. Afterwards we could decide which part of the whole protein to use to find the domain interactions. Then we could start with testing the whole proteins against the different domains.

7.1.1 TraF-TraF Interaction

We found that TraF interacts with other molecules of TraF, so we tested the different TraF domains against the whole TraF protein to find possible interactions.

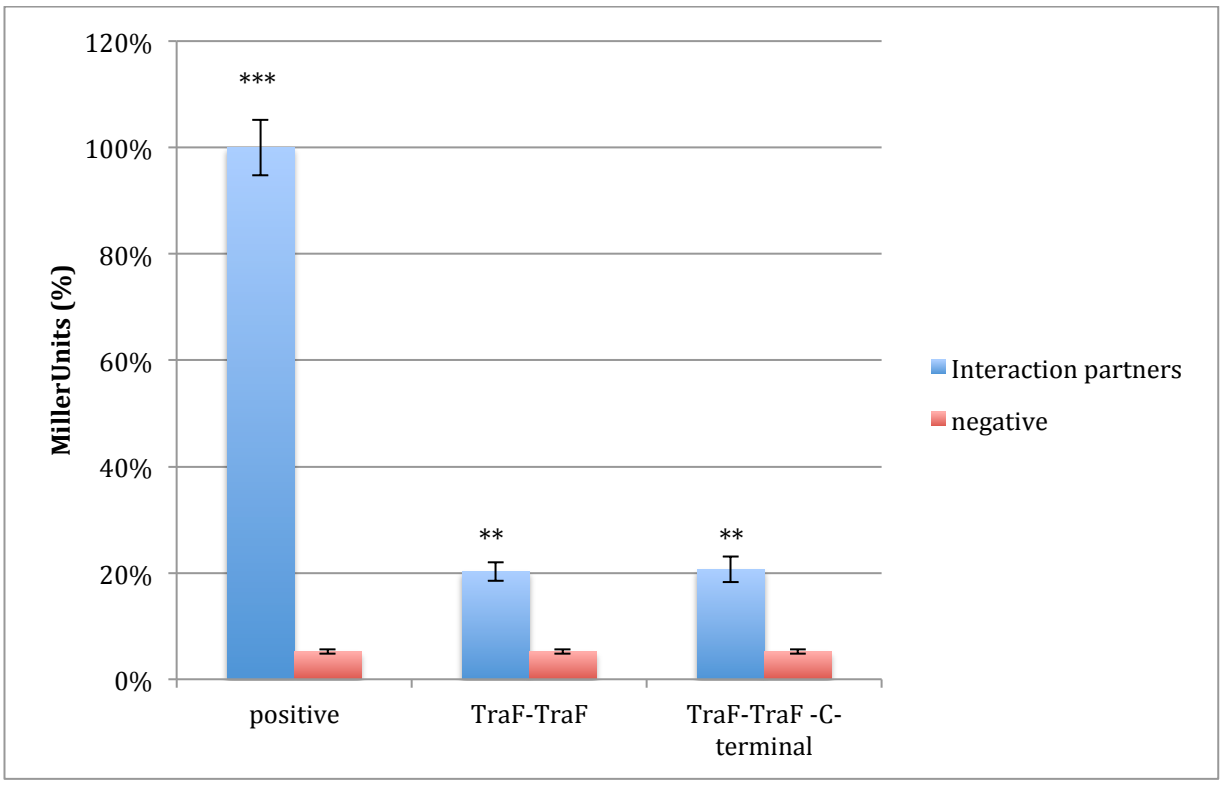

Figure 5: Quantitative interaction studies of TraF-protein. TraF and TraF domain combinations with a significant interaction signal compared to the negative control are depicted. (one way ANOVA,

bonferroni post-hoc test in IBM SPSS or student t-test in IBM SPSS) 16-fold determination of each clone. (All interaction-negative variants are shown in the attachment) This declaration applies to Figure 6 to Figure 19.

There is an interaction of the whole TraF against its C-terminal domain, which is significant. No significant level of interaction was found for the other TraF domains. (Fig.5)

7.1.2 TraF-TraM Interactions

We found interactions of TraM and the TraF and decided to test the domains of TraF against TraM and vice versa.

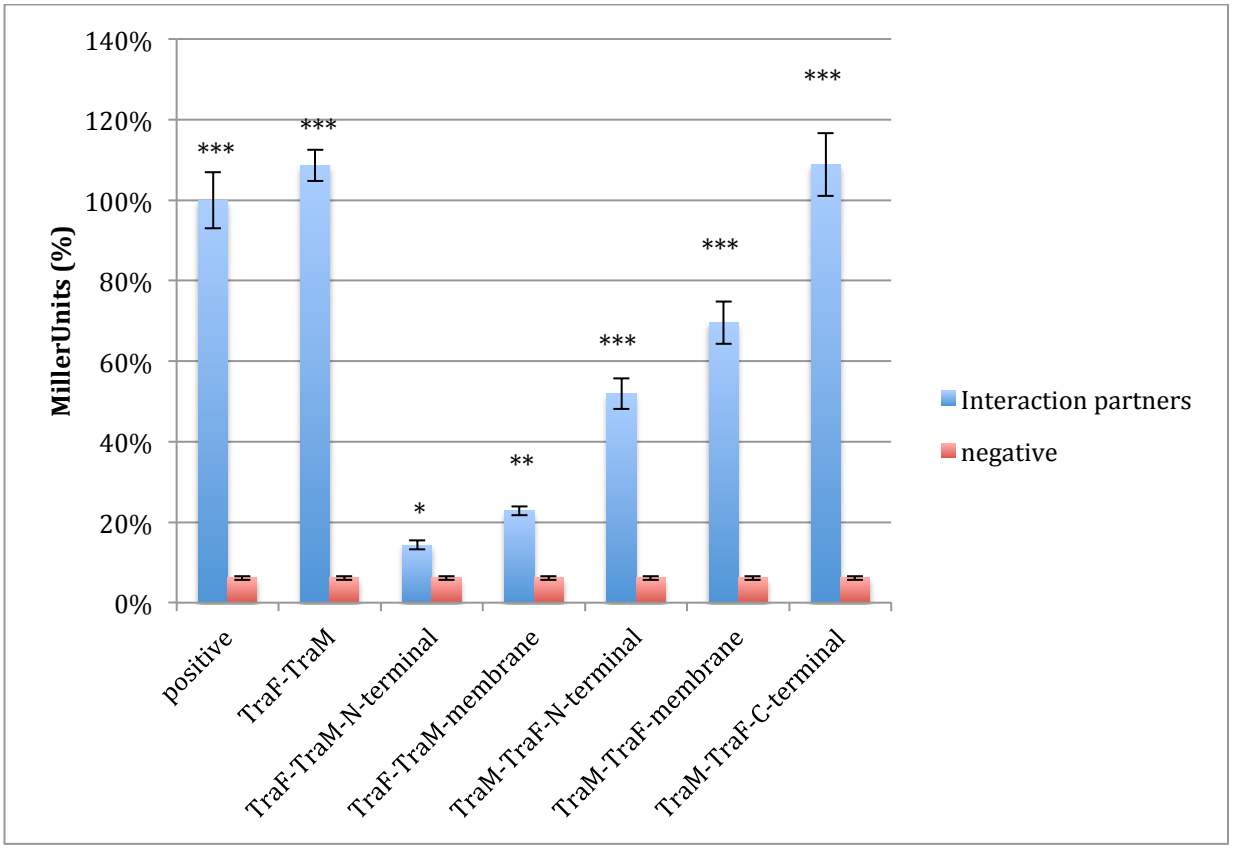

Figure 6: Quantitative interaction studies of TraF.

We found that the N-terminal and the membrane part of the TraM protein are interacting with the whole TraF. Vice versa all parts of TraF, the N-terminal, membrane and C-terminal, are interacting with the whole TraM protein. (Fig.6)

7.1.3 TraG-TraG Interactions

We found TraG-TraG interaction, which has been demonstrated before (Kohler et al unpublished Data). From structural characterizations we propose that TraG is a tetramer – some type of interaction is likely to take place. To find out which domains are involved, we tested it.

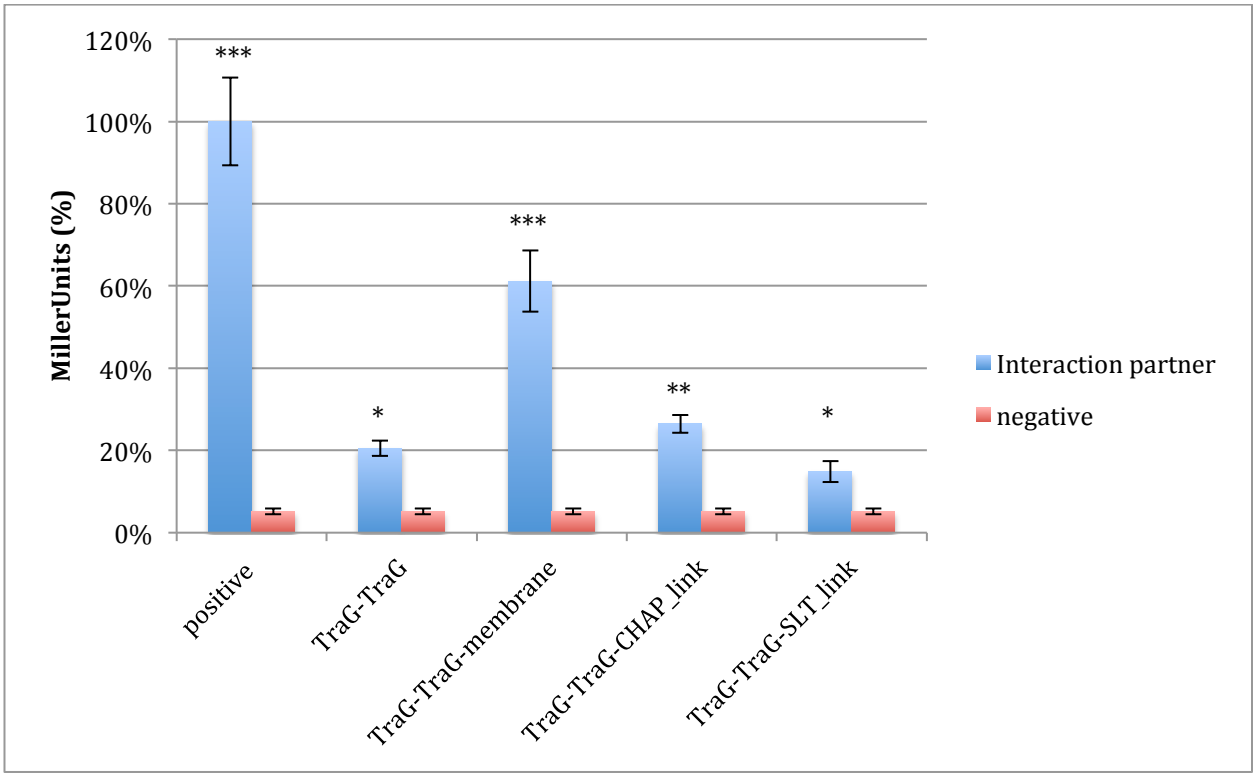

Figure 7: Quantitative interaction studies of TraG-protein.

Suprisingly, all domains of TraG seem to be interacting with the full-length protein to varying degrees. (Fig.7)

7.1.4 TraG-TraM Interactions

Those interactions were found before in a Yeast two hybrid study and were also suspected as part of the core complex. Having been able to reproduce these results, we began to test the different domains of TraG against the full TraM protein and vice versa.

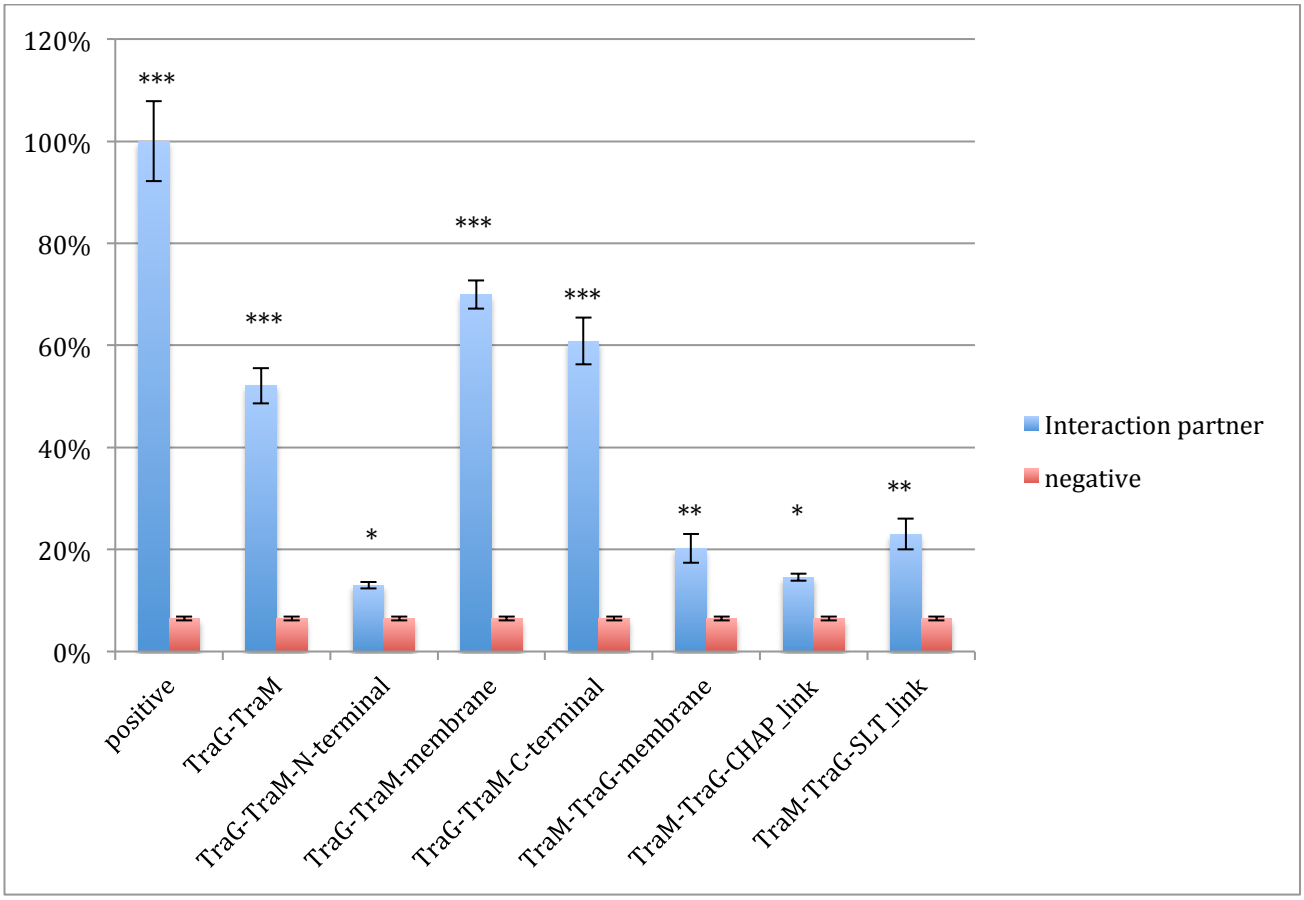

Figure 8: Quantitative interaction studies of TraG.

