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Chapter 1 General introduction

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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 extrachromosomal 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 nonmobilizable 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 Gramnegative *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 μ m in length. The bar = 1 μ m. 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*_T-region, which encodes for all virulence proteins involved in transfer. **B:** The binary system, widely used for AMT, separates the *vir*_T-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 selftransmissible 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 Vir A_{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 Vir G_{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 VirD 2_{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 VirCl_{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 IncPa plasmid (Bryan, van den Elzen & Tseng, 1972; Saunders & Grinsted, 1972; Pansegrau et al., 1994). The plasmid RP4 is part of the IncPa 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.



A. tumefaciens LBA2210 / E. coli DH10B

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 *ori* T_{RP4} (Waters *et al.*, 1991). The *ori* T_{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 $tral_{RP4}$ 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 $TraJ_{RP4}$ 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). Tra H_{RP4} can stabilize the binding of Tra J_{RP4} and Tra J_{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 $VirD2_{Tip}$ 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 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 IncPa 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*_{T1} 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_{T1} 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 fulllength 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_{Tb} 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-tomembrane 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 IncP β R751 T4SS (Lawley *et al.*, 2002). This could be different for the VirB_{Ti}/VirB_{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 RP4pilus 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 multiresistance 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. RP4mediated transfer of genetic information between Agrobacterium and Streptomyces has not been reported vet 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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References

- Achtman M, Morelli G & Schwuchow S (1978) Cell-cell interactions in conjugating *Escherichia coli*: role of F pili and fate of mating aggregates. *J. Bacteriol.* **135**: 1053–1061
- Aguilar J, Cameron TA, Zupan J & Zambryski P (2011) Membrane and core periplasmic *Agrobacterium tumefaciens* virulence Type IV secretion system components localize to multiple sites around the bacterial perimeter during lateral attachment to plant cells. *MBio* **2**: e00218-11
- Alt-Mörbe J, Stryker JL, Fuqua C, Li PL, Farrand SK & Winans SC (1996) The conjugal transfer system of Agrobacterium tumefaciens octopine-type Ti plasmids is closely related to the transfer system of an IncP plasmid and distantly related to Ti plasmid vir genes. J. Bacteriol. 178: 4248–4257
- Alvarez-Martinez CE & Christie PJ (2009) Biological diversity of prokaryotic type IV secretion systems. Microbiol. Mol. Biol. Rev. 73: 775–808
- Aly KA & Baron C (2007) The VirB5 protein localizes to the T-pilus tips in *Agrobacterium tumefaciens*. *Microbiology* **153:** 3766–3775
- Atmakuri K, Cascales E, Burton OT, Banta LM & Christie PJ (2007) Agrobacterium ParA/MinD-like VirC1 spatially coordinates early conjugative DNA transfer reactions. EMBO J. 26: 2540–2551
- Atmakuri K, Cascales E & Christie PJ (2004) Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. *Mol. Microbiol.* **54**: 1199–1211
- Babic A, Guérout A-M & Mazel D (2008) Construction of an improved RP4 (RK2)-based conjugative system. Res. Microbiol. 159: 545–549
- Bakkali M (2013) Could DNA uptake be a side effect of bacterial adhesion and twitching motility? *Arch. Microbiol.* **195:** 279–289
- Balzer D, Pansegrau W & Lanka E (1994) Essential motifs of relaxase (TraI) and TraG proteins involved in conjugative transfer of plasmid RP4. J. Bacteriol. 176: 4285–4295
- Banta LM, Kerr JE, Cascales E, Giuliano ME, Bailey ME, McKay C, Chandran V, Waksman G & Christie PJ (2011) An Agrobacterium VirB10 mutation conferring a type IV secretion system gating defect. J. Bacteriol. 193: 2566–2574
- Barton KA, Binns AN, Matzke AJM & Chilton MD (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. *Cell* **32**: 1033– 1043
- Becker EC & Meyer R (2012) Origin and fate of the 3' ends of single-stranded DNA generated by conjugal transfer of plasmid R1162. *J. Bacteriol.* **194:** 5368–5376
- Beijersbergen AGM, den Dulk-Ras AD, Schilperoort RA & Hooykaas PJJ (1992) Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. Science **256**: 1324–1327
- Bhatty M, Laverde Gomez JA & Christie PJ (2013) The expanding bacterial type IV secretion lexicon. *Res. Microbiol.* **164:** 620–639
- Brencic A & Winans SC (2005) Detection of and response to signals involved in host-microbe interactions by plantassociated bacteria. *Microbiol. Mol. Biol. Rev.* **69:** 155–194
- Bryan LE, Elzen HMVD & Tseng JT (1972) Transferable drug resistance in *Pseudomonas aeruginosa. Antimicrob.* Agents Chemother. 1: 22–29
- Bundock P, Mróczek K, Winkler AA, Steensma HY & Hooykaas PJJ (1999) T-DNA from *Agrobacterium tumefaciens* as an efficient tool for gene targeting in *Kluyveromyces lactis. Mol. Gen. Genet.* **261:** 115–121
- Bushman F (2002) Lateral DNA Transfer: Mechanisms and Consequences Cold Spring Harbor Laboratory Press
- Byrd DR & Matson SW (1997) Nicking by transesterification: the reaction catalysed by a relaxase. *Mol. Microbiol.* **25:** 1011–1022
- Cabezon E, Sastre JI & de la Cruz F (1997) Genetic evidence of a coupling role for the TraG protein family in bacterial conjugation. *Mol. Gen. Genet.* **254**: 400–406

