Trans-kingdom DNA transfer: Comparing the Ti and RP4 systems

Wittleben, F.T.

Chapter 1

General introduction

Felix T. Wittleben

Department of Molecular and Developmental Genetics,
Sylvius Laboratory,
Institute of Biology Leiden, Leiden University,
Sylviusweg 72, 2333 BE Leiden, The Netherlands.

Bacterial conjugation, basis for plant transformation by *Agrobacterium*

Bacteria have developed a myriad of different systems to interact with their environment and each other. Prominent amongst these are systems facilitating the exchange of DNA molecules, such as bacterial conjugation, which promote genetic adaption to the environment. This chapter will introduce the central concepts of bacterial conjugation and furthermore its evolution into a system for plant transformation by the bacterium *Agrobacterium tumefaciens*.

Exchange of genetic information between different organisms is known as horizontal gene transfer. It is a driver in the evolution of prokaryotic (and eukaryotic) organisms and occurs in three different ways: natural genetic transformation, viral-mediated transduction and conjugative transfer (Zatyka & Thomas, 1998). Horizontal gene transfer contributes to the emergence of genetic variation in related species, faster adaptation to environmental changes and stimulation of microbial cooperation (Smillie *et al.*, 2010). The advantage of horizontal gene transfer is the evolution of a bacterial genome without reliance on natural mutation; a freely available dynamic gene pool that can be rapidly shared, provides an immense adaptive potential for an organism (Harrison & Brockhurst, 2012).

The information exchange within horizontal gene transfer is driven by mobile genetic elements. There are different types of mobile genetic elements that can occur intra- or extra-chromosomal within the host organism: plasmids, bacteriophages and integrated conjugative elements (ICE) that include conjugative transposons (Frost *et. al.*, 2005). All elements use the host organism's endogenous replication machinery for propagation. Horizontal gene transfer by bacteriophages can occur via direct transfer of chromosomal or extra-chromosomal regions (e.g. plasmids), along its own DNA during its replication cycle (Canchaya *et al.*, 2003). ICE elements are present in the chromosome of a host cell, but are able to excise themselves and transfer between hosts using its own encoded transfer machinery (Johnson & Grossman, 2015). Both, bacteriophages and ICE will not be discussed in this dissertation. Plasmids are extra-chromosomal circular double-stranded DNA units, with some exceptions of linear double-stranded plasmids (Hinnebusch & Tilly, 1993), containing functional genetic modules organized in such a way to be self-replicating using the host endogenous replication machinery. Further, plasmids can contain genes distinctly different from the host genome, encoding accessory traits that can be utilized by the host organism (Frost *et al.*, 2005) and can be found in *Bacteria*, *Archaea*, and *Eukarya* (Phillips & Funnell, 2004). The focus of this dissertation is on bacterial plasmids. Besides extra-chromosomal existence, plasmids could integrate particular genes, large fragments of itself or completely into the host organism's chromosome (Carroll & Wong, 2018). Plasmids can exhibit different types of host ranges in which they can be active, with some plasmids showing a narrow host range with limitation to one or a few species or a broad host-range being able to be maintained in many different species (Jain & Srivastava, 2013). Plasmids, in general, can be grouped in three distinctive classes according to their mobility: conjugative or self-mobilizable/transmissible plasmids, mobilizable/transmissible plasmids that can be transferred only in the presence of conjugative plasmids, and non-mobilizable/transmissible plasmids (Garcillán-Barcia, Alvarado & de la Cruz, 2011). Conjugative and mobilizable plasmids contain a specific region called origin of transfer (*oriT*) allowing the plasmid to be transferred by a conjugative machinery into another host organism. Non-mobilizable plasmids can be transferred via natural transformation or bacteriophage-mediated transduction (Clark & Adelberg, 1962; Smillie *et al.*, 2010). Another form for a non-mobilizable plasmid to be transferred can be conduction, during which the non-mobilizable plasmid co-integrates with a conjugative plasmid. After transfer, the non-mobilizable plasmid is resolved from the co-integrate within the recipient cell (Clark & Warren, 1979). Each plasmid encodes an intricate system allowing for its own maintenance and propagation within the host organism. A partitioning system controls accurate segregation of plasmid copies into the new daughter cells of the original host organism after cell division

(Frost *et al.*, 2005). There is further a strict regulation of the amount of copies present within the cell, enforced by an encoded negative regulation mechanism. Different plasmids can exhibit a high or a low copy number (Del Solar & Espinosa, 2002). Each plasmid has a specific mechanism for replication control, and different plasmids can contain the same replication mechanism. The replication mechanism acts on as specific region within the plasmid called origin of replication (*oriR*/*ori*), or synonymously origin of vegetative replication (*oriV*). If two plasmids contain the same *oriV*, they can in principle, depending on their copy number, not be stably maintained in the same host cell without external selection (Novick, 1987), as both would not be discriminated by the partitioning machinery. However, a more recent analysis demonstrated that co-existence (of two plasmids with the same *oriV*) could last much longer than previously expected (Velappan *et al.*, 2007). Nevertheless, plasmids can be classified by their incompatibility of being maintained in the same cell at the same time. This trait of incompatibility (Inc) was and is still widely used to distinguish and classify different plasmids (Couturier *et al.*, 1988), and is defined for plasmids of *Enterobacteriaceae*, *Pseudonomdas, Agrobacterium*, and Gram-positive *Staphylococci* (Frost *et al.*, 2005). With the advent of rapid sequencing methods plasmids can now be grouped based on molecular differences in replication-, vegetative maintenance- or conjugative systems (Garcillán-Barcia, Alvarado & de la Cruz, 2011; Shintani, Sanchez & Kimbara, 2015; Orlek *et al.*, 2017). In this dissertation, the following plasmids are discussed and employed: the IncP conjugative broad host range plasmid RP4 (Datta & Hedges 1971), the IncW conjugative broad host range plasmid R388 (Fernández-López *et al.*, 2006), the IncQ mobilizable broad host range plasmid RSF1010 (Rawlings & Tietze, 2001) and IncRh1 Ti (tumor inducing) plasmids of *A. tumefaciens* (Hooykaas *et al.*, 1980).

A key element of conjugative DNA transfer systems is the type-IV secretion system (T4SS) machinery (Guglielmini, de la Cruz & Rocha, 2013). In this dissertation, the focus will be on plasmid-encoded conjugative systems. The T4SS may have evolved from a protein secretion machinery into an apparatus equipped (also) for DNA transfer. Proteins translocated may have a variety of functions in the recipient cell, for instance may be involved in the recircularization of the transferred single-stranded plasmid DNA and in the conversion into a double-stranded form, but may also have functions influencing the growth of the recipient cell (Guglielmini, de la Cruz & Rocha, 2013).

To summarize and provide more detail, the three elements of horizontal gene transfer occurring between organisms are:

1. Natural transformation: where freely available genetic information in the form of DNA molecules can be taken up by naturally occurring competent bacterial cells. Natural transformation is a phenomenon observed in many bacterial species. The percentage of naturally competent cells inside a population can vary from a minuscule fraction to almost the entire group (Dubnau, 1999; Bakkali, 2013). The natural transformation process differs from Gram-positive to Gram-negative bacteria (Chen & Dubnau, 2004). In Gram-negative bacteria with two cell membranes, the captured DNA is transported from the outer membrane into the periplasmic space and then further into the cell. Captured double-stranded DNA is usually converted into single strand molecules, whereby one strand is taken up and the other complementary strand is degraded. If the new DNA is not a plasmid that can replicate, the new genes are either lost or integrated into the genome by homologous recombination (Chen & Dubnau, 2004).

2. Transduction: the direct introduction of genetic information by a viral vector into the organism. In this manner, bacteriophages may be an essential part of spreading genetic material throughout the bacterial community within their reach. A limiting factor in transduction is the ability of bacteriophages to infect only a particular set of host strains within a species (Smillie *et al.*, 2010).