We were able to show that all TraG domains interact with the full-length TraM protein and all parts of TraM interact with the full-length TraG. This could be a hint that both proteins play a central role in the formation or stabilization of the core complex. (Fig.8)

7.1.5 TraG-TraK Interactions

TraK was also assumed to be part of the core complex, so we wanted to know if it interacts with TraG. We found that there is an interaction and we again started to test all TraG domains against the full-length TraK and the other way around. (Fig.9)

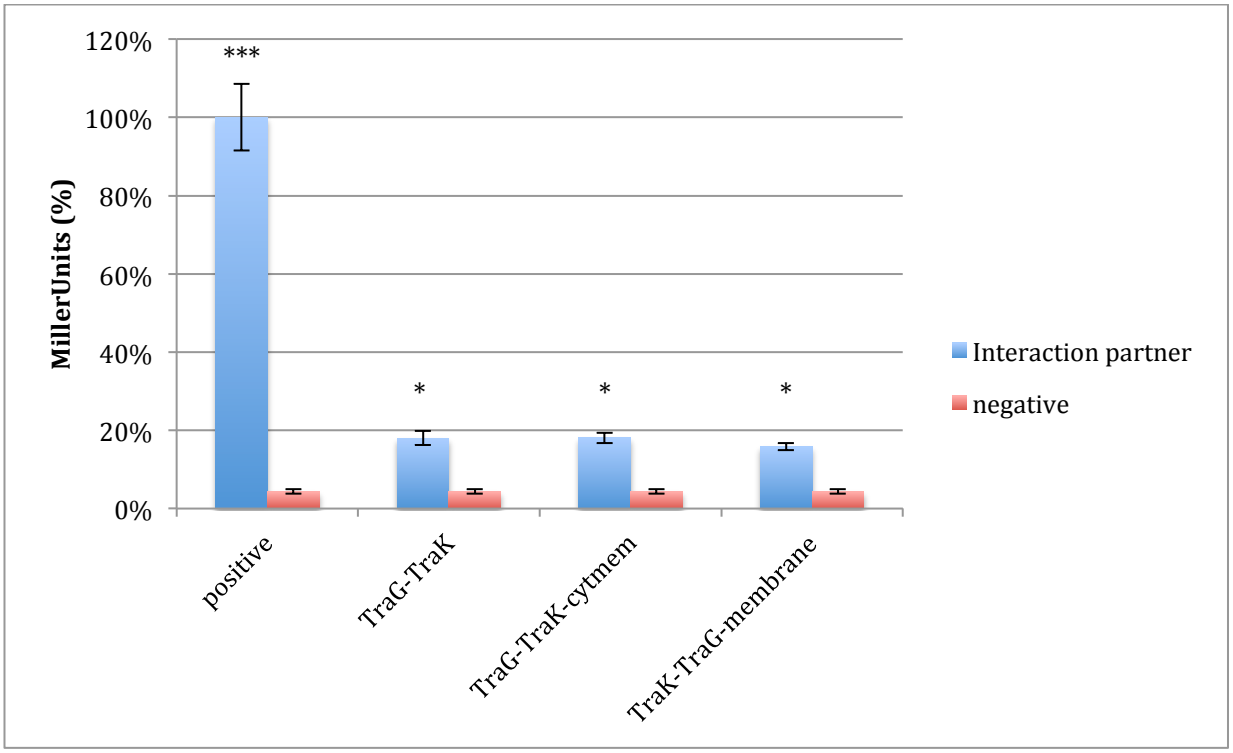

Figure 9: Quantitative interaction studies of TraG-protein.

7.1.6 TraI-TraI Interactions

Interaction between TraI monomers has been shown before, so we looked to expand this data by screening for interactions between the different domains of TraI and the full-length TraI protein.

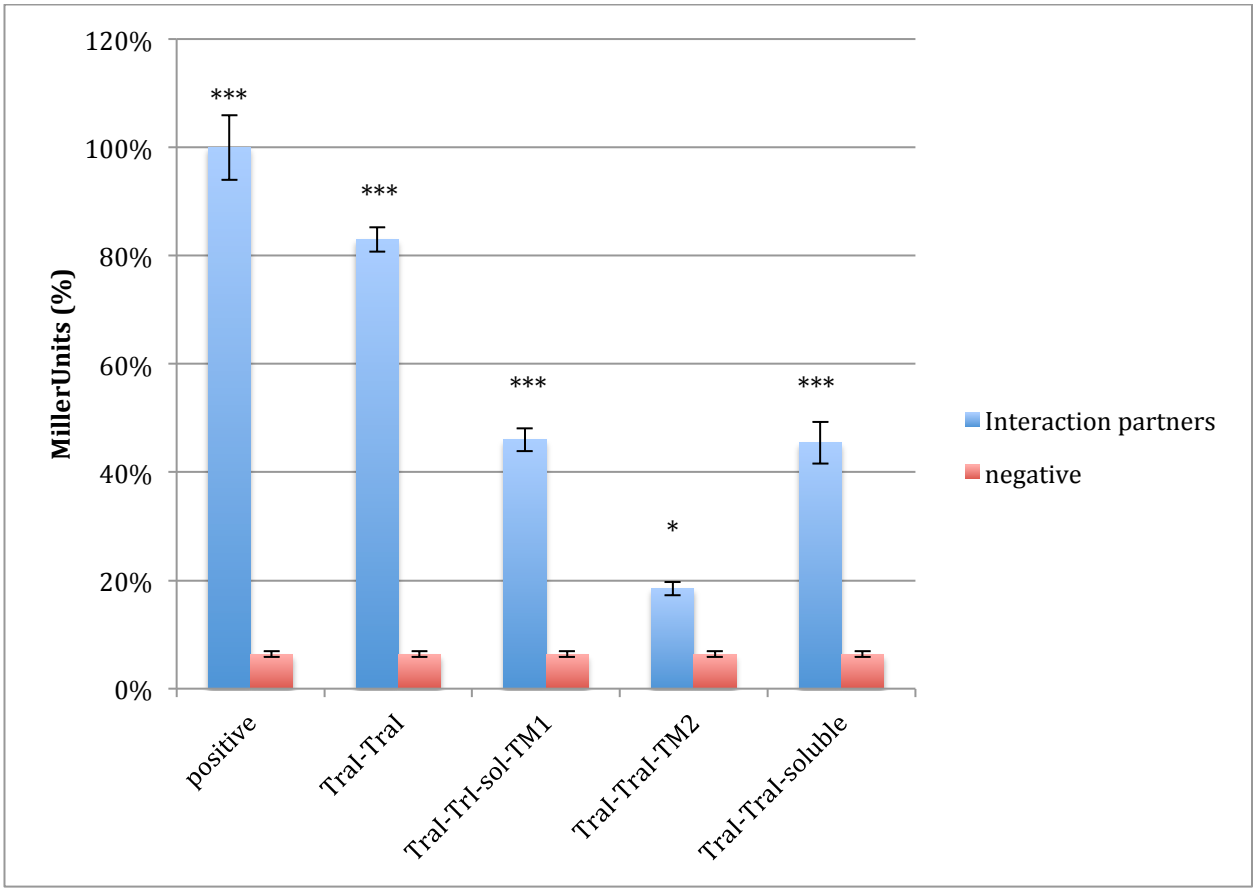

Figure 10: Quantitative interaction studies of TraI.

We found a possible interaction between the TM2 and the full-length TraI as well as with its soluble domain. (Fig.10)

7.1.7 TraI-TraG Interactions

As possible part of the core complex of the T4SS we further tested the interaction between the full-length TraI and TraG, as well as all three of their respective domains.

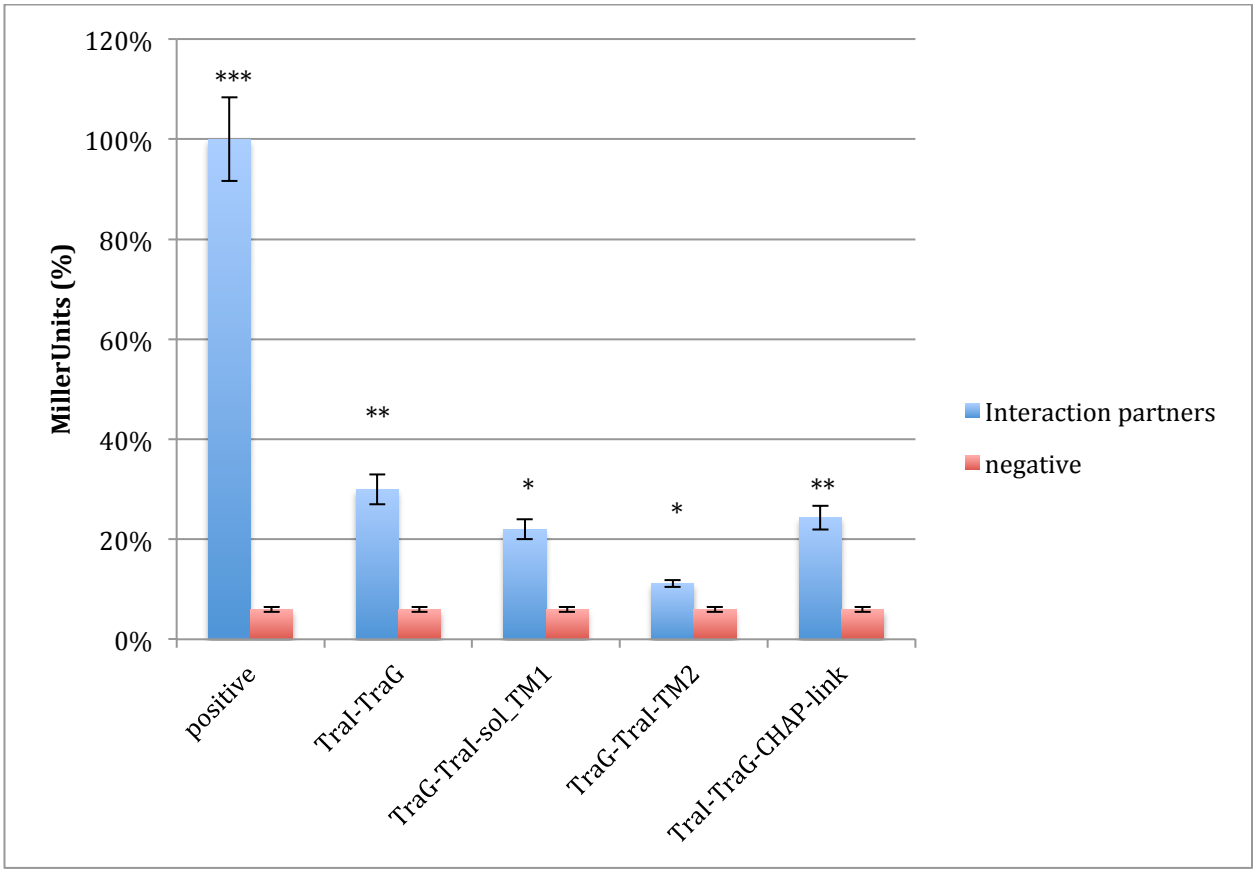

Figure 11: Quantitative interaction studies of TraI and TraG.

We found interactions between TraI and TraG and also between TraG and the sol_TM1 part of TraI and TraI and the CHAP-link of TraG. (Fig.11)

7.1.8 TraI-TraK Interactions

Previously, interaction between TraK and TraI has been shown, so we sought to reproduce these results and further tested the three domains of TraI against TraK and the two TraK domains against TraI.

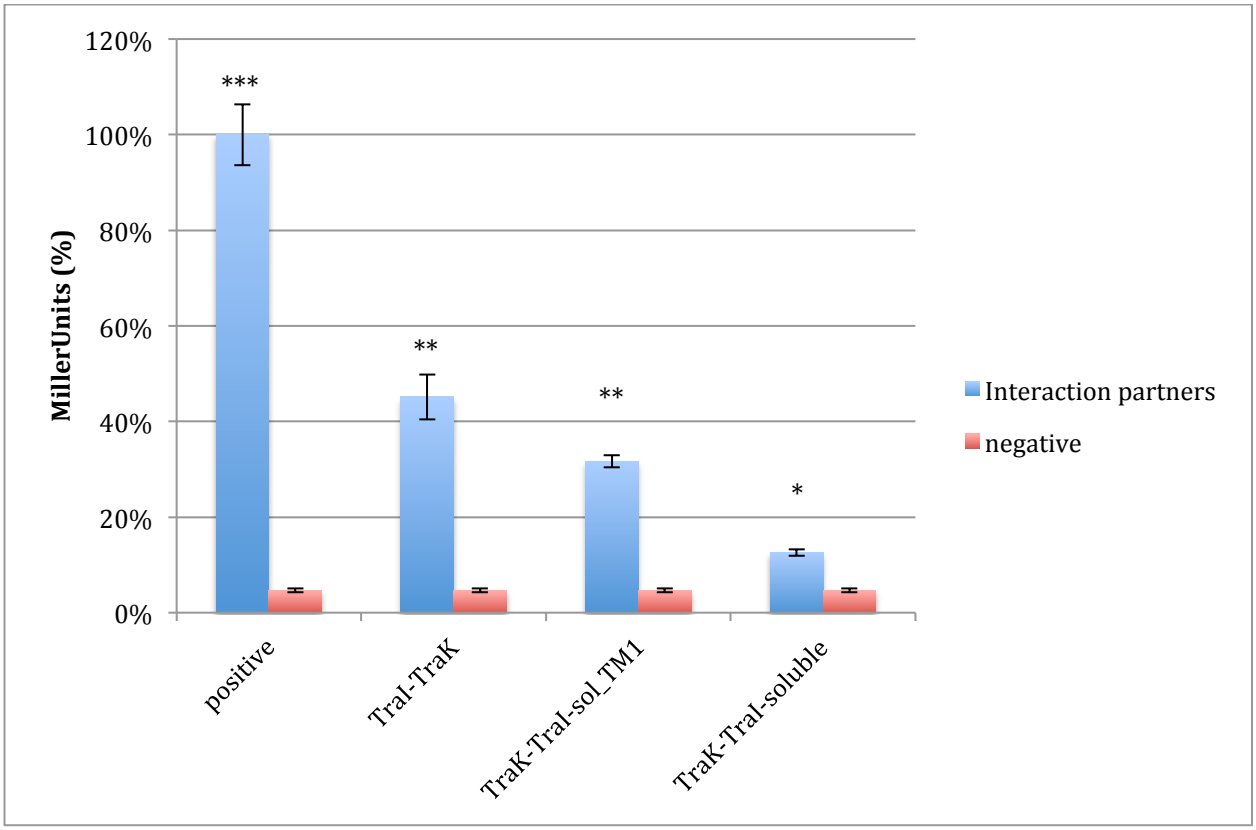

Figure 12: Quantitative interaction studies of TraI and TraK

We found interactions between the soluble TraI part and TraK as well as the TraI sol_TM1 domain and TraK, but no significant interaction between TraK and the TM2 part of TraI. We also found no significant interaction between TraI with any part of TraK. (Fig.12)

7.1.9 TraI-TraM Interactions

As we mentioned before, we suspect TraI as part of the membrane-integrated core complex, so we also tested it against TraM and the different domains of TraM and vice versa.

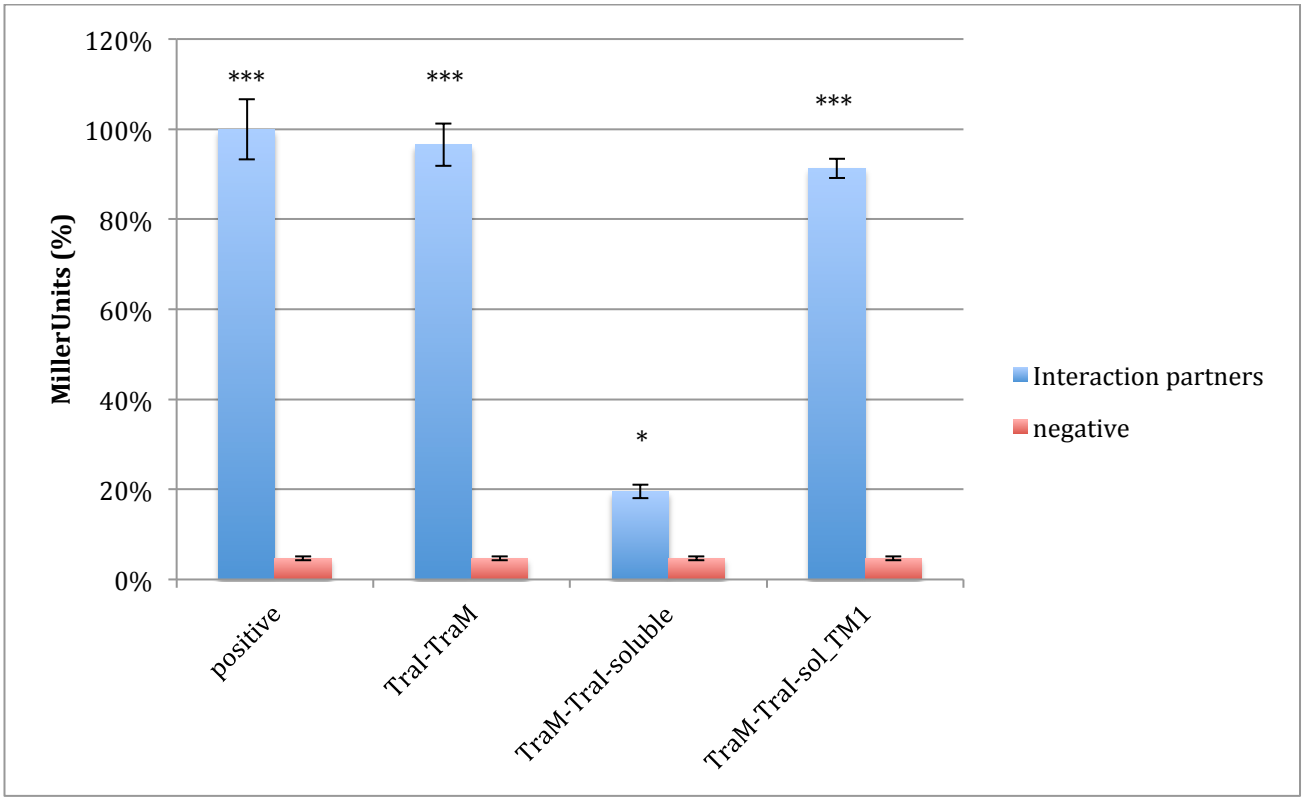

Figure 13: Quantitative interaction studies of TraI and TraM.

We found a highly significant interaction between the sol_TM1 part of TraI and the whole TraM protein, as well as interactions between TM2 and the soluble part of TraI with TraM. So far, we could not find interactions between the domains of TraM and TraI. (Fig.13)

7.1.10 TraK-TraK Interactions

TraK showed interaction with other TraK molecules, which was the reason why we tested it against its own two domains.

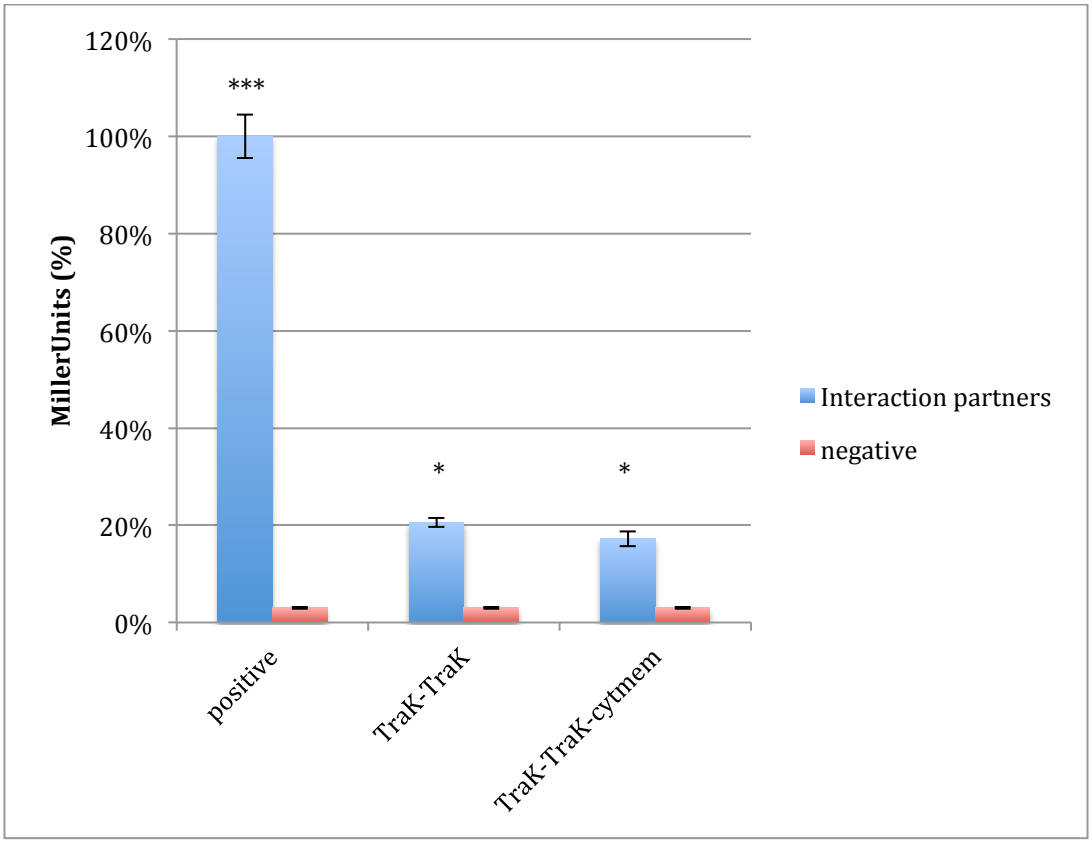

Figure 14: Quantitative interaction studies of TraK.

Interaction occurred between the cytoplasmic membrane domain and the whole TraK protein. All other options tested negative. (Fig.14)

7.1.11 TraK-TraM Interactions

As we found a highly significant interaction between TraK and TraM, we tested all TraK domains against TraM and vice versa.

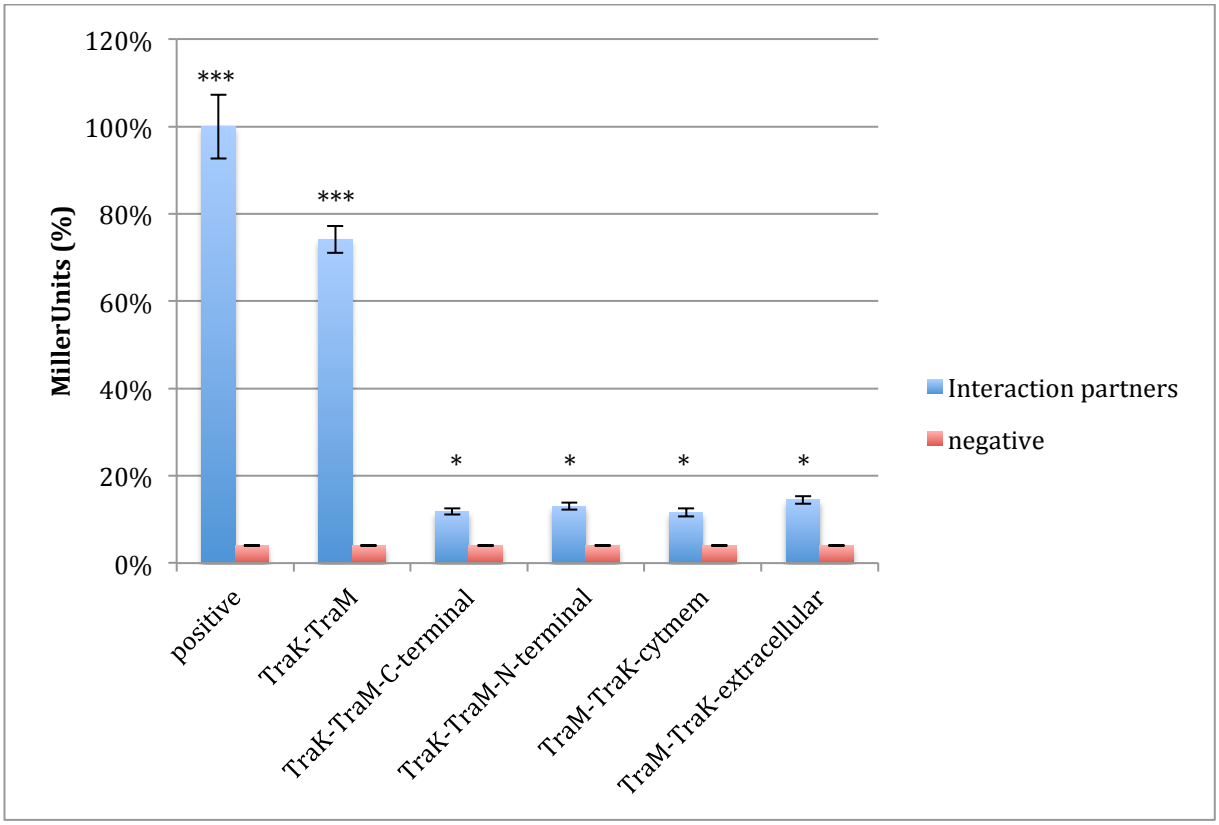

Figure 15: Quantitative interaction studies of TraK and TraM.

We found interactions between TraK and the N-terminal and C-terminal part of TraM, but no interaction with the membrane part of TraM. TraM showed interactions with both parts of TraK, the membrane-integrated and the extracellular part. (Fig.15)

7.1.12 TraM-TraM Interactions

TraM has been thought to form a tetramer in the membrane. Unsurprisingly, we found a significant interaction between TraM molecules, which lead us to screen for interactions between the full-length TraM and its individual domains.

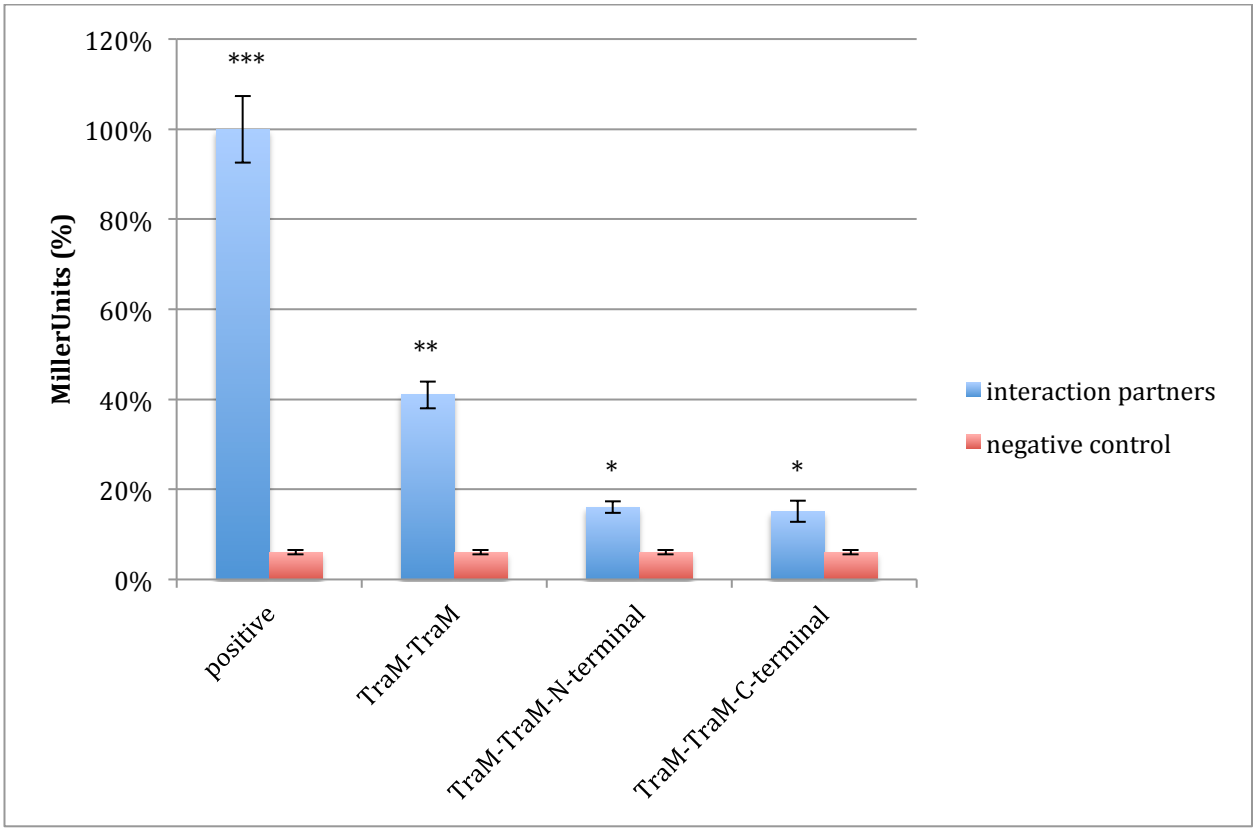

Figure 16: Quantitative interaction studies of TraM-protein.

We found interaction of the full-length TraM with all three parts of TraM – the N-terminal, the C-terminal and the membrane part. (Fig.16)

7.1.13 TraJ Interactions

TraJ did not only show interaction with other TraJ molecules, but also with TraI and other Tra-proteins. Because of this we tested it against the domains of TraI and found interactions with the TM2 part of TraI, but not with the sol_TM1 or the soluble part. (Fig.17)

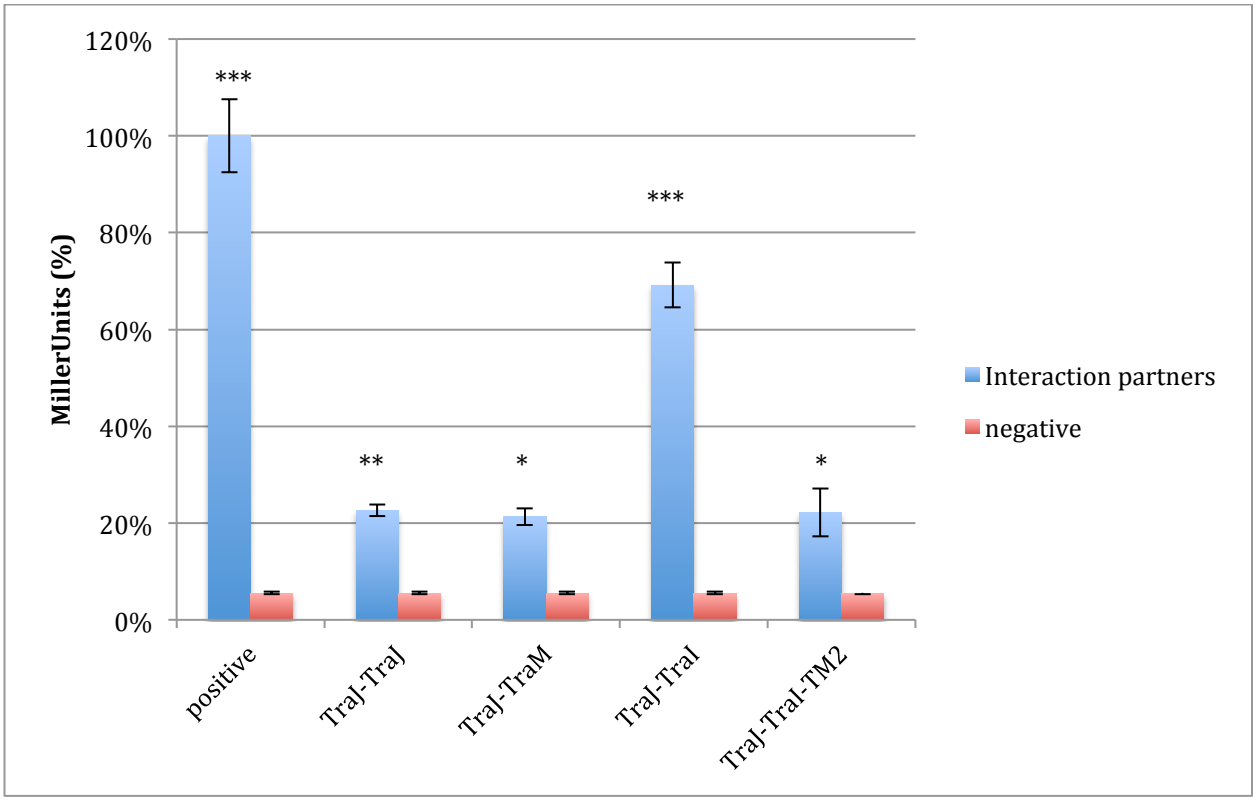

Figure 17: Quantitative interaction studies of TraJ-protein.

As mentioned before, we found that TraJ has an additional interaction partner in TraM. As such, we tested all domains of TraM against TraJ. We found no significant interaction between TraJ and the domains of TraM – all variants tested negative.