3. Conjugative transfer: the exchange of genetic information between cells during direct physical contact. Conjugation is the imperative mode of terrestrial-based horizontal gene transfer and is more prevalent than viral transduction (Bushman, 2002; Volkova *et al.*, 2014; Nazarin, Tran & Boedicker, 2018). An extensive network analysis study of 111 bacterial genomes and hundreds of thousands of environmental DNA samples of plasmids and viruses from various databases provided evidence of the importance of plasmid driven exchange (Halary *et al.*, 2010). Horizontal gene transfer also occurs in marine-based environments, but little is known about prevalence between conjugation and transduction (Hermansson & Lindberg, 1994; Sobecky & Hazen, 2009) The exchanged genetic material is almost invariably mediated by conjugative plasmids, extra-chromosomal DNA elements in the possession of conjugative transfer (*tra*) genes as well as genes regulating its own replication. Conjugative elements can provide the host with specialized traits to settle in specific niches that could previously not be colonized (Kado, 1998). The initial cell-to-cell (donor to recipient) contact can be established by the conjugative pilus structure (Willetts & Wilkins, 1984). For a successful conjugational process, cell-to-cell contact is required for unidirectional exchange of genetic material (Achtman, Morelli & Schwuchow 1978). During conjugation, single-stranded DNA is transferred between bacterial cells (Guglielmini, de la Cruz & Rocha, 2013). There is an exception with the Gram-positive bacterium *Streptomyces spp*., as double-stranded DNA has been found to be transferred in a chromosomal segregation type transfer mechanism in this organism (Possoz *et al.*, 2001; Vogelmann *et al.*, 2011).

Conjugation

Lederberg and Tatum (1946) first described the process of bacterial conjugation in the Gram-negative *Escherichia coli* K12. The conjugative F-plasmid of *E. coli* K12 then became the model system for bacterial conjugation. For simplification and categorization, the general process of bacterial conjugation can be broken down into four phases: DNA substrate processing and substrate recruitment to the transfer apparatus in the donor, the actual translocation itself by the T4SS, re-circularization of the transferred single-stranded plasmid DNA and conversion into double-stranded DNA in the recipient (Grohmann, Muth & Espinosa, 2003; Thomas & Nielsen, 2005; Alvarez-Martinez & Christie 2009; Cabezon *et al.*, 2015). The fourth phase will not be discussed in more detailed as the fate of the transferred DNA depends on the type of DNA transferred as well as on the type of recipient cell. These phases are orchestrated by three discernable systems:

1. The DNA transfer and replication system (**Dtr**), mediating the enzymatic processing of DNA substrate to be transferred into the donor. The primary enzyme of the Dtr is the relaxase, which forms a nick (single-strand break) at a specific *nic* site within the *oriT*. Auxiliary proteins assist in this step.

2. The coupling protein (**CP**), linking the processing (Dtr) and transport (Mpf) of the substrate during conjugation.

3. The mating pair formation system (**Mpf**) creating and mediating a physical bridge between two cells, that allows for substrate transfer. Mpf systems are large multi-protein complexes that create a *trans*-membrane-spanning channel (Schröder & Lanka, 2005). The

actual physical cell-to-cell contact that is necessary for substrate transfer is still a poorly understood process. A cell junction is created during this event, and specific signals are triggered to initiate the transfer process (Samuels, Lanka & Davies, 2000; Lang *et al.*, 2011; Bhatty, Laverde Gomez & Christie, 2013). Nowadays the Mpf is often referred to as the T4SS. Although the T4SS is best known from its role in conjugation, it has also evolved and specific T4SS have been found to play a role in transformation (secretion and uptake of DNA molecules from the environment) and also in virulence, by facilitating the transfer of virulence proteins and sometimes also DNA molecule into eukaryotic host cells (plant, animal and human) contributing to disease development (Christie, 2001; Llosa *et al.*, 2002; Cabezon *et al.*, 2015).

Mobilizable plasmids do not encode and Mpf transport system, but may still be transmitted by use of the Mpf encoded by another plasmid. Such mobilizable plasmids encode their own Dtr system that is mostly referred to as Mob system, and may or may not encode their own CP (Szpirer, Faelen & Couturier, 2000; Smillie *et al.*, 2010).

To summarize these three pillars of conjugation, a simplified overview is given in **figure 1**. All components acting together make the transfer of DNA to a host cell possible. In some instances, the T4SS only transfers effector proteins without DNA transfer (Cascales & Christie, 2003).

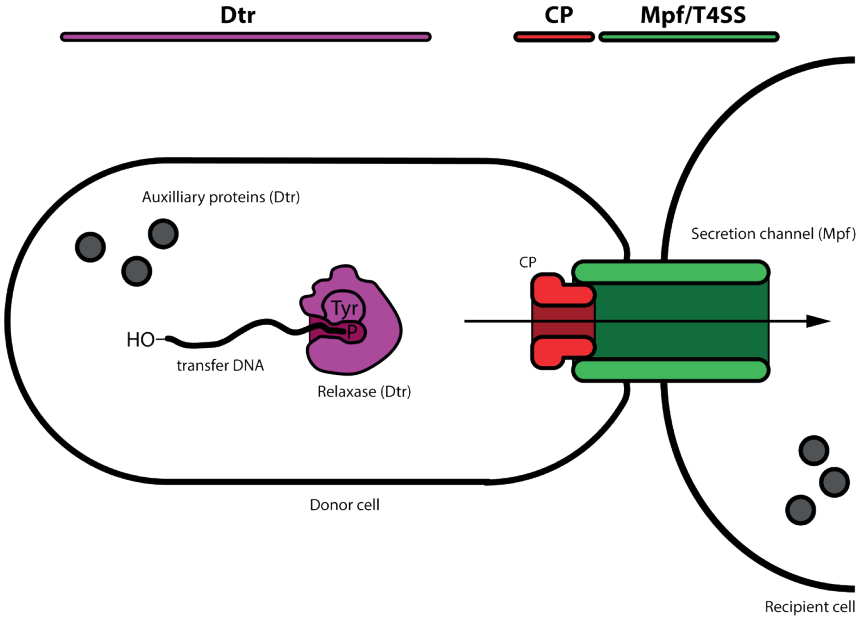

Figure 1. The three elements of bacterial conjugation in a simplified representation. The designated transfer DNA can be either a full-length plasmid or a specific DNA sequence on a plasmid. The DNA transfer and replication system (Dtr) and its auxiliary proteins process the transfer DNA. A relaxosome complex binds to a specific double-stranded DNA sequence and initiates nicking of one of the DNA strands via the relaxase (purple). The relaxase binds during this process covalently to the 5'-end of the transfer DNA, and a single-strand still bound to the relaxase is displaced. The single-strand associates via the translocation signal of the relaxase with the coupling protein (CP; red). The CP thus recognizes the specific substrate and initiates the transfer via the type-IV secretion system (T4SS) or mating pair formation (Mpf; green) channel into the recipient cell. Besides the relaxase, other proteins may also be recognized by the CP and transported via the T4SS into the recipient cell, aiding in further DNA processing, for example in the conversion from the single-stranded into a double-stranded form.

For easier understanding of the nomenclature, the system established by Guglielmini, de la Cruz & Rocha (2013) will be used throughout this dissertation. Proteins and genetic elements will be noted by **GI_{GE}**, referring first to the protein name or gene identification (**GI**), and then the genetic element (**GE**) where it is derived from in subscript. For example, $VirD2_{Ti}$ is the relaxase VirD2 protein encoded in the virulence system of the Ti plasmid of *A. tumefaciens*, or $traI_{RP4}$ the gene encoding the relaxase TraI on the conjugational plasmid RP4. Proteins are noted in roman letters with the first letter in capital. Genetic elements will be given in italicized letters with lower case first letters. For example, the protein $VirD2_{Ti}$ is encoded in the gene $virD2_{Ti}$. Plasmid names are always given in roman letters and are not italicized. For consistency, the abbreviation of the type-IV secretion system will be T4SS.