7.1.14 TraL-Interactions

On the basis of the structural shape of TraL and its relation to VirB6, we thought it could play a role in the formation or the stability of the core complex, which is the why we tested it not only against itself, but also against other Tra proteins. We could demonstrate interactions between TraL molecules, as well as between TraL and TraG and other Tra proteins.

Another interaction partner of TraL is TraK – both TraK domains were tested against TraL. Last but not least we found interactions between TraL and TraM, causing us to test the TraM domains against the full-length TraL. (Fig.18)

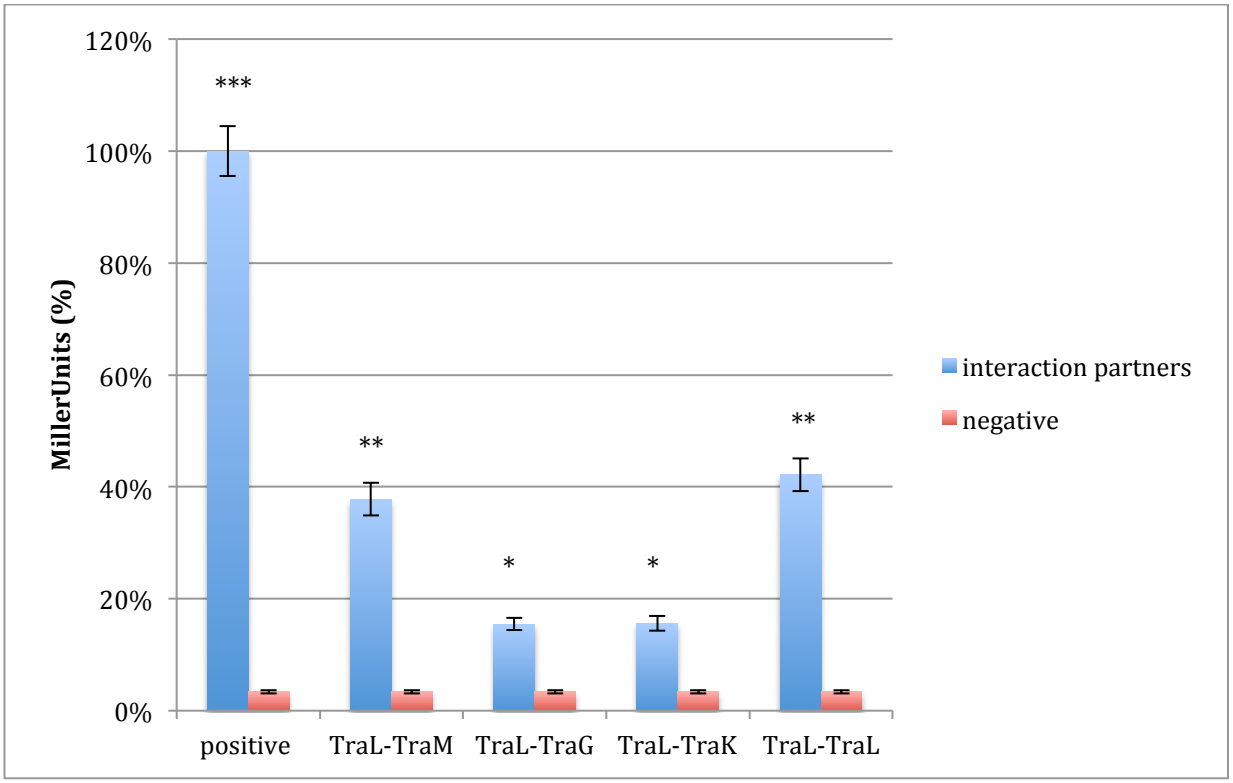

Figure 18: Quantitative interaction studies of TraL-protein.

7.1.15 TraD and TraE Interactions

We further found interactions between TraD and TraD as well as TraE and TraD and among TraE molecules. (Fig.19)

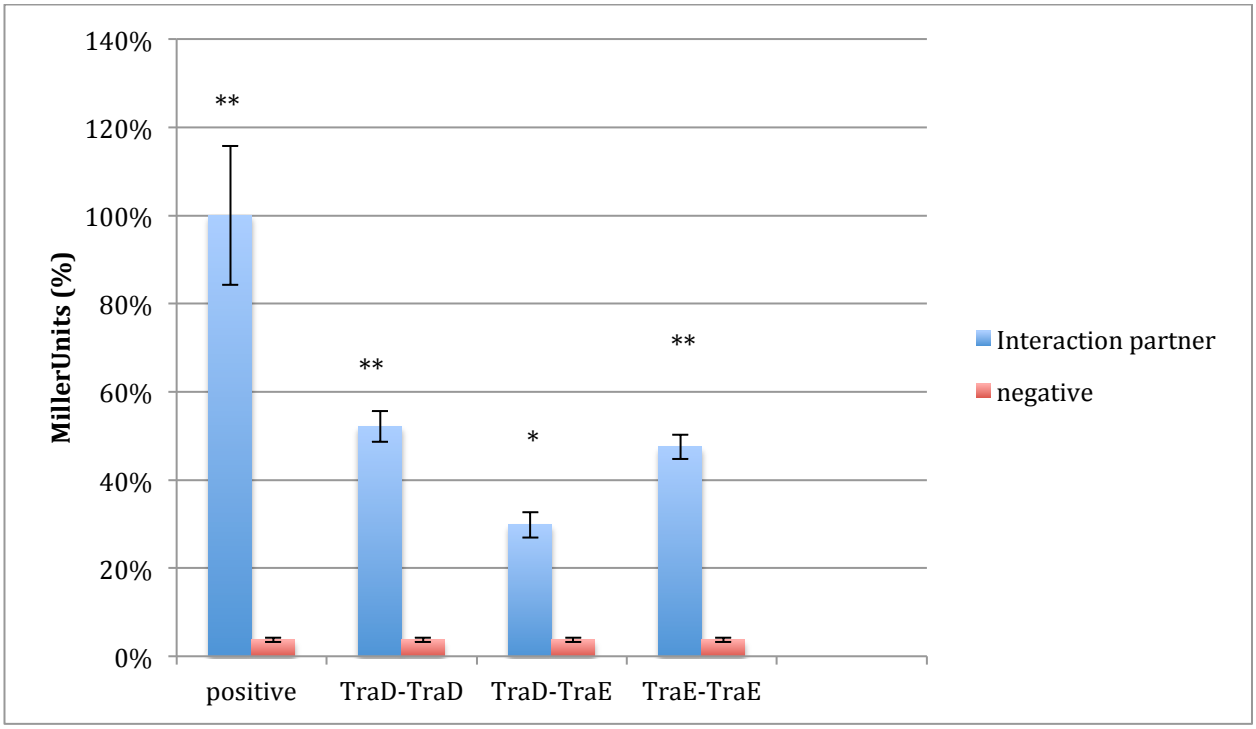

Figure 19: Quantitative interaction studies of other TraD and TraE proteins.

7.2 In vivo Crosslinking

To further verify the interactions of the tra proteins demonstrated in the bacterial two hybrid, we wanted to replicate them directly in *Enterococcus faecalis* using in vivo crosslinking. The bacterial two hybrid is a robust assay, but it is done in the gram-negative *E.coli*, making it a bit artificial. Hence we wanted to reproduce these interactions in *Enterococcus faecalis*, not only because it is a Gram-positive bacteria; it is also a natural host of the pIP501.

As the crosslinking agent we used para-formaldehyde in different concentration levels to see a shift from the monomeric protein to the dimer or different interaction partners and oligomers. The small molecular weight of para-formaldehyde allows us to selectively crosslink only closely associated proteins and to stabilize protein-protein interactions in our host cell. Another advantage of formaldehyde is the high permeability, which makes it possible to use intact *Enterococcus faecalis* cells for our experiments. With Western blotting techniques and *tra*-specific antibodies we hope to get a detailed interaction map of all proteins from the *tra*-operon. We do not use reducing techniques for our crosslinked probes, because these could possibly harm our interprotein-links. (Klockenbusch, O´Hara and Kast 2012)

As negative control we use *E. faecalis* cells not harboring the pIP501, one time treated with para-formaldehyde and once without treatment. As additional control we use *E. faecalis* cells

harboring the pIP501 not treated with para-formaldehyde.

7.2.1 In vivo crosslinking of TraK

During our B2H assay we found interactions between TraM and TraK, which we wanted to show directly in *E. faecalis*.

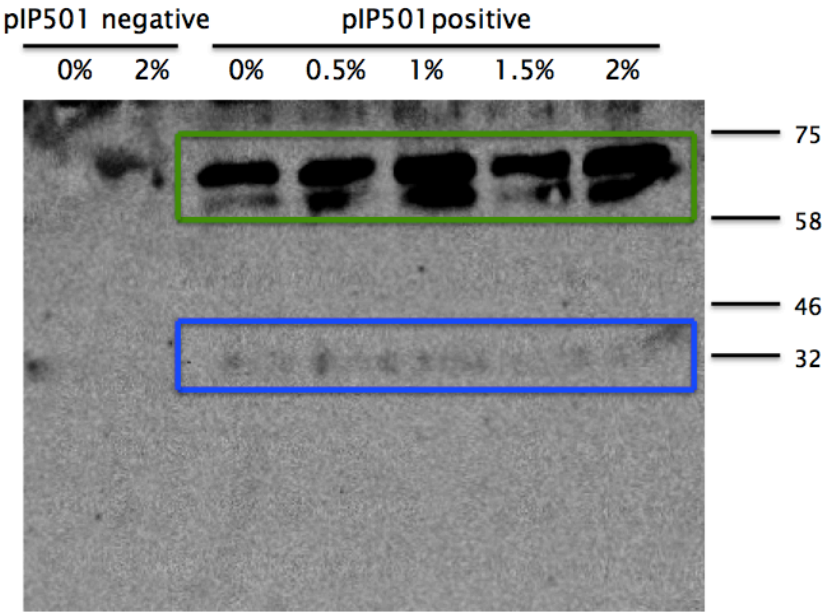

Figure 20: In vivo crosslinking of TraK (32kDa). TraK was probed with specific antibodies. The bands in the blue box represent the monomeric form of TraK, the bands in the green box show higher molecular, crosslinked forms. pIP501 negative = *Enterococcus* cells not harbouring pIP501. Crosslinking between 0.5% and 2% formaldehyde.

The higher molecular bands of TraK (Fig.20) could be the dimeric form of it, which is about 64kDa. There could be an interaction with TraM (70kDa), which might explain the second bigger band (green box). We also found TraK-TraK and TraK-TraM interactions in other complementary interaction studies and in the B2H, making either variant seem plausible. Stripping and reprobing with a TraM-antibody did not give viable results.

7.2.2 In vivo crosslinking of TraN

We attempted in vivo crosslinking for TraN, because there were previously shown interactions, which we were unable to reproduce in the B2H experiments.

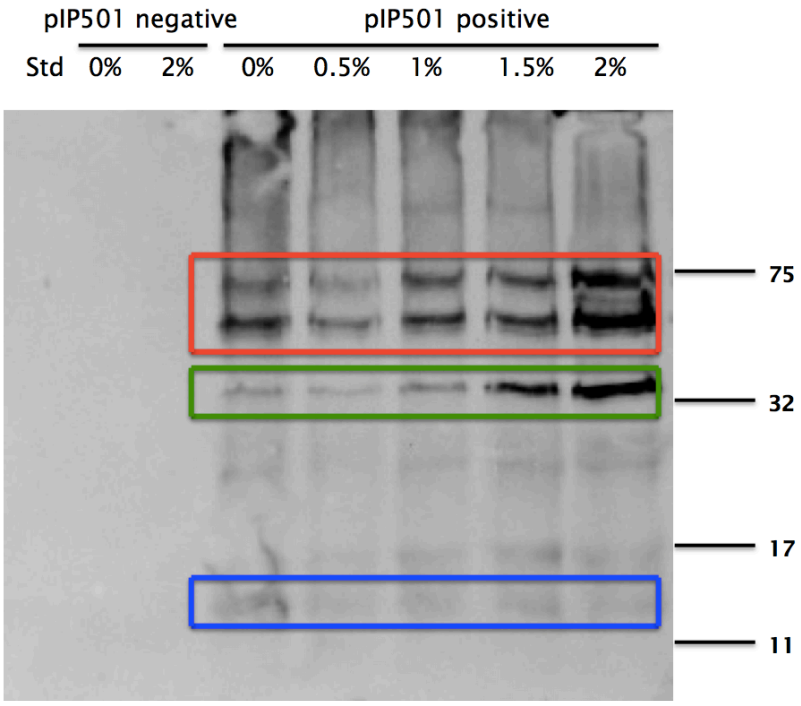

Figure 21: In vivo crosslinking of TraN (13kDa). TraN was probed for with specific antibodies. The bands in the blue box represent the monomeric form of TraN, the bands in the green and red box show possible oligomeric forms of TraN. pIP501 negative = *Enterococcus* cells not harboring pIP501. Crosslinking between 0.5% and 2% formaldehyde.

TraN shows several crosslinked bands (Fig.21), which may correspond to a di-or trimeric form (green box) or interactions with other *tra*-proteins (red box). The band at the height of approximately 75 kDa may be an indicator for an interaction of TraN with TraJ (61kDa), which was first shown in a yeast-2-hybrid assay.(Abajy, Kopec et al. 2007) To verify this, we stripped the membrane and reprobed it with a TraJ-specific antibody. (Fig.22)

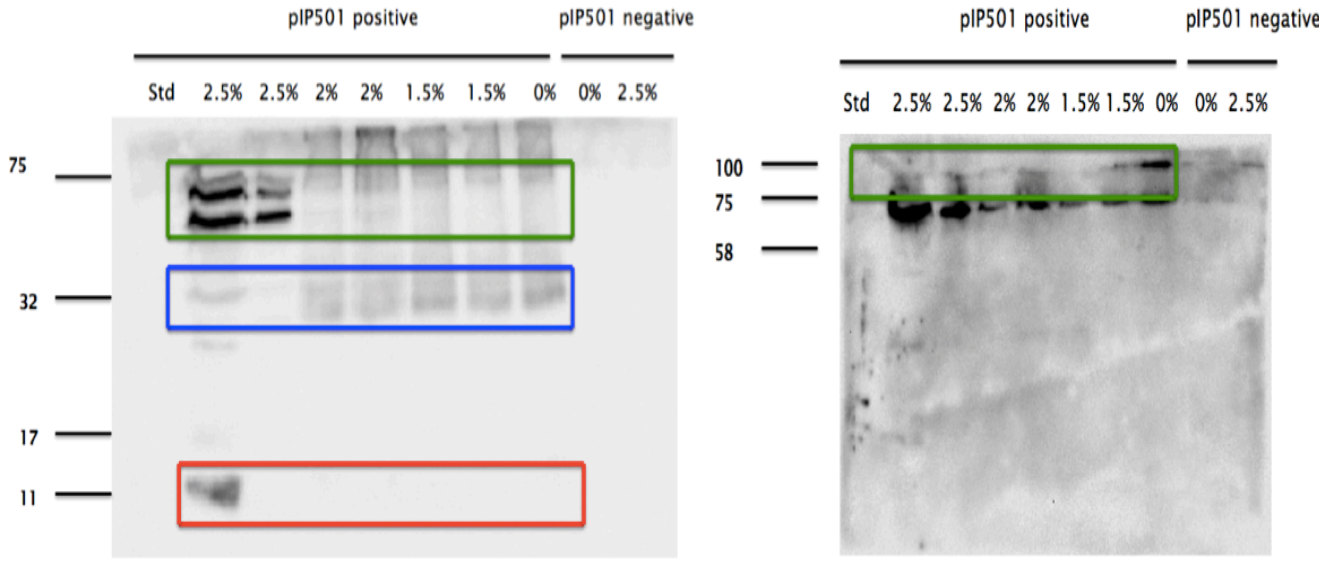

Figure 22: In vivo crosslinking of TraN (13kDa) and TraJ (61kDa). Left: TraN was probed for with specific antibodies. The bands in the red box represent the monomeric form of TraN, the bands in the green and blue box show possible oligomeric forms of TraN. Right: In vivo crosslinking of TraJ (61kDa) after stripping and reprobing of the membrane. TraJ was probed for with specific antibodies. The bands in the green box represent the possible interaction between TraN and TraJ. pIP501 negative = *Enterococcus* cells not harbouring pIP501. Crosslinking between 0.5% and 2.0% formaldehyde.