Agrobacterium tumefaciens - plant pathogen

This dissertation studies gene and protein transfer from *A. tumefaciens*, a Gram-negative bacterium of the rhizosphere (**Figure 2**). It is the causative agent of crown gall disease, a formation of neoplastic growth in a variety of plant species (De Cleene & De Ley, 1976). Specific DNA is transferred into plant cells, which in turn are transformed by the DNA that further leads to tumor growth. This *trans*-kingdom gene transfer is carried out by a set of virulence (Vir_{Ti}) proteins able to transport oncogenes, encoded on transfer-DNA (T-DNA) into host cells. Integration into the host cells genome leads to stable transformation. This process resembles bacterial conjugation (Lessl & Lanka, 1994). The induced neoplastic plant growth produces a vast amount of specific compounds called opines that can be utilized by *A. tumefaciens.* The virulence genes (vir_{Ti}), as well as the opine catabolic genes, are located in the tumor-inducing plasmid (Ti plasmid; **Figure 3A**). The most studied strain is the pathogenic *A. tumefaciens* strain C58, on which many laboratory strains are based. It has a genomic size of around 5 Mbp, split on a circular and linear chromosome. It harbors the cryptic plasmid pAtC58, sized around 550 kbp, and the nopaline-type Ti plasmid pTiC58 with an approximate size of 220 kbp (Goodner *et al.*, 2001).

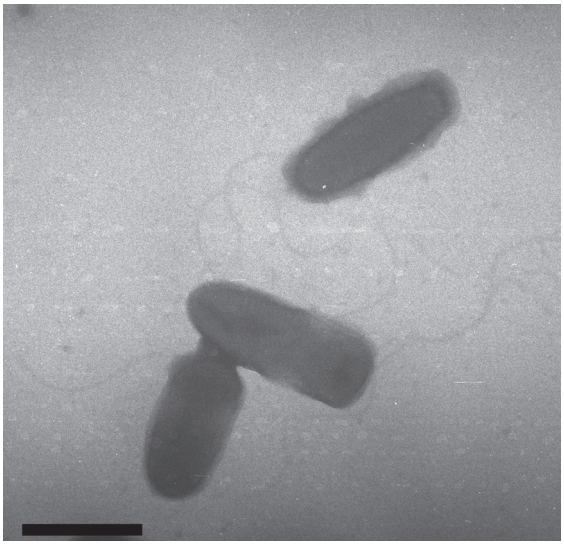

Figure 2. Electron micrograph of cells of *Agrobacterium tumefaciens* strain LBA1100. Bacterial cells were negatively stained with 3% ammonium molybdate. The cell is approximately 1.2 μm in length. The bar = 1 μm. Magnification is 15000×.

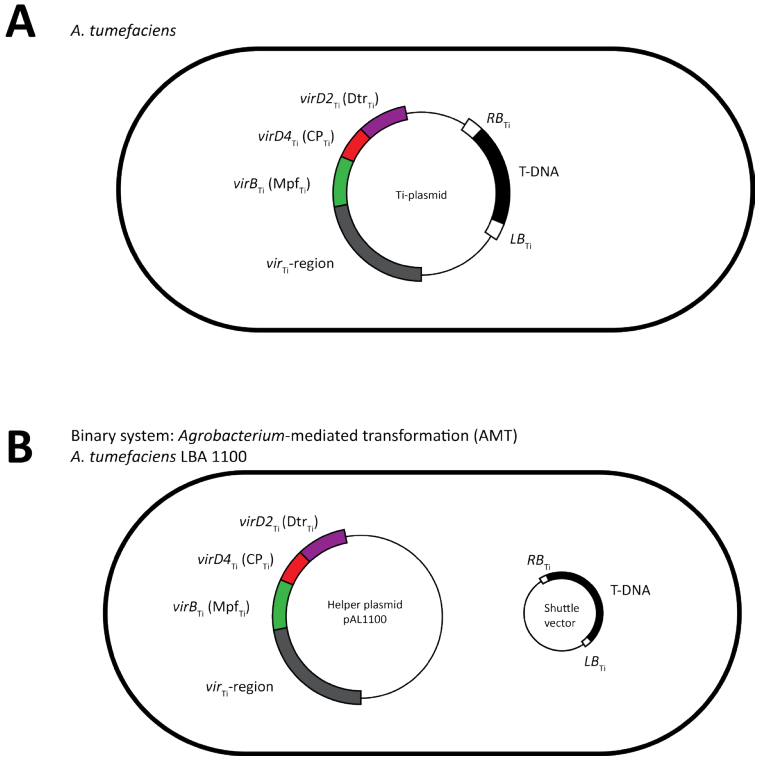

Figure 3. General representation of the *A. tumefaciens*-mediated transformation system. The graphic is a general overview as shown in **figure 1**, including Dtr with relaxase (purple), CP (red), T4SS/Mpf channel (green). **A:** The Ti plasmid in the wild-type configuration. All parts essential for plant transformation are present on the Ti plasmid: the transferred T-DNA and vir_{Ti}-region, which encodes for all virulence proteins involved in transfer. **B:** The binary system, widely used for AMT, separates the vir_{Ti}-region including Dtr, Mpf and CP on the helper plasmid from the T-DNA that is provided on a separate plasmid (shuttle vector). The figure is not a true spatial representation.

The cryptic plasmid pAtC58 is not essential for transfer but has a positive effect on it, is self-transmissible and contains genes for metabolic regulation (Kado, 1998; Nair *et al.*, 2003). The pTiC58 belongs to the incompatibility group IncRh1 (Hooykaas *et al.*, 1980). Within Ti plasmids, several functional regions have been recognized: replication, virulence (vir_{Ti} region), opine uptake and catabolism, self-conjugation (tra_{Ti}/trb_{Ti} region), and the region designated to be transferred to the recipient cells (T-DNA region). Additionally, the Ti plasmid contains the $oriV_{Ti}$ for plasmid maintenance within *Agrobacterium*. Ti plasmids, in general, have a host range limited to the family of *Rhizobiaceae* that includes the genus *Agrobacterium* (Hooykaas & Beijersbergen, 1994). The tra_{Ti}/trb_{Ti} region, which mediates the transfer of the Ti plasmid in-between *Agrobacteria*, shares similarities with the conjugative systems of the RP4, RSF1010 and F plasmids (Farrand, Hwang & Cook, 1996). All these features are likely to reflect the evolutionary history of the Ti plasmid (Alt-Mörbe *et al.*, 1996).

To infect and transform plants, *A. tumefaciens* must become virulent by expressing its virulence proteins. These are encoded in the vir_{Ti} region containing 8-10 operons that are together essential for tumorigenesis (Hooykaas & Beijersbergen, 1994). The release of sugars and phenolic compounds present in the sap of wounded plant cells and stable low pH more acidic level (Brencic & Winans, 2005), caused by the apoplastic fluid release, can be sensed by the two-component system $VirA_{Ti}/VirG_{Ti}$ detecting these physiological signals. Within

laboratory settings, the phenolic compound acetosyringone is used to stimulate virulence of *Agrobacteria*. Phenolic compounds are bound by the *trans*-membrane-spanning histidine kinase $VirA_{Ti}$. The chromosomally encoded virulence protein $ChvE_{Ti}$ binds to released sugar molecules and enhances the induction signal of $VirA_{Ti}$ (Hu *et al.*, 2013; Gao & Lynn, 2005). Both $ChvE_{Ti}$ and $VirA_{Ti}$ can detect an environmental change to acidic conditions, at around a pH of 5.5, to induce virulence (Rogoswky *et al.*, 1987; Melchers *et al.*, 1989; Liu *et al.*, 1993; Turk *et al.*, 1994; Gao & Lynn, 2005; Hu *et al.*, 2013). When $VirA_{Ti}$ is activated, it auto-phosphorylates and in return phosphorylates the cytoplasmic transcriptional activator $VirG_{Ti}$ (Jin *et al.*, 1990). Active $VirG_{Ti}$ binds to vir_{Ti}-box regions on the Ti plasmid and induces vir_{Ti} gene expression (Gao & Lynn, 2005). In the plant wound, *Agrobacterium* attaches to the plant cells (Matthysse, 2014). The induced $VirB_{Ti}$ proteins form a core channel-structure ($T4SS_{Ti}$) spanning through the membrane of *A. tumefaciens* contacting with the host cell, and together with the CP_{Ti} $VirD4_{Ti}$ facilitating the transfer of genetic material and auxiliary/effector proteins into the recipient cell (Cascales & Christie, 2004; Schröder & Lanka, 2005; Aguilar *et al.*, 2011). The T-DNA, the transfer vehicle for tumor-inducing genes (auxin & cytokinin) as well as opine synthesis genes, is flanked by *cis*-acting recognition sequences comparable in function to *oriT* sequences, called right and left border (RB_{Ti} and LB_{Ti} respectively; **Figure 3A**; Bundock *et al.*, 1999). The relaxase $VirD2_{Ti}$ recognizes the border sequences marking the T-DNA region on the T-region present on the Ti plasmid. $VirD2_{Ti}$ nicks one of the DNA strands at the border sequences and attaches to it covalently at its 5'-end. Several auxiliary proteins aid $VirD2_{Ti}$ in binding and nicking the border sequences. The nicking function of $VirD2_{Ti}$ is aided when $VirD2_{Ti}$ is associated with $VirD1_{Ti}$ (Relić *et al.*, 1998), and binding of $VirD2_{Ti}$ to the designated DNA sequences is aided by $VirC1_{Ti}$ and $VirC2_{Ti}$ (Toro *et al.*, 1988). Ensuing DNA synthesis at the 3'-end of the nick site restores the original T-DNA region, and helps to displace the single-stranded T-strand. After displacement, the T-strand bound to $VirD2_{Ti}$ associates with the CP_{Ti} $VirD4_{Ti}$, which then facilitates the transfer of the T-strand complex via the $T4SS_{Ti}$, comprised of $VirB_{Ti}$ proteins, into the host cell (Vergunst *et al.*, 2000). Inside the host cell, the T-strand bound to $VirD2_{Ti}$ is forming a so-called T-complex with $VirE2_{Ti}$. The effector protein $VirE2_{Ti}$, another transferred virulence protein, is able to bind along the transferred single-stranded T-strand inside the host cell (Citovsky, de Vos & Zambryski 1988). Nuclear localization signals are present in $VirD2_{Ti}$ (Rossi, Hohn & Tinland, 1993) and $VirE2_{Ti}$ (Zupan, Citovsky & Zambryski, 1996), that guide the T-complex into the host cell nucleus. In the nucleus, host endogenous DNA processing systems convert the single-stranded T-strand into a double-stranded form (Liang & Tzfira, 2013). The T-DNA can be integrated into the host cell genome (Tzfira *et al.*, 2004), creating stable transformation and tumor formation. Transient expression of the T-DNA, when not integrated, is also possible (Narasimhulu *et al.*, 1996). In biotechnological research, disarmed (no tumorigenic T-DNA) *Agrobacterium* can be used to create transgenic plants (Barton *et al.*, 1983). The discovery that vir_{Ti} genes located *in trans* on a separate plasmid-vector still allowed for T-strand transfer (Hoekema *et al.*, 1983), prompted the development of the binary vector system (**Figure 3B**). The binary vector system is now routinely used for *Agrobacterium*-mediated transformation (AMT) of plants. AMT is also possible, within laboratory conditions, to transform yeast and fungi. (Bundock *et al.*, 1995; de Groot *et al.*, 1998). That the *Agrobacterium* virulence system is evolutionary related to bacterial conjugation systems became apparent, when it was shown that it could still mediated conjugative plasmid transfer from *Agrobacterium* to other *Agrobacterium* and *E. coli* (Beijersbergen *et al.*, 1992).

The broad host range conjugative plasmid RP4

The RP4 plasmid was initially identified to be an agent of antibiotic drug resistance spread between bacteria (Saunders & Grinsted, 1972). It was found in *Pseudomonas aeruginosa*

isolated from a clinical sample in Birmingham (UK) at the Birmingham Accident Hospital (Ingram, Richmond & Skyes, 1973). Other conjugative plasmids R18, R68, RK2 and RP1 isolated in the same hospital in the same period were identified to be identical to RP4, and are also referred to as Birmingham IncPα plasmid (Bryan, van den Elzen & Tseng, 1972; Saunders & Grinsted, 1972; Pansegrau *et al.*, 1994). The plasmid RP4 is part of the IncPα incompatibility group of conjugative plasmids together with plasmids such as R702 (Hedges & Jacob, 1974). RP4 is a broad range host plasmid, able to be maintained for instance in *E. coli* and *A. tumefaciens,* conferring antibiotic resistance to its hosts (Datta & Hedges, 1971; Levin *et al.*, 1976). It has a size of 60 kbp and contains around 74 genes, of which only 60 have been functionally identified. It is a self-transmissible plasmid with conjugative transfer encoded within the *tra1*$_{RP4}$ region (Dtr$_{RP4}$, CP$_{RP4}$) and the *tra2*$_{RP4}$ region (Mpf). The Mpf$_{RP4}$ system used by RP4 is a T4SS$_{RP4}$. The *oriT*$_{RP4}$ is located in the *tra1*$_{RP4}$ region. The *oriV*$_{RP4}$ and replication genes, regulating plasmid maintenance, are situated around 6000 bp upstream of the *tra1*$_{RP4}$ region (Pansegrau *et al.*, 1994). After its discovery and realization of its strong promiscuity, the conjugative system of RP4 was adapted for use in genetic engineering (**Figure 4**; Jacob & Grinter, 1975; Ditta *et al.*, 1980; Paget *et al.*, 1990; Babic, Guérout & Mazel, 2008).

The conjugative system encoded by plasmid RP4 and the core of the virulence system of the Ti plasmid share a close structural and functional homology, and are likely derived from one ancestral conjugative system (Lessl, Pansegrau & Lanka, 1992; Pansegrau *et al.*, 1994; Lessl & Lanka, 1994; Alt-Mörbe *et al.*, 1996; Hamilton *et al.*, 2000). Each conjugational system is specialized for each host organism or its function. The Ti plasmid, using a T4SS$_{Ti}$ can transport and integrate DNA into plant cells as well as transfer auxiliary proteins aiding in this process. The RP4 plasmid, on the other hand, is highly effective in moving itself as well as other transmissible plasmids to a multitude of Gram-positive and -negative species. The function of the different elements of both conjugative systems will be discussed in detail and compared to each other in context below.