We were able to again detect a protein band pointing towards a possible interaction between TraN and TraJ. (Fig.22) To ensure that this was not an artifact, we wanted to analyze it with mass spectrometry, which did not work out.

7.3 Expression Levels of the different Tra-Proteins

For quantitative real time PCR total RNA from wild type *Enterococcus faecalis* pIP501 cells was isolated and reverse transcribed to cDNA to measure the different expression levels of the Tra-proteins. Theoretically, the expression level of all should be the same, because the tra-operon (Fig.23) is transcribed and expressed polycistronically.

Figure 23: *Tra*-Operon of the conjugative model plasmid pIP501. (Provided from research group Walter Keller)

Possible differences between the expression levels of the Tra-proteins could mean that the mRNA takes on secondary structures that influence the transcription level.

7.3.1 Expression of the Wildtype proteins

To determine the transcription level, we performed quantitative real time PCR. We obtained the same level for all proteins, because they are expressed polycistronically.

Figure 24: Expression analysis on a transcriptional level. For all *tra* genes, specific primers were used.

All proteins showed the same or nearly the same expression level. (Fig.24) We were unable to demonstrate any influence of secondary structures on the expression levels. To get further information about the transcriptional levels of the proteins, we started to use different *tra* gene knockout *E. faecalis* pIP501 strains and compared their *tra* operon mRNA expression levels to the wildtype.

7.3.2 Expression of the delta TraN knockout mutant compared to the Wildtype

We suspect that TraN acts as repressor for the *tra* operon (Fig.25), as Footprinting assays revealed the *oriT*-proximate location of its specific DNA-binding site. It may even be an accessory protein of the relaxase TraA. (Goessweiner-Mohr, Eder et al. 2014)

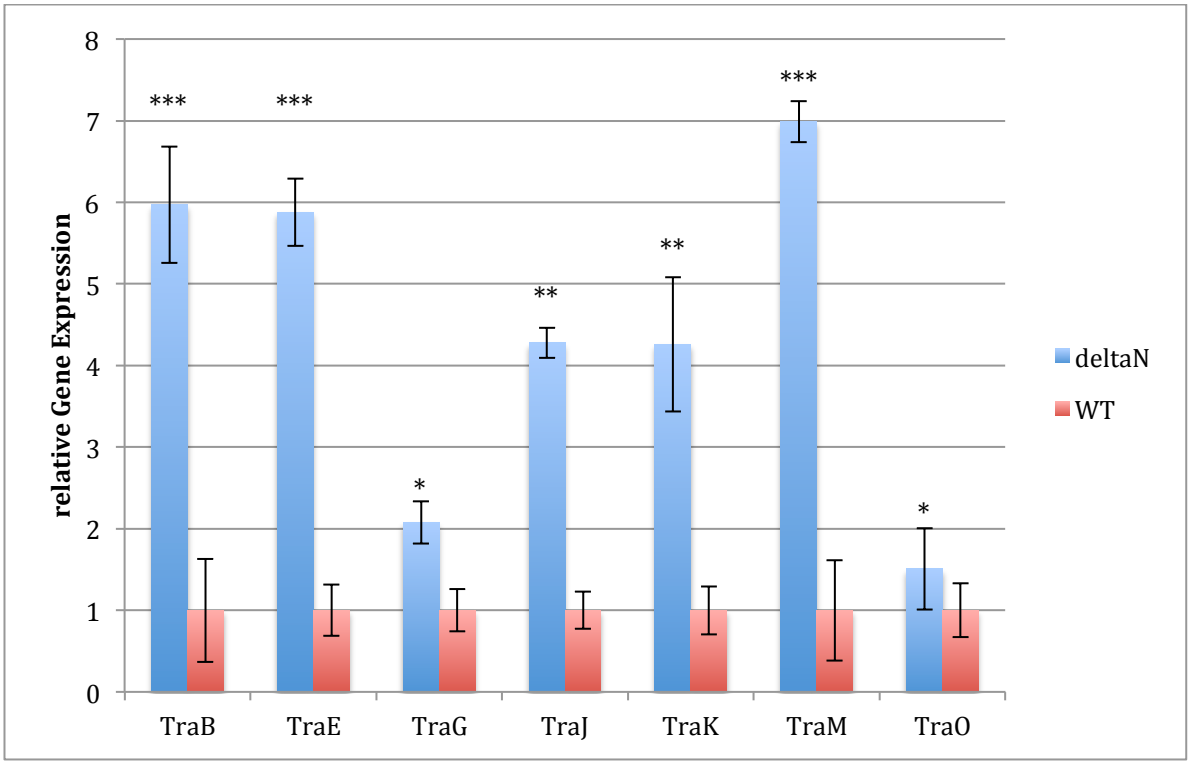

Figure 25: Expression analysis on a transcriptional level – normalized to GAPDH. For all *tra* genes, specific primers were used. For normalization we used the housekeeping gene GAPDH. All *tra* genes show an overexpression in the Δ*traN* mutant compared to the wild type. WT=Wildtype

We found strong evidence that TraN may have a repressor function targeted at the pIP501 conjugative model plasmid's *tra* operon, because all *tra* genes were overexpressed in the pIP501Δ*traN* strain compared to the wild type.

8 Discussion

8.1 Novel protein-protein interactions between the transfer proteins of pIP501

The bacterial two hybrid assay has some notable advantages over other interaction assays, making it a viable method to investigate interactions between bacterial proteins. It can be used for membrane proteins, it is a bacterial system and no auto-activators have been found thus far.(Battesti and Bouveret 2012) Our B2H studies have provided us with new insight into the interactions between transfer proteins of pIP501. On the basis of the generated data, we propose a potential scenario for a protein-protein interaction map of the pIP501. Not only have we found novel protein-protein interactions, but also between single domains of the tra-proteins.

TraM, the translocation channel protein, is membrane-associated and surface-exposed. We propose that it is an important part of the secretion apparatus, as well as part of the core complex. Not only because of its affinity to the cell envelope – TraM also showed structural similarity to the VirB8 protein and has a transmembrane motif. A key role for VirB8 homologues as a putative cell wall spanning component has been postulated. Based upon the structure similarities, TraM may exhibit a scaffolding role. (Waksman and Fronzes 2010) However, there was no evidence for its involvement as part of the core complex previously. In a yeast two hybrid and a pull-down assay, TraM showed no significant interactions. (Goessweiner-Mohr, Grumet et al. 2013, Abajy, Kopec et al. 2007). As such, discovering its interaction with the TraG protein, which is also suspected as a major part of the membrane-integrated core complex, came as a surprise. We also found domain interactions for both proteins. Of the pIP501-encoded *tra* operon, these are the only two proteins whose respective domains all interact with the full-length proteins. This could hint that both play a vital role in the formation of the secretion apparatus. Even more surprising was the fact that TraM showed interactions with nearly all putative core complex Tra-proteins, such as TraF and its N-terminal domain. TraM also showed interactions with itself, precisely with its N-terminal and C-terminal domain. TraM further interacts with TraI and TraJ, another VirB/VirD4 homologue.

TraJ is a VirD4-like, putative coupling protein and could be aided and brought to the cell membrane by TraI. It also has ATPase activity. (Goessweiner-Mohr, Arends et al. 2014, Alvarez-Martinez and Christie 2009) We found interactions between TraJ and TraI, which supports this

theory, as well as between TraJ and the TM2 part of TraI. In a yeast two hybrid experiment, TraJ also showed also interactions with TraN. (Abajy, Kopec et al. 2007) This could not be reproduced in the bacterial two hybrid assay, but our in vivo crosslink experiments produced compatible results. TraJ also showed interactions with other TraJ molecules. It might be an oligermic protein.

For the VirB1-like enzyme TraG, a modular architecture is suggested. The N-terminus contains a putative transmembrane helix, followed by a soluble lytic transglycosylase (SLT) domain and a putative cystein-, histidin-dependent amidohydrolase/peptidase (CHAP) domain at the N-terminus. (Arends, Celik et al. 2013) It is also postulated that TraG locally hydrolyzes peptidoglycan and through its interactions with TraE, TraL as well as the putative two-component coupling protein TraI/TraJ, to aid in their proper localization. (Abajy, Kopec et al. 2007, Goessweiner-Mohr, Arends et al. 2013) We found interactions between TraL and TraG, as well as TraI and TraG. No interactions between TraG and TraE were found via B2H assay so far.

TraL has been denoted as a putative VirB6 homologue. According to a prediction-based analysis, TraL belongs to the smaller VirB6-like proteins with six transmembrane motifs. TraL is assumed to play an important role as a component of the inner membrane secretion channel or a scaffolding protein. TraL, together with TraG and TraM, could be the key players of the membrane-spanning channel, but no interactions had been found until now. (Alvarez-Martinez and Christie 2009, Goessweiner-Mohr, Arends et al. 2013, Bhatty, Gomez et al. 2013) TraL not only interacts with TraM and TraG, but also TraK. These are novel protein-protein interactions among the pIP501's transfer proteins.

Not much is known about TraK presently other than it is a cell envelope located protein and/or it might be an integral component of the core complex. TraK only shows a very limited number of structural relatives. So far, we only know of some *Enterococcal* T4SS, which encode a putative Tra-protein with a TraK-like secondary structure. Some of them lack a corresponding gene product, like pCF10. (Goessweiner-Mohr, Fercher et al. 2014, Bhatty, Laverde Gomez et al. 2013) TraK showed interaction with TraM as well as its C- and N-terminal domains; vice versa both domains of TraK showed interactions with TraM. It further interacts with TraG, with TraL and TraI. Additionally, it interacts with other TraK molecules. TraK might play an important role during the formation of the core complex.

TraI is proposed to be a part of the two-component coupling protein TraJ/TraI. (Goessweiner-Mohr, Arends et al. 2013) As mentioned above, this hypothesis is founded in its interaction

with TraJ. Additionally, it interacted with the VirB8-homologue TraM, wherein it showed interactions with its soluble domain and its soluble_TM1 domain. It also showed interactions with itself, possibly making it an oligomeric protein. We further found interactions with the VirB1-homologue TraG and TraK.
TraE, which is a VirB4-like protein and another ATPase of pIP501, showed interaction with itself and TraD. (Fronzens, Christie et al. 2009) No further interactions were found using the B2H assay. In a yeast two hybrid experiment, TraE was shown to interact with TraG as well as TraN. These interactions we were unable to verify. (Abajy, Kopec et al. 2007)

In summary, we discovered several novel protein-protein and protein–domain interactions. Due to these findings, we propose the following interaction model for the Tra-proteins of the pIP501. (Fig.26)

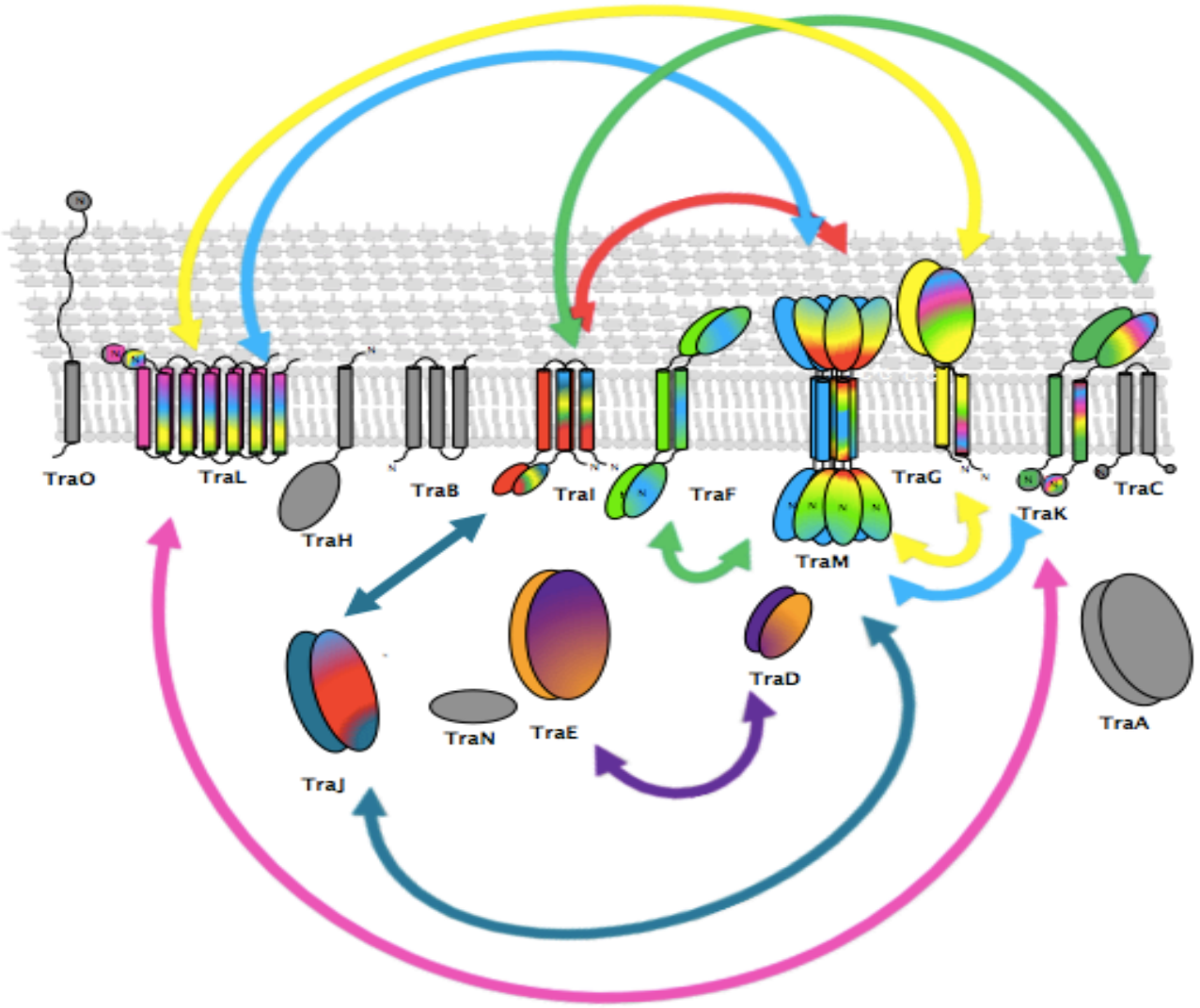

Figure 26: Protein-protein interactions in a Gram-positive Type IV Secretion System. Tra-protein interactions according to qualitative and quantitative B2H approaches are depicted in the appropriate color. Grey = no significant interaction detected. (basic figure was provided from research group Walter Keller)

We did not find a notable amount of interactions with the soluble proteins of the *tra* operon, which might be a methodical problem. Additional interaction studies may be required.

8.2 Interactions found directly in *Enterococcus faecalis* using in vivo crosslinking techniques

In previous experiments we identified different interacting potential key players in the membrane-integrated core complex of the pIP501-encoded T4SS. These Tra-proteins were

identified via bacterial two hybrid assay. We further wanted to verify these interactions directly in *Enterococcus faecalis*, using in vivo crosslinking.

TraK is among the number of Tra-proteins of the pIP501, which we know very little about. With our B2H assay, we found several novel interaction partners, which is why we sought to reproduce these interactions with in vivo crosslinking approaches. TraK weighs 32kDa in its monomeric version. We additionally found a shift to a higher molecular version, which could be the TraK-TraK or TraM-TraK interaction. As can be deduced from Fig. 20, there are two bands at about 60kDa. These may correspond to a putative dimer of TraK-TraK (approximately 64kDa) and the proposed TraK-TraM (aproximatly 70kDa) heterodimer. However, the *tra*K locus also encompasses a secondary start codon, therefore alternatively the two bands may correspond to homodimers of the two different *tra*K gene products, which vary 4.1 kDa in mass. So far, no functional explanation for the presence of the secondary start codon has been found. (Goessweiner-Mohr, Fercher et al. 2014) Since the two different bands between 58 kDa to 70 kDa may have been a result of the alternative start codon, we excised the bands and ran them through mass spectrometry using a nitrocellulose membrane. Unfortunately, the amount of protein was insufficient, so we could not detect any protein or dimeric versions of TraK or possible interaction partners.