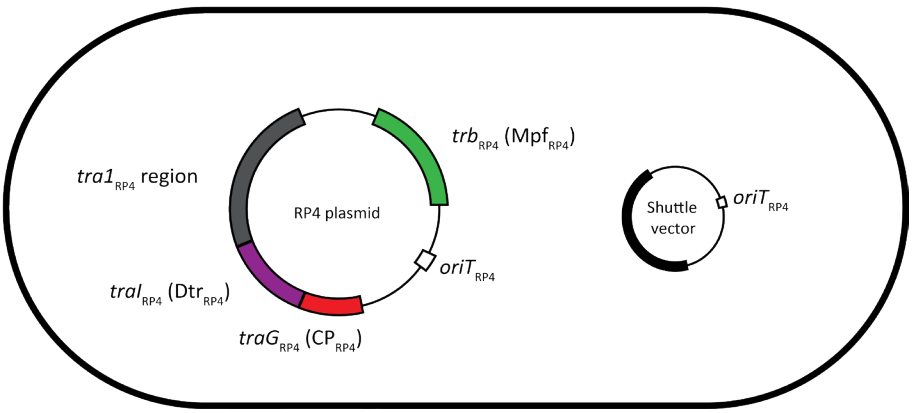

Figure 4. General representation of the RP4 plasmid-based transfer system. This is a general overview as shown in **figure 1**, including Dtr with relaxase (purple), CP (red), the T4SS/Mpf channel (green). The plasmid itself, and a shuttle vector to be transferred, both contain an *oriT*$_{RP4}$ that is recognized by TraI$_{RP4}$. The Dtr, CP and Mpf machinery will transfer both RP4 and the shuttle plasmid into a recipient cell. The plasmid RP4 can be maintained in *A. tumefaciens* as well as in *E. coli*. The figure is not a true spatial representation.

DNA transfer and recognition system (Dtr)

Regardless of the further details, the transferred DNA must contain at least one recognition site for the Dtr system. This *cis*-acting sequence, the *oriT*, is essential for conjugative transfer and contains the site where the relaxase forms a nick (Fuqua & Winans, 1996). The relaxase requires the assistance of auxiliary proteins able to open the double-stranded DNA sequence at the *oriT* recognition site to provide access of the relaxase to the nicking site.

The Dtr_{RP4} system of the RP4 plasmid consists of the relaxase $TraI_{RP4}$ and its auxiliary proteins $TraH_{RP4}$, $TraJ_{RP4}$ and $TraK_{RP4}$. Plasmids that can be mobilized by the conjugative RP4 plasmid contain a specific $oriT_{RP4}$ (Waters *et al.*, 1991). The $oriT_{RP4}$ is recognized by the relaxase $TraI_{RP4}$ and by the auxiliary proteins $TraH_{RP4,}$ $TraJ_{RP4}$ and $TraK_{RP4}$, collectively forming the relaxosome complex. All these proteins are encoded in the Dtr_{RP4} operon in the $tra1_{RP4}$ region of RP4 (Ziegelin *et al.*, 1991). The $oriT_{RP4}$ contains 19 bp inverted repeat sequences that are recognized by $TraJ_{RP4}$, recruiting the relaxase $TraI_{RP4}$ to bind to its nicking site (Ziegelin, Fürste & Lanka, 1989). The binding site for $TraI_{RP4}$ is called *sri*, for $TraJ_{RP4}$ *srj*, and for $TraK_{RP4}$ *srk* (Pansegrau & Lanka, 1996). $TraH_{RP4}$ can stabilize the binding of $TraJ_{RP4}$ and $TraI_{RP4}$ as a kind of scaffold protein and does not bind DNA by itself (Pansegrau & Lanka, 1996). $TraK_{RP4}$ is thought to unwind superhelical DNA for easier access of $TraI_{RP4}$ to the $oriT_{RP4}$. The DNA strand is wrapped around a multimeric complex of $TraK_{RP4}$ that can recognize *srk*, a site 180 bp downstream of the $oriT_{RP4}$-nic_{RP4} site (Ziegelin *et al.*, 1992). $TraK_{RP4}$ is essential for the conjugative process *in vivo* (Fürste *et al.*, 1989). The relaxases are the key enzymes in DNA processing. Once recruited to the transfer DNA recognition site, they cut the nic_{RP4} site in the recognition sequence, creating a single-strand DNA break in one of the strands (Vogel & Das, 1992; Pansegrau *et al.*, 1990; Pansegrau *et al.*, 1993). The DNA backbone is cleaved by a nucleophilic attack on the phosphate backbone of the DNA strand, started by the hydroxyl group of the tyrosine residue within the active center of the relaxase. The cleavage leads to a nick and the covalent attachment of the 5'-end to the relaxase. As a reverse reaction, the relaxase can again ligate the 5'- and 3'-end of the DNA strand of the $oriT_{RP4}$ without additional energy. This so-called transesterification process could play an important biological role in single-stranded DNA re-circularization in the recipient host cell (Becker & Meyer, 2012). The essential tyrosine of $TraI_{RP4}$ is the Tyr22 residue (Pansegrau *et al.*, 1993).

The Ti plasmid contains two distinct Dtr_{Ti} systems, one for conjugative plasmid transfer of the Ti plasmid, encoded in the tra_{Ti} and trb_{Ti} regions, and one for T-DNA transfer encoded in the vir_{Ti} regions (Christie & Gordon, 2014). This study focused on the Dtr_{Ti} system involved in T-DNA transfer. This Dtr_{Ti} system consists of the relaxase $VirD2_{Ti}$, and its auxiliary proteins $VirD1_{Ti}$, $VirC1_{Ti}$ and $VirC2_{Ti}$. They are encoded in the $virD_{Ti}$ and $virC_{Ti}$ operon. The T-DNA is defined by two 25 bp imperfect direct repeats, the right-border (RB_{Ti}) and the left-border (LB_{Ti}) sequences. The border sequences contain the nicking sites nic_{Ti} for the relaxase $VirD2_{Ti}$. Within the extensively studied octopine Ti plasmids, the octopine RB_{Ti} is flanked by an upstream enhancer domain. The enhancer is also called overdrive (OD_{Ti}) aiding to increase the nicking by the relaxase (Toro *et al.*, 1988). The OD_{Ti} domain is recognized by $VirC1_{Ti}$ (Toro *et al.*, 1989) and together with $VirC2_{Ti}$ they facilitate the nicking reaction of $VirD2_{Ti}$ and $VirD1_{Ti}$ (Lu *et al.*, 2009). $VirC2_{Ti}$ contains a conserved ribbon-helix-helix (RHH) DNA binding domain to initiate the contact with the recognition site (Lu *et al.*, 2009). The $VirD2_{Ti}$ nicking of the border sequences is facilitated by the essential tyrosine 29 (Tyr29) in its active center (Vogel & Das, 1992). After DNA nicking, $VirC1_{Ti}$ seems involved in helping the T-strand, coupled to $VirD2_{Ti}$, to be recruited to cellular membrane and associate to the coupling protein $VirD4_{Ti}$ for the subsequent transfer (Atmakuri *et al.*, 2007).

The *nic* sites in RB_{Ti}, $LB_{Ti,}$ as well as in the $oriT_{RP4}$ show similarity and share the common relaxase binding site YATCCTG*Y, with the nicking site marked with an asterisk (Pansegrau & Lanka, 1991). In addition, the relaxases $TraI_{RP4}$ and $VirD2_{Ti}$ are closely related, and belong to the same P-type group of relaxase-*nic* systems (Pansegrau & Lanka, 1991), which is clearly distinct from the Dtr systems of other plasmids such as IncF, IncW and IncQ plasmids (Lanka & Wilkins, 1995).

Coupling protein (CP)

The translocation of the nucleo-protein complex via the T4SS is determined by the CP (**Figure 1**). The CP particulates the specificity of the conjugational system in what precisely is recognized and transported. Although CP are formally not part of the multi-protein complex of the transport channel, they are sometimes described as if being a T4SS protein. The CP is not required for the assembly of the T4SS and the pilus structure (Hamilton *et al.*, 2000). CPs range typically in size of around 600-700 amino acid residues (AA) and possess a minimum of two predicted N-terminal *trans*-membrane domains, with an intervening periplasmic loop of 30-50 residues. The CP contains three domains to ensure functionality: an N-terminal part for association with the membrane, a nucleotide-binding domain, and a C-terminal all-alpha domain (Alvarez-Martinez & Christie, 2009). All CPs have a nucleotide-binding domain with a conserved Walker motif, involved in ATP hydrolysis. The ATPase function could drive DNA transport in conjunction with two other ATPases $VirB4_{Ti}$ and $VirB11_{Ti}$ associated with the $T4SS_{Ti}$ (Llosa *et al.*, 2002; Alvarez-Martinez & Christie, 2009; Cascales *et al.*, 2013). The CP is the first factor contacted by the T-strand/$VirD2_{Ti}$ complex (Cascales & Christie, 2004). The mode of action of CP is not known, but it is thought that that the DNA/protein complex is moved through the middle of a hexameric ring structure comprised of multiple $VirD4_{Ti}$ proteins present at the inner membrane structure of the $T4SS_{Ti}$ channel (Alvarez-Martinez & Christie, 2009). This so-called 'shoot and pump' model for CP mediated substrate transfer via the T4SS seems the most likely pathway (Llosa *et al.*, 2004; Atmakuri, Cascales & Christie, 2004; Cascales *et al.*, 2013). The 'shoot and pump' model refers to the act of the T4SS initially 'shooting' the relaxase bound to the transfer DNA across several membranes and then 'pumping' the rest of the DNA via the CP into the recipient cell (Llosa *et al.*, 2004). The pathway for the transfer of single proteins is still unclear. Although there is substrate selection of the transport system, a yet unknown cell-to-cell contact derived activation signal via the CP is required to mediate the direct transfer of the DNA into the recipient cell (Cascales *et al.*, 2013).

The CP of the RP4 conjugative system is $TraG_{RP4}$, encoded in the $tra1_{RP4}$ region (Ziegelin *et al.*, 1991; Pansegrau *et al.*, 1994), while the CP of the Ti plasmid virulence system is $VirD4_{Ti}$, encoded in the $virD_{Ti}$ operon (Porter *et al.*, 1987).

In certain cases, CP of different conjugative systems can be exchanged and continue their function within other systems. The CP_{RP4} $TraG_{RP4}$ and CP_{R388} $TrwB_{R388}$ can substitute each other's function, but with reduced the efficiency in between plasmids IncW R388 and IncPα RP4. In chimeric systems of both plasmids, the RSF1010 plasmid can be translocated (Cabezon *et al.*, 1997). Within *Agrobacterium*, transfer of the plasmid RSF1010 is dependent on the CP_{Ti} $VirD4_{Ti}$for transfer as it lacks its own CP (Beijersbergen *et al.*, 1992), but transfer of CloDF13 is possible in absence of $VirD4_{Ti}$ as CloDF13 encodes it's on $CP_{CloDF13}$ (Escudero *et al.*, 2003).

Mating pair formation (Mpf) and architecture of the T4SS

After the DNA substrate is successfully processed and associated with the CP, via the covalently bound relaxase, the final step is the transfer from the donor to the recipient cell. The transfer is ensured by the T4SS, a transmembrane spanning protein channel. The structure is essential for the successful transport of the nucleoprotein-complex. Along the transmembrane spanning T4SS an extracellular pilus structure is present, visible by microscopic observation. This pilus structure may be involved in making the initial cell-to-cell contact, but is not necessary for actual DNA transfer.

The T4SS is one of the most abundant secretion systems present in bacteria. The core elements of the T4SS are highly conserved (Segal *et al.*, 1999). The most studied T4SS are those encoded by the conjugative transfer genes of the conjugative F and RP4 plasmids, and the virulence *virB*$_{Ti}$ genes of *A. tumefaciens*. To illustrate the architecture of the T4SS, first the T4SS$_{RP4}$ of the RP4 plasmid is briefly discussed followed by the more detailed functional analysis of the T4SS$_{Ti}$ of *A. tumefaciens*. Other T4SS, their functions and more detailed experimental findings, are excellently reviewed by Alvarez-Martinez & Christie (2009) and Fronzes, Christie & Waksman (2009).

The T4SS$_{RP4}$ channel of the conjugative plasmid RP4 is made up of the Trb$_{RP4}$ proteins TrbB$_{RP4}$-TrbL$_{RP4}$ encoded in the *trb*$_{RP4}$ operon of the *tra2*$_{RP4}$ region, except for TrbF$_{RP4}$, which is encoded in the *tra1*$_{RP4}$ region (Pansegrau *et al.*, 1994). The elements of T4SS$_{RP4}$ are largely homologous in function to elements of the T4SS$_{Ti}$ encoded by the *virB*$_{Ti}$ operon (Grahn *et al.*, 2000): TrbC$_{RP4}$ (VirB2$_{Ti}$), TrbD$_{RP4}$ (VirB3$_{Ti}$), TrbE$_{RP4}$ (VirB4$_{Ti}$), TrbF$_{RP4}$/TrbJ$_{RP4}$ (VirB5$_{Ti}$), TrbL$_{RP4}$ (VirB6$_{Ti}$), TrbH$_{RP4}$ and TrbK$_{RP4}$ (both possibly VirB7$_{Ti}$), TrbG$_{RP4}$ (VirB9$_{Ti}$), TrbI$_{RP4}$ (VirB10$_{Ti}$), and TrbB$_{RP4}$ (VirB11$_{Ti}$). There are no readily discernable functional homologues for the function of VirB1$_{Ti}$ and VirB8$_{Ti}$ with RP4 proteins (Fernandez *et al.*, 1996; Eisenbrandt *et al.*, 1999; Lawley *et al.*, 2003; Souza *et al.*, 2012). The extracellular pilus structure of RP4 is composed of TrbC$_{RP4}$ and creates a rigid and brittle structure (Haase *et al.*, 1995; Eisenbrandt *et al.*, 1999; Grahn *et al.*, 2000). The Ti plasmid T4SS$_{Ti}$ similarly encodes a pilin subunit VirB2$_{Ti}$ that is used for building a T-pilus on the cell surface.

The T4SS$_{Ti}$ of *A. tumefaciens* is encoded by the *virB*$_{Ti}$ region on the Ti plasmid. The channel structure is made up of 11 Vir$_{Ti}$ proteins (Fronzes, Christie & Waksman, 2009), that can be divided into energy using components (VirB4$_{Ti}$ and VirB11$_{Ti}$) responsible for the physical transfer of the substrate or for morphogenesis of the T-pilus structure (Sagulenko *et al.*, 2001), components of the inner membrane pore (VirB3$_{Ti}$, VirB6$_{Ti}$, VirB8$_{Ti}$ and VirB10$_{Ti}$), and proteins forming the outer membrane complex including the extracellular protruding T-pilus structure (VirB2$_{Ti}$, VirB5$_{Ti}$, VirB7$_{Ti}$ and VirB9$_{Ti}$). The T4SS$_{Ti}$ pilus associated protein VirB1$_{Ti}$ is not part of the structure, but is a transglycosylase that is supposed to aid in the T4SS$_{Ti}$ channel formation as well as the T-pilus biogenesis by opening the cell wall (Zupan *et al.*, 2007). Besides this simplified positional classification of the different Vir$_{Ti}$ proteins, the actual architecture of the transport channel can be deduced using structural data obtained for the closely related T4SS$_{pKM101}$ from the IncN plasmid pKM101 plasmid (Pohlman, Genetti & Winans, 1994; Mortelmans, 2006; Fronzes *et al.*, 2009; Chandran *et al.*, 2009; Rivera-Calzada *et al.*, 2013). Using near atomic scale cryo-electron microscopy a self-assembled core complex (VirB7$_{pKM101}$, VirB9$_{pKM101}$, and VirB10$_{pKM101}$) was observed that is made up of an inner layer and an outer layer, reaching from the inner membrane to the outer membrane, respectively. This core complex is thought to be the central structure of the T4SS$_{pKM101}$, where all other Vir$_{pKM101}$ proteins assemble around, possibly acting as a scaffold. It has an estimated size of 185 Å in diameter and height. The outer layer of the core complex is made up of the full-length VirB7$_{pKM101}$ and the C-terminal parts of VirB9$_{pKM101}$ and VirB10$_{pKM101}$. It is shaped as a helical barrel made up of several copies of the C-terminal part of VirB10$_{pKM101}$. VirB7$_{pKM101}$