Additionally, we attempted to verify the TraK-TraM interaction by stripping the membrane. As mentioned above, we were unable to detect TraM after stripping and reprobing with the TraM-antibody. This might indicate that the shifted bands truly arose from the dimeric interaction of the two different gene products of *tra*K.

With our B2H assay, we could not detect interactions of soluble proteins like TraN. However, due to the results of previous interaction assay approaches, we postulate that TraN does have interaction partners. TraN not only has a specific DNA-binding site; it may also be an accessory protein of the relaxase TraA. (Goessweiner-Mohr, Eder et al. 2014) TraN is localized in the cytoplasm and binds almost exclusively to dsDNA. It also contains two helix-turn-helix-like fold domains, which also support the presence of a DNA-binding function. Additionally, it has been shown to interact with the coupling protein TraJ. We wanted to demonstrate TraN-TraJ binding with the in vivo crosslinking method. (Goessweiner-Mohr, Fercher et al. 2012) Our results support the hypothesis of the existence of such an interaction, which points towards a role of TraN as repressor. (Goessweiner-Mohr, Eder et al. 2014)

8.3 Expression levels of the different Tra-proteins

The entire T4SS of the pIP501 is encoded by the *tra* operon coding for 15 different transfer proteins expressed from a single, polycistronic mRNA. The expression of the whole operon is controlled by the transfer initiator protein, TraA and is not influenced by the different growth phases of *Enterococcus faecalis.* (Grohmann, Goessweiner-Mohr et al. 2016) These data match with our data from the quantitative real time PCR.

As mentioned above, we propose that TraN plays a role as a negative regulator of the *tra*-operon in the pIP501. Not only because of the nature of its interaction partners, but also because of its structural similarity to proteins of the merR family. The merR family is a group of transcriptional regulators that have a similar helix-turn-helix DNA-binding structure at the N-terminus and an N-terminal effector binding region. MerR-like regulators have been found in a wide range of bacteria and the majority of them respond to environmental stimuli, such as oxidative stress. (Brown, Stoyanov et al. 2003) It has been suggested that TraN works as a transfer regulator by preventing the pIP501 plasmid DNA from being nicked by the relaxase without the establishment of cell-to-cell contact and formation of the conjugative core complex. Interestingly, TraN has been shown to interact with several key components of the pIP501 T4SS, despite being localized in the cytoplasm. Through these interactions, for example with the ATPase TraE, the muramidase TraG and the coupling protein TraJ, (Fig.27) (Abajy, Kopec et al. 2007) TraN could be prompted to release the plasmid DNA and would subsequently allow the relaxase to nick the plasmid DNA at the oriT site. The proposed mechanism would ensure that the relaxase-dependent processing of the conjugative plasmid is only initiated once the transfer apparatus has been assembled. If TraN functions as negative regulator, it has to have some sort of influence on the expression level of the Tra-proteins. One of the questions that arose was if TraN was knocked out, whether this would alter the expression level of the remaining Tra proteins. We found that every Tra-protein, which was tested for was upregulated, which complements the suppressor function postulated for TraN. (Grohmann, Goessweiner-Mohr et al. 2016)

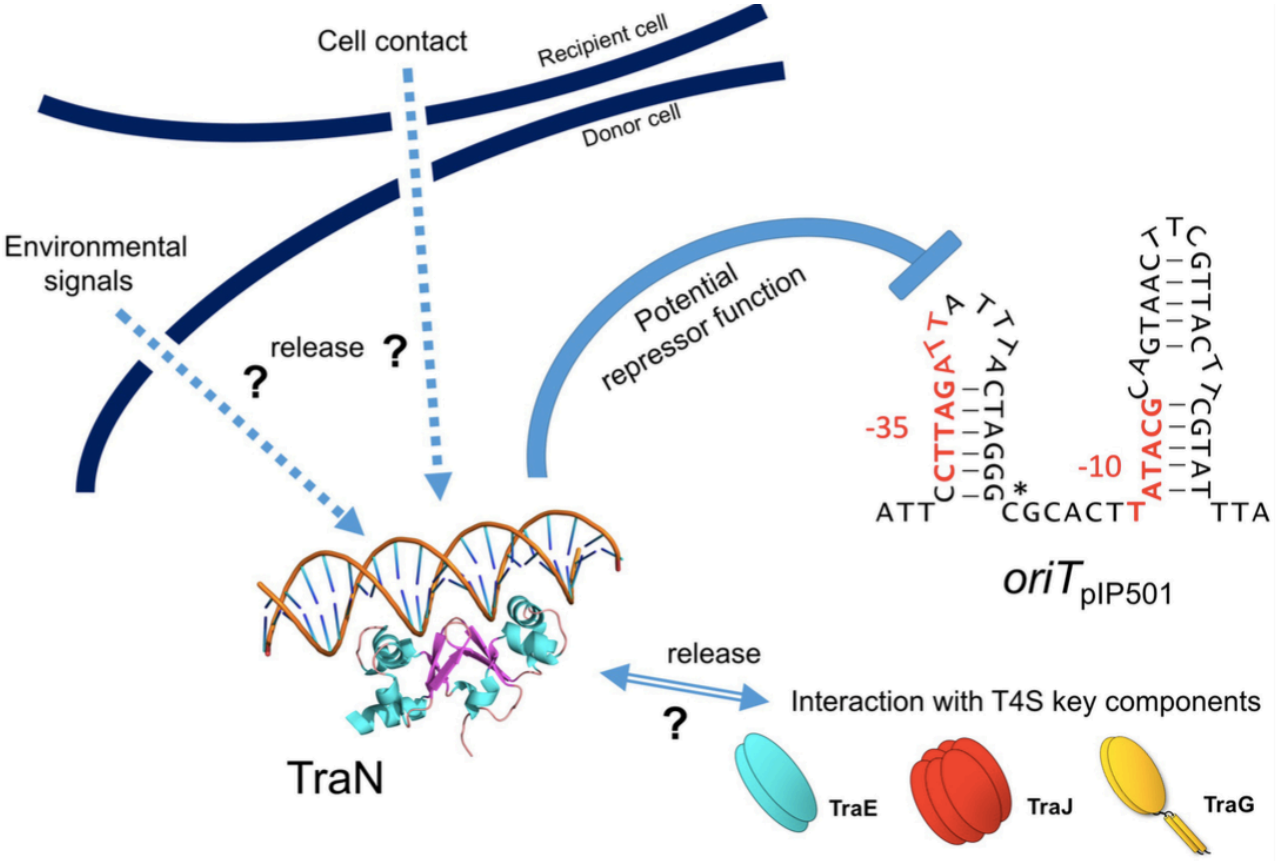

Figure 27: Model for negative *tra* operon regulation by transcriptional repressor TraN. Suggested interaction and activation of TraN as negative transfer regulator. TraN is shown docked to a random DNA-strand with the proposed binding mode. Colored in red were the -35 and -10 *tra* promotor region of the oriT. Pronounced nic-sites are lined with a star. (Grohmann, Goessweiner-Mohr and Brantl 2016)

With the upregulation of the Tra-proteins in the knockout strain compared to the wild type and the putative interaction with TraJ, we have found additional evidence that TraN plays a role as a repressor for the conjugative plasmid model pIP501.

9 Outlook

Utilizing a bacterial two hybrid system, we identified new interactions between possible key players of the membrane-integrated core complex of the pIP501-encoded T4SS, responsible for the actual transfer of the DNA strand during conjugation. To obtain a more detailed picture, domain-domain interaction experiments will need to be done.

Additionally, we attempted to analyze in vivo crosslinked Tra-proteins via mass spectrometry, but were unable to identify the found interactions due to insufficient protein amounts. Single Tra-proteins will need to be tagged and subjected to pull-down assays. Furthermore, co-crystallization of the different domains of a Tra-protein and interacting proteins are an option.

The *tra* operon of the pIP501 has a well titratable polycistronic expression in a gram-positive expression host, so we aim for assembly and membrane integration of its core complex in the right stoichiometry. For purification of the core complex, affinity chromatography and pull-down assays will be utilized. Structural characterization of the complex will be attempted using x-ray crystallography and cryo-electron microscopy methods.

To further investigate the overall composition of the conjugation complex, immuno-cryo-electron microscopy, where intact bacterial cells carrying pIP501 are nanogold-labeled with specific Tra-protein antibodies, is a possibility. This approach was used to identify the structure of the Gram-negative core complex of the T4SS encoded by a conjugative plasmid from *E. coli*, R388. (Low, Gubellini et al. 2014)

Additional ways of cross-confirming these findings and getting a better insight into the core complex are immunostaining and fluorescence microscopy. Both the pIP501-harboring wild type strain of *Enterococcus faecalis* as well as different *tra* knockout strains may be analyzed using these methods. To identify which of the domain-protein interactions are essential for the complex formation, complementation could be done with the different domains of the knocked-out proteins.

For our expression analysis, all available knockout strains had to be subjected to quantitative real time PCR. To find out which of the Tra-proteins are essential for conjugative transfer and which are not. Determining how the knockout of different Tra-proteins influences the expression levels of others may help to refine and better understand the generated data. Additionally, quantitative immunoblotting with Tra-protein levels normalized to a housekeeping gene to ascertain whether the expression of the proteins is transcriptionally and/or translationally regulated may provide insightful data.

To obtain more information about the Gram-positive conjugation, a combined effort utilizing structure determination methods and co-expression of several potential key players will be necessary.

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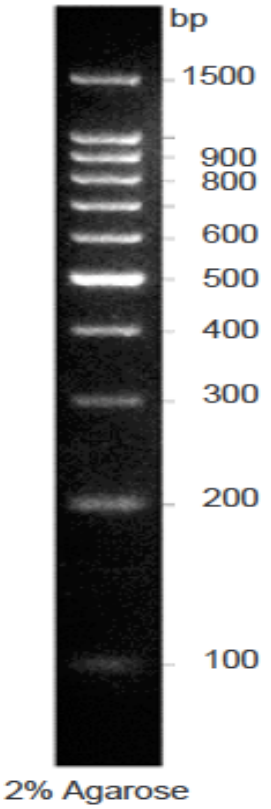
11 Attachment

11.1 Index of Abbrevations

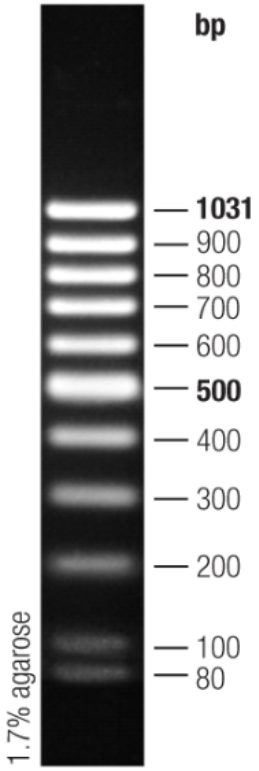
AA	Amino acid
BCP	1-bromo-3-chloropropane
bp	Base pairs
BSA	Bovine serum albumin
B2H	Bacterial Two Hybrid
C-terminal	Corboxy terminus of a protein
cAMP	Cyclic AMP
CAP	Catabolite activator protein
CHAP_link	Cystein-,histidin-dependent amidohydrolase/peptidase linker
CM	Cytoplasm membrane
CP	Coupling protein
cytmem	Cytomembrane domain of a protein
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
dso	Double strand origin
extracellular	Extracellular domain of a protein
f-factor	Fertility factor
Fw	Forward
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hfr	High frequency of recombination
ICE	Integrative conjugative elements
IMC	Inner membrane complex
Inc-family	Incompatibility family
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo-Dalton
kb	Kilobasepairs
LacZ-Assay	β-Galactosidase encoded by the *lacZ* gene of the *lac* operon in *E. coli*.
LB	Luria Bertani

membrane	Membrane domain of a protein
merR-family	Group of transcriptional activators
mpf	Mating pair formation
mRNA	Messenger RNA
mrs	Multimer resolution system
N-terminal	Amino-terminus of a protein
OMC	Outer membrane complex
ONPG	*o*-Nitrophenyl-β-D-galactopyranosid
Orf	Open reading frame
oriR	Origin of Replication
oriT	Origin of transfer
PAGE	Polyacrylamide gel electrophoresis
PG	peptidoglycan
PCR	Polymerase chain reaction
RC	Rolling circle
rev	Reverse
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SLT_link	Soluble lytic transglycosylase linker
Sol_TM1	Soluble-transmembrane helix 1
soluble	Soluble domain of a protein
ssDNA	Single stranded DNA
ssi	Single stranded origin region
sso	Single stranded origin
T4CP	Type IV coupling proteins
T4SS	Type IV secretion system
TM2	Transmembrane helix 2
Tra-Protein	Transfer Protein
WT	Wildtype
X-Gal	5-Bromo-4-Chloro-3-Indolyl B-D-Galactopyranoside

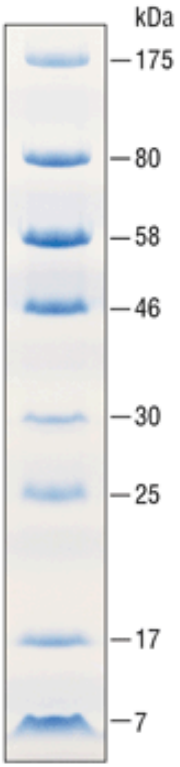
11.2 DNA-Standards and Protein-Standards