anchors the C-terminus of $VirB9_{pKM101}$ into the inner leaflet of this barrel structure (Fronzes *et al.*, 2009; Chandran *et al.*, 2009). The outer layer connects to the distinct inner layer with small and thin linkers. The channel exhibits a constriction at the height of the linker region in between both layers. The diameter of the channel structure varies from 55 Å at its base to a 10 Å constriction at its tip (Rivera-Calzada *et al.*, 2013). These size changes also indicate that a conformational change of the structure is necessary for the substrate transport. It is thought that upon activation $VirB10_{Ti}$ undergoes a conformational change (Cascales & Christie, 2004), and mediates the full conformational change of the pore (Rivera-Calzada *et al.*, 2013). $VirB10_{Ti}$ is not involved in direct contact with the substrate, but indirectly orchestrates its movement through the $T4SS_{Ti}$. The ATPase proteins are located at the cytoplasmic side of the inner membrane, allowing for interaction with the T-complex for transport (Jakubowski *et al.*, 2009). **Figure 5** shows a simplified version of the channel structure.

Besides the transmembrane spanning channel structure, the $VirB_{Ti}/VirD4_{Ti}$ $T4SS_{Ti}$ further contains a structure called the T-pilus (**Figure 5**). The T-pilus forms an extra-cellular protruding structure of $VirB2_{Ti}$, with $VirB5_{Ti}$ forming the tip. It is thought that the T-pilus is not involved in the actual T-DNA transfer but in the interaction with the recipient cell (Christie, 2004; Aly & Baron, 2007; Grohman *et al.*, 2018). The extra-cellular T-pilus structure is long and semi-rigid and seems to be more flexible than the RP4 pilus (Eisenbrandt *et al.*, 1999; Lai & Kado, 2002).

The interaction path of the T-strand/$VirD2_{Ti}$ complex within the $T4SS_{Ti}$ was measured by using a specific transfer DNA immunoprecipitation (TrIP) assay (Cascales & Christie, 2004). After processed T-strands interact with the coupling protein $VirD4_{Ti}$, the second ATPase $VirB11_{Ti}$ binds to the single-strand DNA molecule. The subsequent steps during the transfer process are not fully understood yet. It is proposed that T-strands interact further with $VirB7_{Ti}$, $VirB8_{Ti}$ and $VirB9_{Ti}$ (Atmakuri, Cascales & Christie, 2004). As mentioned before, $VirB10_{Ti}$ guides the conformational change of the channel, but so far, this has only been measured indirectly. A specific mutation in $VirB10_{Ti}$ and its homolog $TraF_{pKM101}$ showed a 'gating' defect of the T4SS, allowing unregulated protein leakage in the extracellular space (Banta *et al.*, 2011).

From a broader perspective, the T4SS can be differentiated into three groups according to their function (Christie and Vogel, 2000; Cascales & Christie, 2003), without taking the phylogenetic distribution of all different T4SS into account (Guglielmini, de la Cruz & Rocha, 2013). All three groups can be found in Gram-negative bacteria (Alvarez-Martinez & Christie, 2009). First, the group participating in conjugative systems, for example, the conjugative plasmids F, R388 and RP4. This group of T4SS is the only one also present in Gram-positive bacteria as well as in archaea (Grohmann, Muth & Espinosa, 2003; Alvarez-Martinez & Christie, 2009). Secondly, the DNA uptake and release systems, that either import free DNA from the surrounding environment and provide for a natural competence, as it occurs for example in *Helicobacter pylori*, or excrete DNA to its environment, as *Neisseria gonorrhoea* is capable of. The third category consists of effector translocator systems, playing a profound role in the pathogenicity and infection of many microorganisms (Cascales & Christie, 2003). The T4SSs of *Helicobacter pylori* and of *Legionella pneumophila* belong to this group. The $T4SS_{Ti}$ encoded by the *A. tumefaciens* virulence system is special, as besides DNA, also a set of effector proteins are translocated (not bound at the T-complex), attributing to the bacterial virulence to infect and transform plant tissue. Nevertheless, the fundamental process of T-DNA transfer by $T4SS_{Ti}$ is similar to that by the true conjugative group, with the difference that effector proteins are translocated independently of DNA transfer. This grouping does not provide a complete and definite categorization of the different T4SS, but manages to divide the vast number of different systems into three defined by function. An earlier classification of the T4SS grouped the systems into a general IVA class with systems

similar to the *A. tumefaciens* $VirB_{Ti}/VirD4_{Ti}$ $T4SS_{Ti}$ (that included conjugative and effector translocator systems), class IVB with systems similar to the T4SS of IncI plasmids and the *Legionella pneumophila* Dot/Icm system, and the class 'others' for unclassified systems (Christie & Vogel, 2000; Christie *et al.*, 2005). A more recent study based on a phylogenic analysis of T4SS proposed to create a new classification based on the highly conserved VirB4 protein present in most T4SS (Guglielmini, de la Cruz & Rocha, 2013).

T4SS are able to transfer effector proteins with or without DNA transfer (Christie, 2004). In general, protein translocation requires a full T4SS and a CP as well as cell-to-cell contact. There is one exception with the *Bordetella pertussis* toxin translocation. The pathogenic *B. pertussis* contains a T4SS named Ptl, that has no $VirD4_{Ti}$ homolog, hence does not contain a CP. The pertussis toxin is transferred through the inner membrane via the general secretory (*sec*) pathway and from there it is translocated via the $T4SS_{Ptl}$ (Locht, Coutte & Mielcarek, 2011; Christie, Whitaker & González-Rivera, 2014). Toxin secretion does not require cell-to-cell contact; the pertussis toxin is rather secreted in the vicinity of host cells and taken up by cells due to an intrinsic property of the toxin.

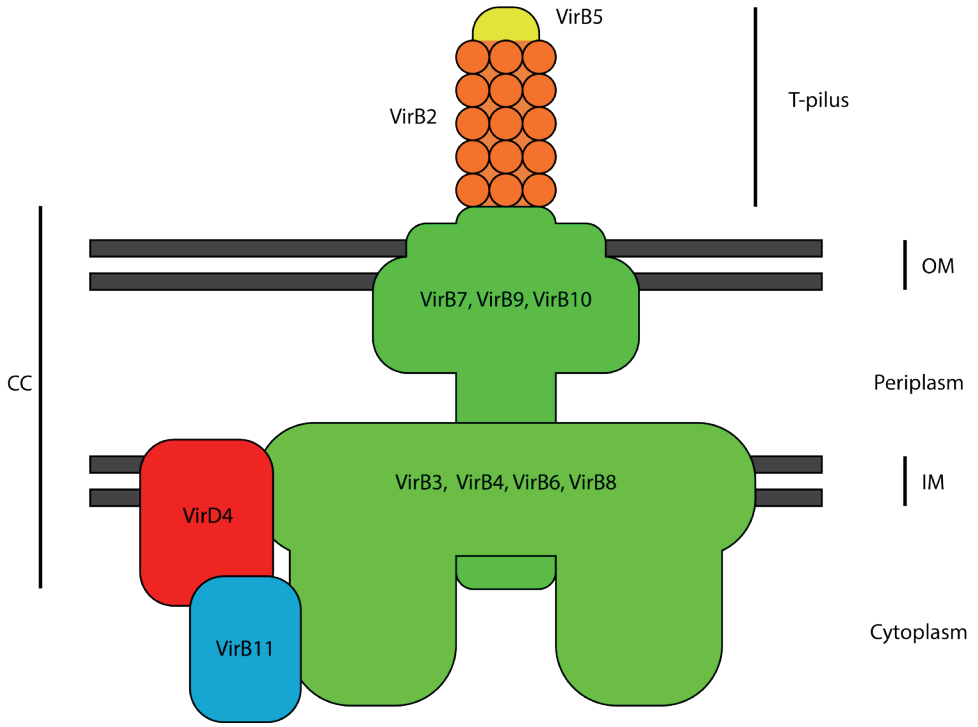

Figure 5. Simplified structure of a VirB/VirD4-type T4SS channel and pilus. This overview depicts the structural assembly across a Gram-negative cell wall spanning both across the inner membrane (IM) and outer membrane (OM). The core complex (CC) reaches across both membranes and comprises of VirB7, VirB9 and VirB10 on top and the central chamber complex of VirB3, VirB4, VirB6 and VirB8. Within the inner membrane, the CP VirD4 is connecting with an ATPase VirB11 to the main complex ATPase VirB4. The extracellular pilus structure is assembled from VirB2 and VirB5 aided by VirB1 (not shown as it is not part of the final assembled structure). The figure is not a true spatial representation.

Trans-cellular transport

The physical transfer of the conjugational substrate in between donor and recipient cell is still not fully determined. The initial cell-to-cell contact is concerted by the extracellular conjugative pilus structure (Willetts & Wilkins, 1984). However, the function of the extracellular conjugative pilus during the transfer is not clear. There are two different hypotheses for the transfer. In both cases, a mating group, an accumulation of cells of donor and recipient cells, must form first. The proximity of the cells then triggers the conjugative process. In one case, it is thought that two cells attached to each other, form a mating-bridge/pore for transfer of DNA and proteins by fusion of both membranes. In the other case, it is thought that the extracellular pilus structure is responsible for the DNA and protein transfer between two not necessarily touching cells. Experiments showed that conjugation could occur when cells were physically separated. DNA transfer, mediated by the F plasmid, was shown to be successful when cells were not interacting via their membranes. Those experiments led to the conclusion that the DNA must have been transferred via the extracellular F-pilus structure (Ou & Anderson, 1970; Achtman, Morelli & Schwuchow, 1978; Willetts & Wilkins, 1984; Harrington & Rogerson, 1990). On the other hand, a clear association of mating pairs via membrane-to-membrane seemed to be the pre-requisite for a successful DNA transfer using a T4SS of the F plasmid and RP4 plasmid (Panicker & Minkley, 1985; Samuels, Lanka & Davies, 2000). This indicated that DNA transfer was not performed via the pilus structure. Further evidence was provided by direct visualization of transformed cells using light microscopy. This experiment showed that a direct cell-to-cell contact was necessary for successful DNA transfer mediated by IncPβ R751 T4SS (Lawley *et al.*, 2002). This could be different for the $VirB2_{Ti}$/$VirB5_{Ti}$ Ti plasmid T-pilus as it contains an inner cylindrical lumen (Haase *et al.*, 1995; Eisenbrandt *et al.*, 1999; Fronzes, Christie & Waksman, 2009) that could allow for substrate translocation. So far, contrary to the F-pilus function as discusses above, DNA transfer via the Ti- and RP4-pilus structure has not been experimentally proven yet. While an early idea considered the formation of a mating bridge/pore by pore-forming proteins of the Mpf system to be the conjugational system (Willetts & Wilkins, 1984), it is now evident that the transmembrane spanning core complex of the VirB/virD4-like T4SS (for Tra_{RP4} as well as for the $VirB_{Ti}$) must be the mating pore able to deliver the T-complex into the recipient cell (Grohmann *et al.*, 2018). It is still unknown how the membrane of the recipient cell is manipulated for the conjugative transfer. So far, no evidence is available showing that a T4SS complex protrudes inside the recipient cell membrane.

Bacterial conjugation in biotechnology and implication for future research

With the background of increasing occurrences of human pathogens acquiring multi-resistance against antibiotic treatment, there is not only a need to research antibiotics but also a need for understanding the spread of antibiotic resistance through conjugational systems. Bacterial conjugation research was very active during the 1970s and 1980s, but stagnated in recent years. Understanding the interaction of individual parts of T4SS allows for identifying suitable targets to optimize the process, or if needed to efficiently disrupt the process. Furthermore, there can be more straightforward biotechnological applications of conjugative systems. Conjugative systems allow for a speedy transfer of large quantities of genetic information into a recipient cell. However, most methods need to be tailor-made for each particular task. Incompatibility of donor and recipient cell and material to be transferred commonly occurs. To find solutions to those problems, easy to handle and easy to combine conjugational systems are essential for efficient future applications.

Outline of this dissertation

Agrobacterium-mediated transformation is a robust system for plant transformation in academia and industry. Up to date, only a few improvements have been made to this methodology, for example adjustments of cultivation conditions, identification of high virulence mutations of *A. tumefaciens* or use of site-specific nucleases to achieve targeted insertions. A goal in plant transformation would be the use of protein therapy, enabling genomic modification without integration of foreign DNA, by just transferring effector molecules into a recipient cell. Protein therapy could be achieved for example by transferring non-integrative T-strands, from modified T-DNA, which will transiently lead to the expression of desired proteins. **Chapter 2** explores for the first time a direct modification of T-DNA termini, aimed to influence the transfer and integration of the T-DNA. A mutation in one of the T-DNA end-modifications was discovered, that influenced T-strand end processing within the recipient cells after transfer via the $T4SS_{Ti}$. In plant cells, this mutation facilitated faster protein expression, and in yeast, the mutation negatively influenced T-DNA integration.

In order to study single components of AMT more thoroughly, it would be advantageous to find an easy to handle model organism for high-throughput screening. Commonly, plant or yeast cells are used for AMT research, but all showed to be cumbersome and inefficient. *Streptomyces*, on the other hand, a Gram-positive bacterium from the same biome as *Agrobacterium*, shares a similar size, cultivation conditions as well as growth rates. Those attributes could be beneficial to create a new screening platform for AMT research. **Chapter 3** explores this model organism as a potential recipient for AMT and compared transfer by AMT with RP4-mediated transformation. Studying these two conjugative systems with their specific functions side by side in the same model organism is highly favorable for in-depth comparative analysis. Unfortunately, AMT to *Streptomyces* was not successful within the tested conditions, challenging previously published results. Nevertheless, for the first time, an RP4-mediated transformation from *A. tumefaciens* to *Streptomyces* was described. RP4-mediated transformation between *E. coli* strains and between *E. coli* and *Streptomyces* has been described previously, and is routinely used for biotechnological application. RP4-mediated transfer of genetic information between *Agrobacterium* and *Streptomyces* has not been reported yet in literature.

The plasmid RP4 was shown to be a very robust and capable translocation system for large DNA sequences. The encoded $T4SS_{RP4}$ is in comparison to the $T4SS_{Ti}$ of the Ti plasmid easier to handle in laboratory conditions. **Chapter 4** describes the characterization of the RP4 relaxase translocation signal. The signal is further tested for possible applications of protein and DNA transport. Here, for the first time, protein only transport from RP4 to recipient cells was shown; indicating that RP4 could be further modified and used efficiently in biotechnological applications.

The hybridization of both conjugational systems of the Ti plasmid and RP4 plasmid is described in **Chapter 5**. This segment ventures in the creation of a modular conjugational Dtr system that could be easily adapted for biotechnological adaptions. The hybridization of the relaxases was studied, as well as the exchange and use of the channel proteins between both systems and modification of the recognition sequence for DNA transfer. Single modules of the hybrid relaxases have been created and tested to be able to be transferred via T4SSs. Further, a fully functional operon expressing essential auxiliary proteins as well as the $VirD2_{Ti}$-$TraI_{RP4}$ hybrid relaxes was created. No transfer was detected so far, but a limited proof of principle for the modular conjugational system could be delivered.

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