100 bp DNA *Ladder*



Low range DNA *Ladder*

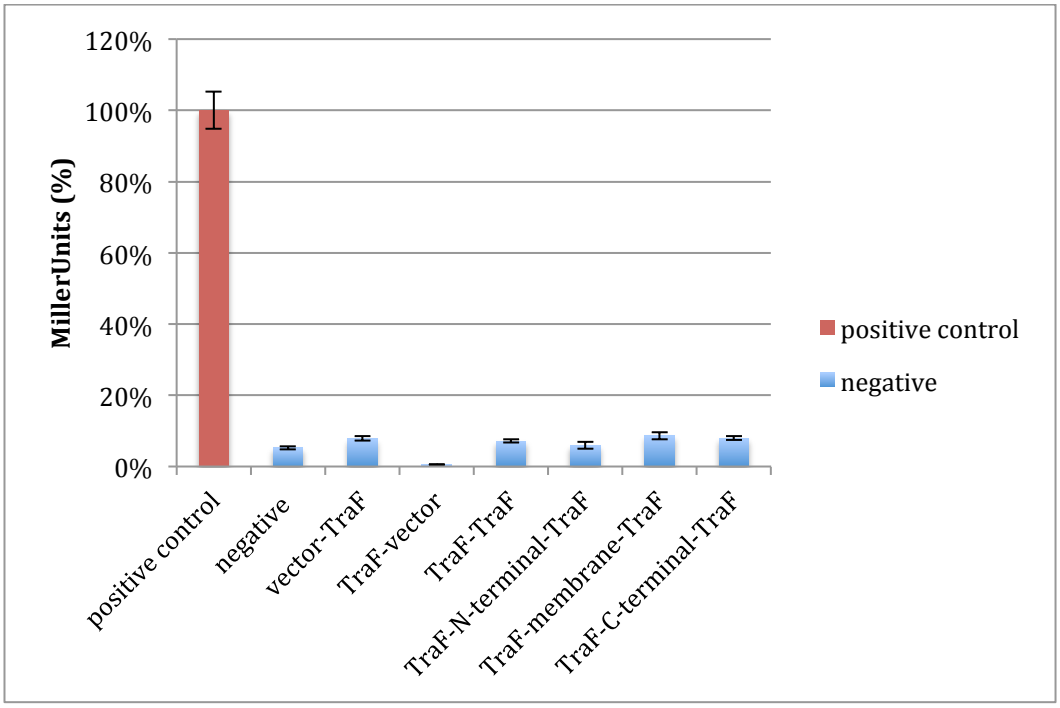


mid range prestained protein standard

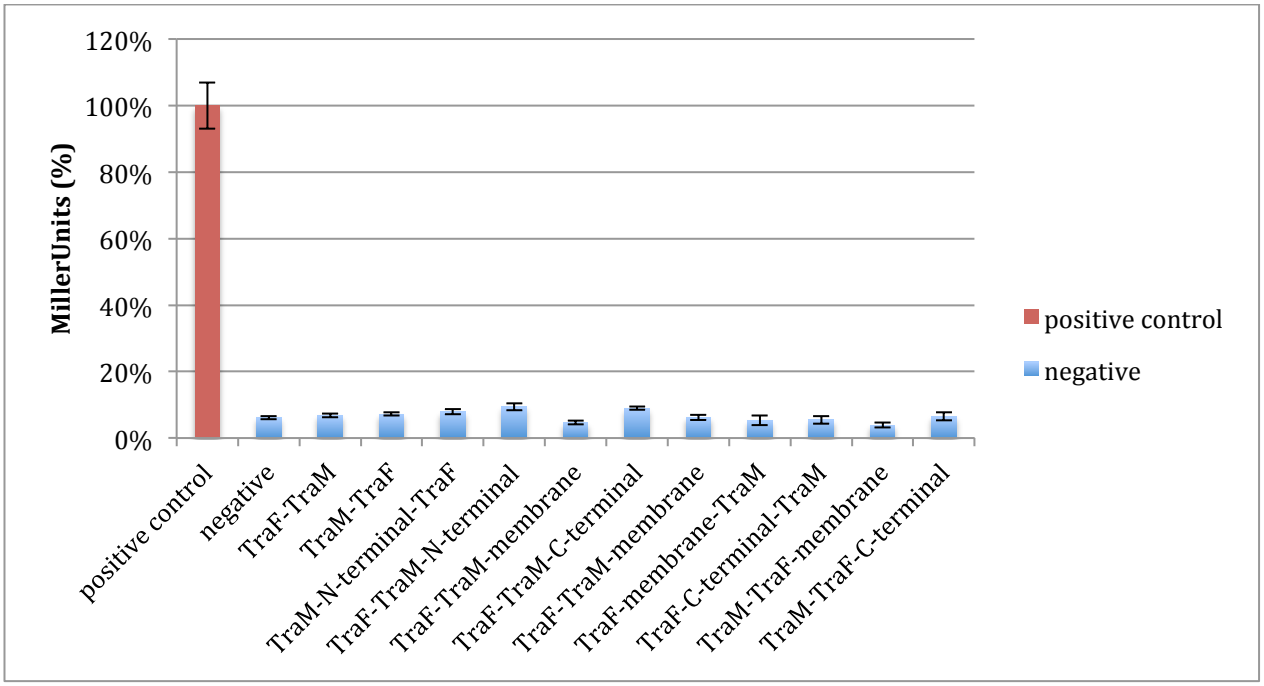
11.3 Negative interactions of the B2H experiments

Listed below are all negative interaction data, which were obtained as part of the B2H experiments. Any additional control data are also included.

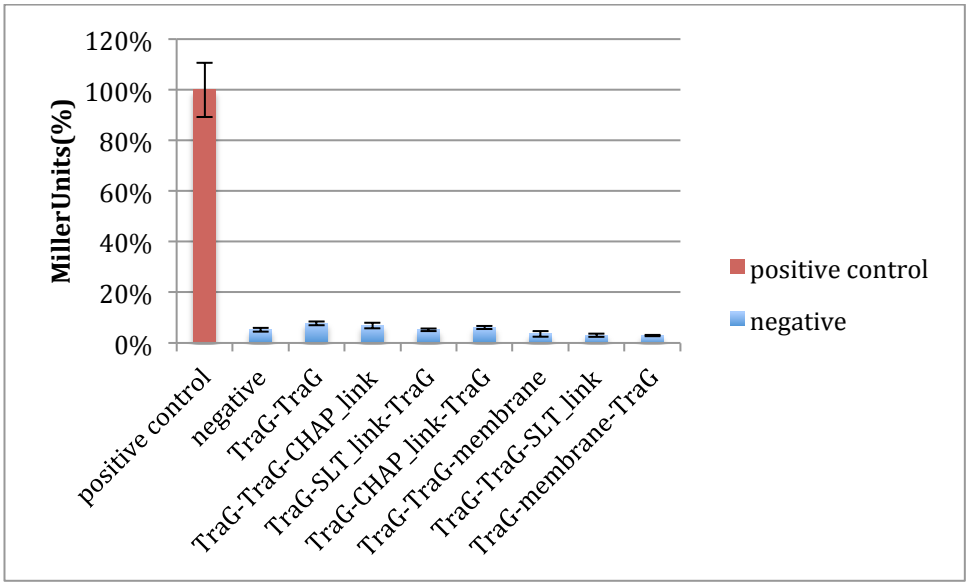
TraF-TraF



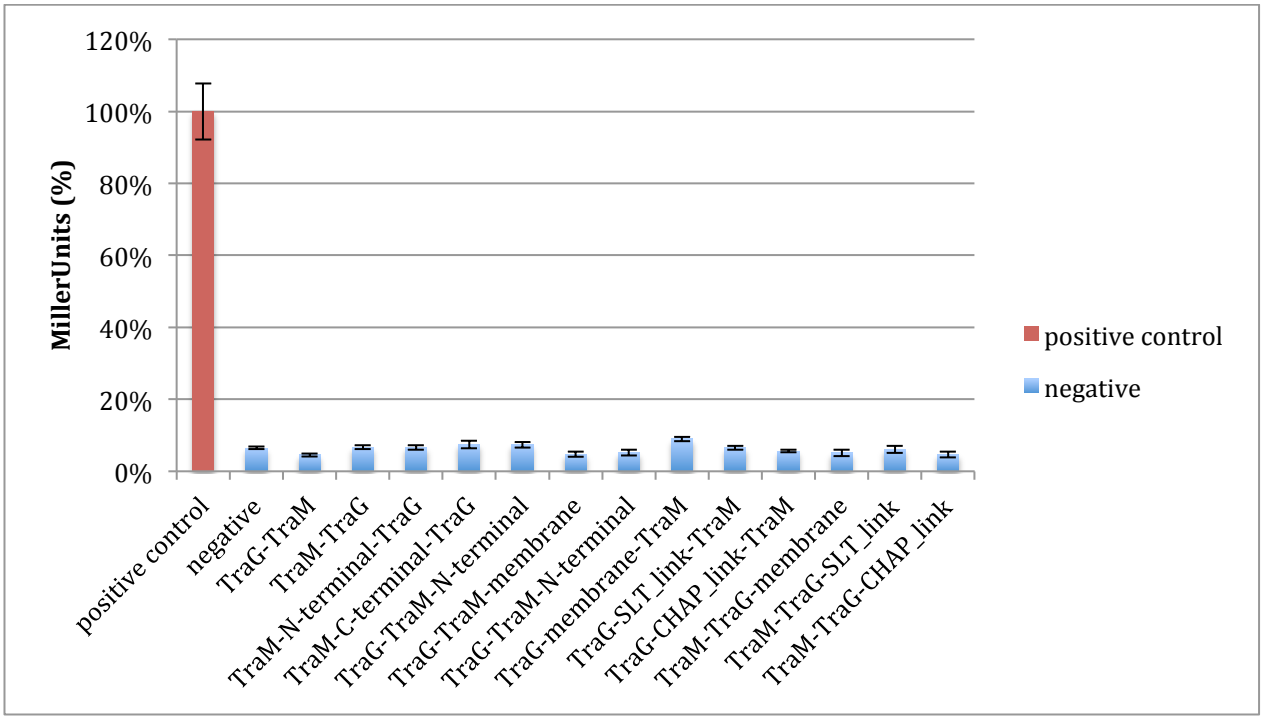
TraF-TraM



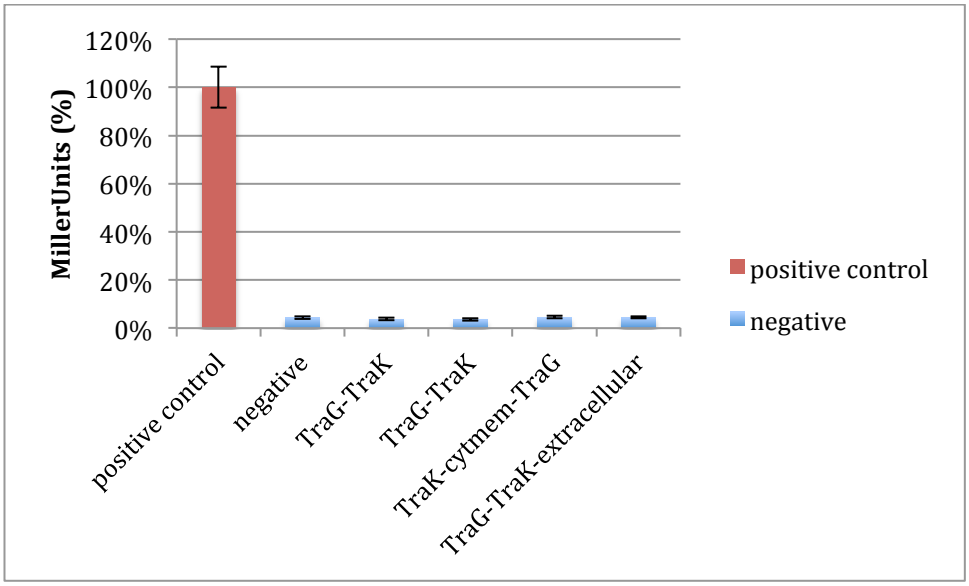
TraG-TraG



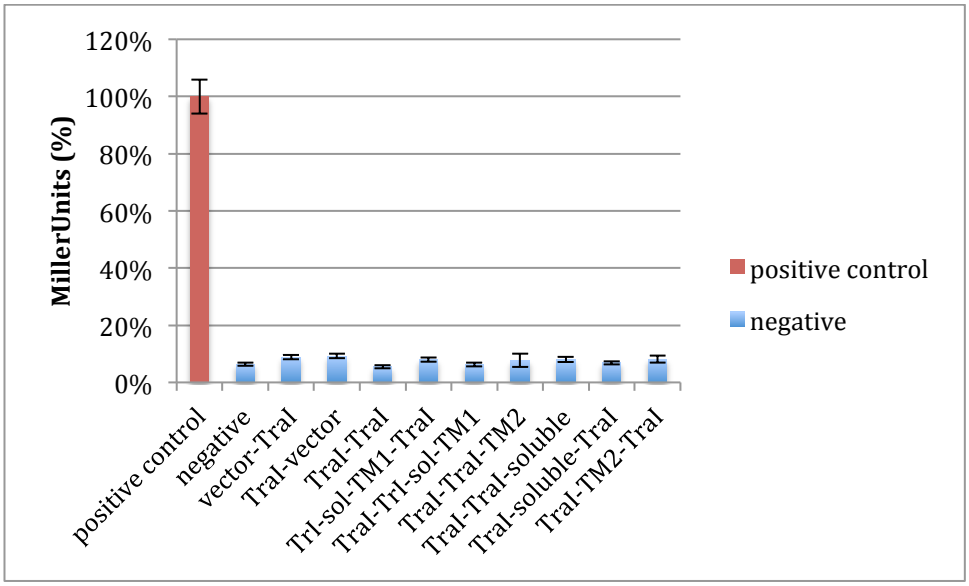
TraG-TraM



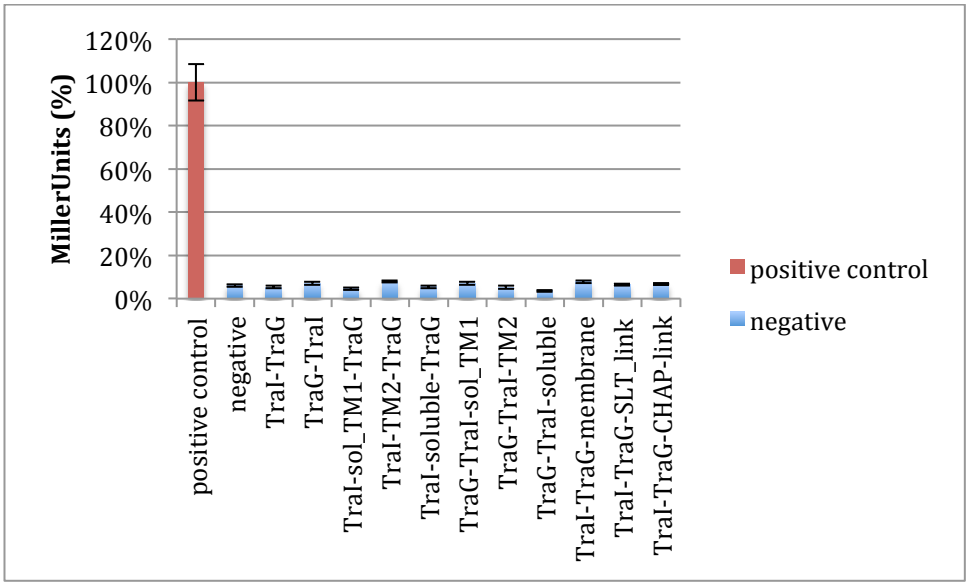
TraG-TraK



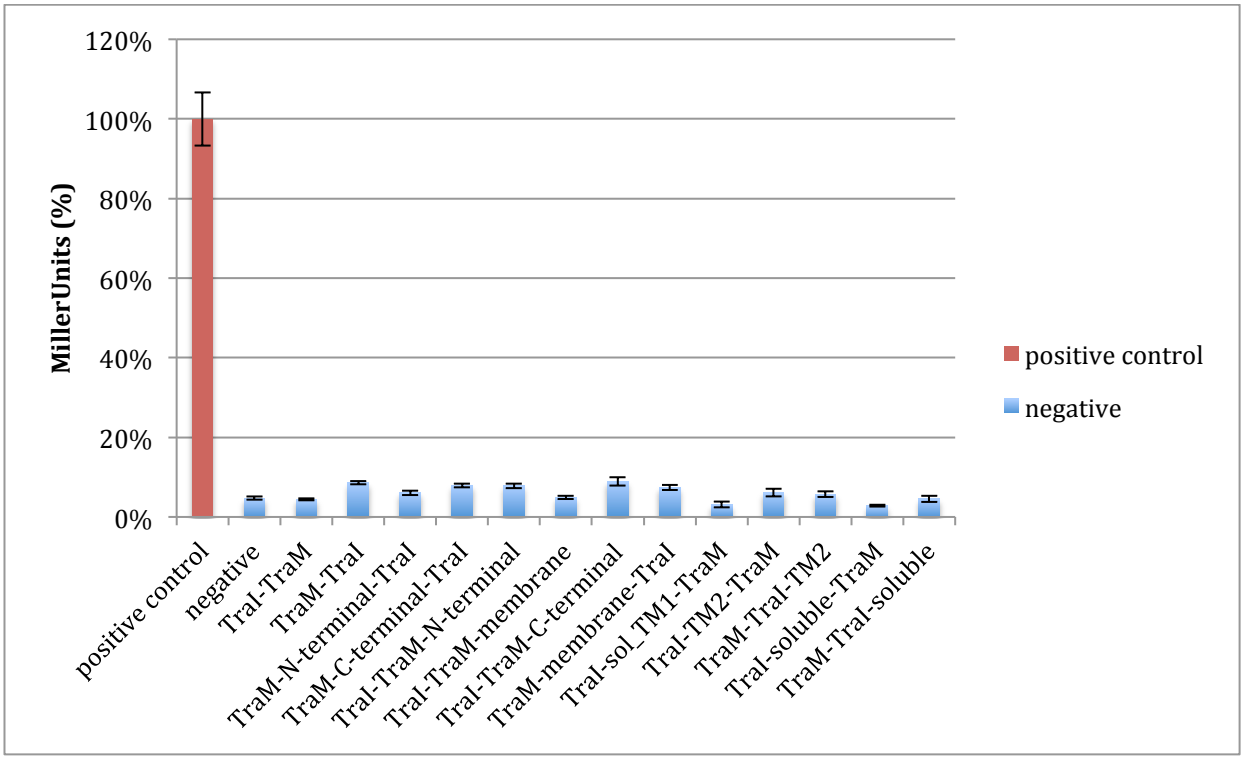
TraI-TraI



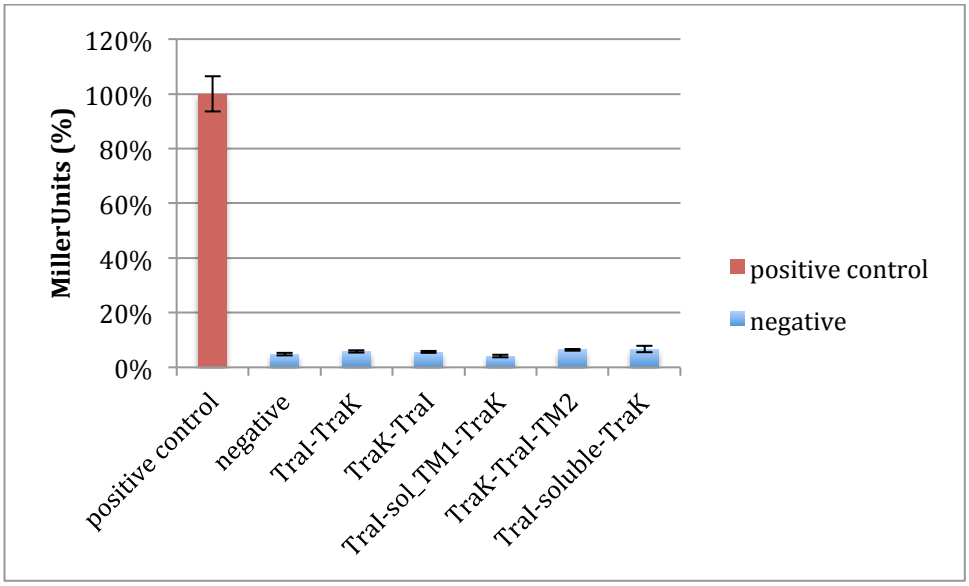
TraI-TraG



TraI-TraM



TraI-TraK



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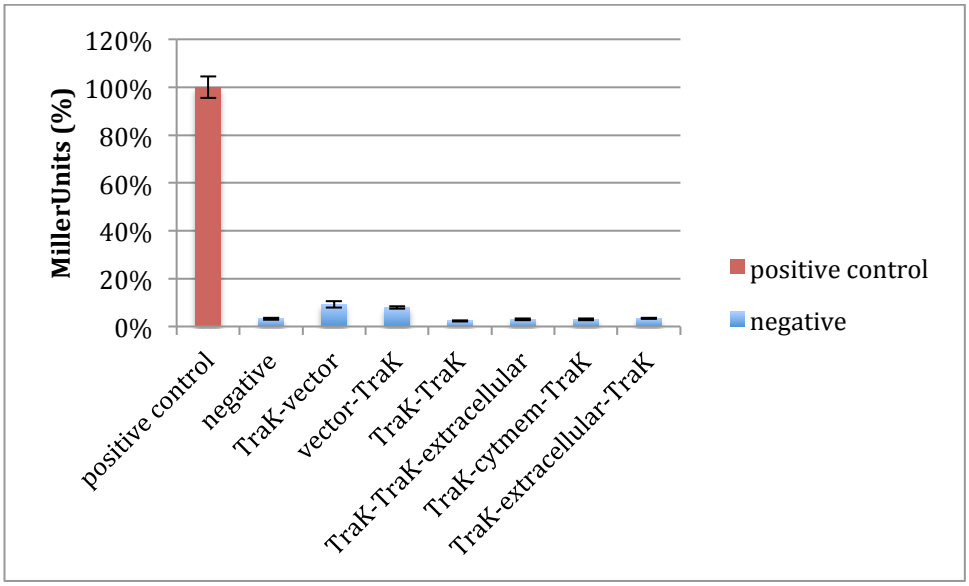

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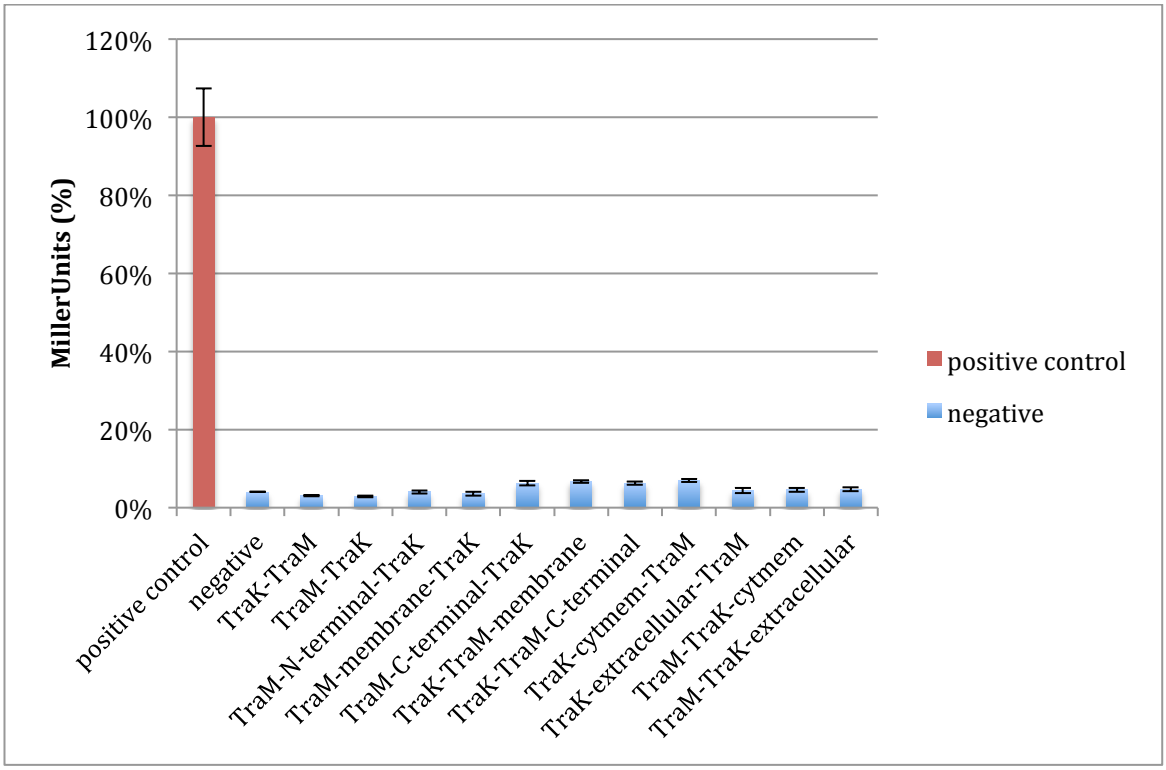

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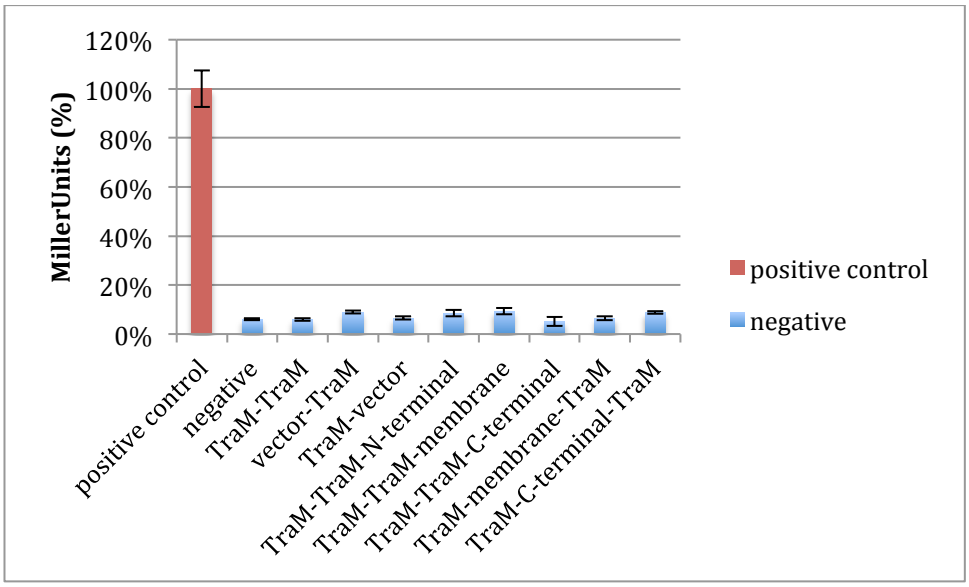
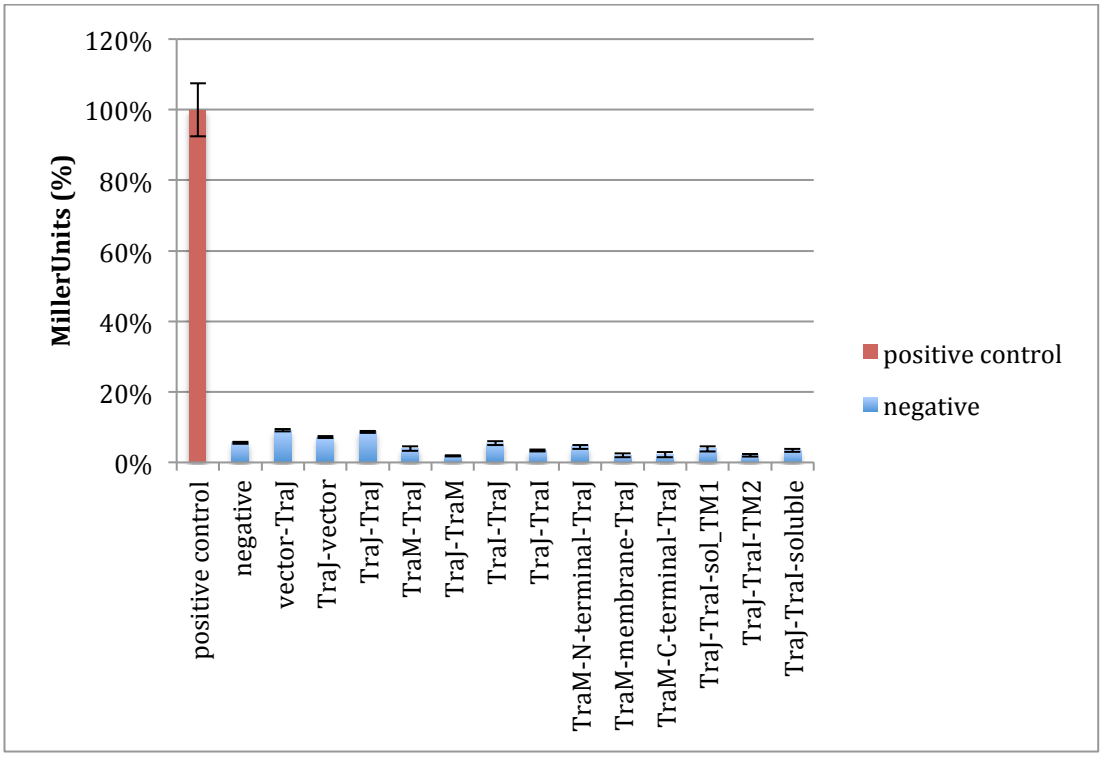


TraJ


120%
100%
80%
60%
40%
20%
0%
MillerUnits (%)
positive control
negative
positive control
negative
vector-TraJ
TraJ-vector
TraJ-TraJ
TraM-TraJ
TraJ-TraM
TraI-TraJ
TraJ-TraI
TraM-N-terminal-TraJ
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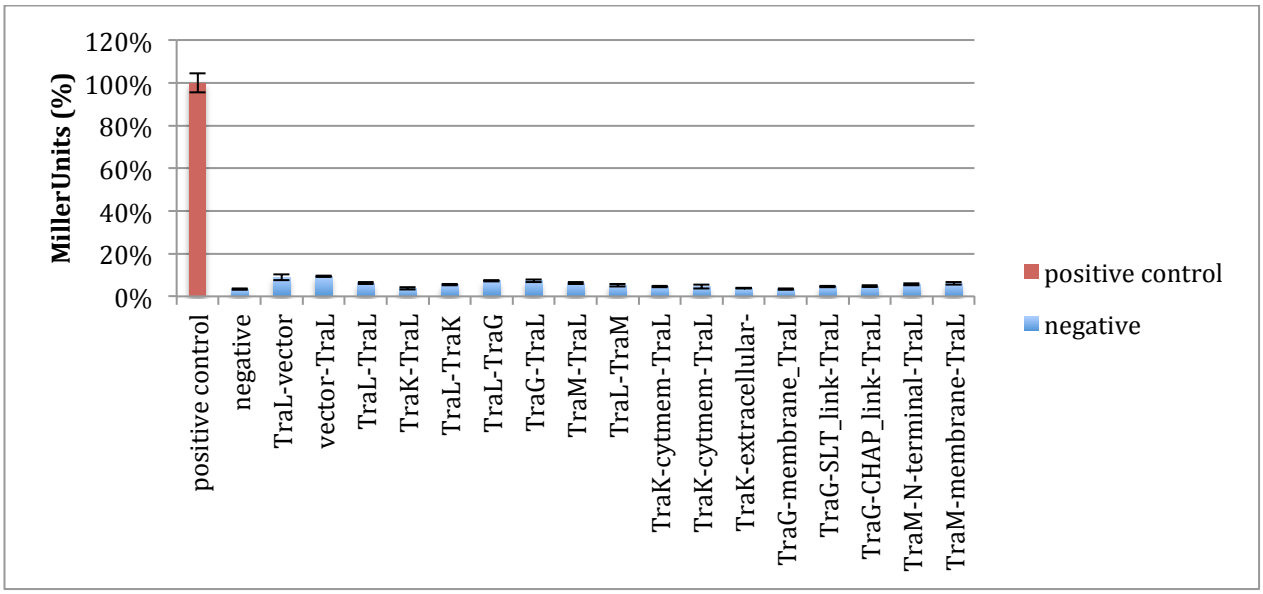
TraL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vector-TraL
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TraK-TraL
TraL-TraK
TraL-TraG
TraG-TraL
TraM-TraL
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TraK-cytmem-TraL
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TraK-extracellular-
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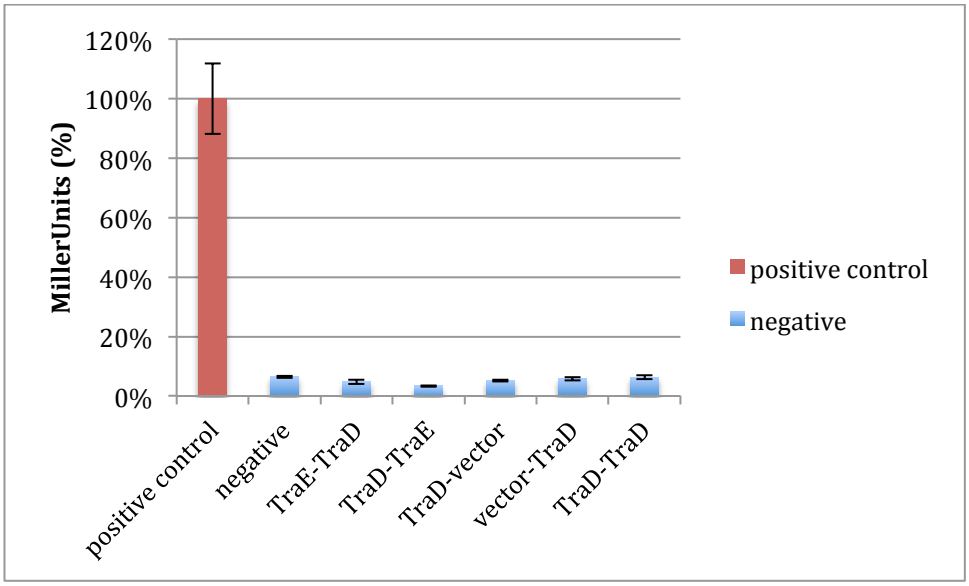
TraD-E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positive control
negative
TraE-TraD
TraD-TraE
TraD-vector
vector-TraD
TraD-TraD
positive control
negative