- Cabezon E, Ripoll-Rozada J, Pena A, de la Cruz F & Arechaga I (2014) Towards an integrated model of bacterial conjugation. *FEMS Microbiol. Rev.* **39**: 81–95
- Canchaya C, Fournous G, Chibani-Chennoufi S, Dillmann M-L & Brüssow H (2003) Phage as agents of lateral gene transfer. *Curr. Opin. Microbiol.* **6:** 417–424
- Carroll AC & Wong A (2018) Plasmid persistence: costs, benefits, and the plasmid paradox. Can. J. Microbiol. 64: 293-304
- Cascales E, Atmakuri K, Sarkar MK & Christie PJ (2013) DNA substrate-induced activation of the *Agrobacterium* VirB/VirD4 type IV secretion system. *J. Bacteriol.* **195:** 2691–2704
- Cascales E & Christie PJ (2003) The versatile bacterial type IV secretion systems. Nat. Rev. Microbiol. 1: 137-149
- Cascales E & Christie PJ (2004) Definition of a bacterial type IV secretion pathway for a DNA substrate. *Science* **304**: 1170–1173
- Chandran V, Fronzes R, Duquerroy S, Cronin N, Navaza J & Waksman G (2009) Structure of the outer membrane complex of a type IV secretion system. *Nature* **462**: 1011–1015
- Chen I & Dubnau D (2004) DNA uptake during bacterial transformation. Nat. Rev. Microbiol. 2: 241-249
- Christie PJ (2001) Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol. Microbiol.* **40**: 294–305
- Christie PJ (2004) Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. *Biochim Biophys* Acta **1694**: 219–234
- Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S & Cascales E (2005) Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu. Rev. Microbiol.* **59**: 451–485
- Christie PJ & Gordon JE (2014) The Agrobacterium Ti plasmids. Microbiol. Spectr. 2: 295-313
- Christie PJ & Vogel JP (2000) Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol.* 8: 354–360
- Citovsky V, de Vos G & Zambryski P (1988) Single-stranded DNA binding protein encoded by the *virE* locus of *Agrobacterium tumefaciens*. *Science*. **240**: 501–504
- Christie PJ, Whitaker N & González-Rivera C (2014) Mechanism and structure of the bacterial type IV secretion systems. *Biochim. Biophys. Acta Mol. Cell Res.* **1843:** 1578–1591
- Clark AJ & Adelberg EA (1962) Bacterial conjugation. Annu. Rev. Microbiol. 16: 289-319
- Clark AJ & Warren GJ (1979) Conjugal transmission of plasmids. Annu. Rev. Genet. 13: 99-125
- Costa TRD, Ilangovan A, Ukleja M, Redzej A, Santini JM, Smith TK, Egelman EH & Waksman G (2016) Structure of the bacterial sex F pilus reveals an assembly of a stoichiometric protein-phospholipid complex. *Cell* **166**: 1436–1444
- Couturier M, Bex F, Bergquist PL & Maas WK (1988) Identification and classification of bacterial plasmids. *Microbiol. Rev.* **52**: 375–395
- Datta N & Hedges RW (1972) Host ranges of R factors. J. Gen. Microbiol. 70: 453-460
- De Cleene M & De Ley J (1976) The host range of crown gall. Bot. Rev. 42: 389-466
- Del Solar G & Espinosa M (2002) Plasmid copy number control: an ever-growing story. Mol. Microbiol. 37: 492-500
- Ditta G, Stanfield S, Corbin D & Helinski DR (1980) Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. 77: 7347–3751
- Dubnau D (1999) DNA uptake in bacteria. Annu. Rev. Microbiol. 53: 217-244
- Eisenbrandt R, Kalkum M, Lai EM, Lurz R, Kado CI & Lanka E (1999) Conjugative pili of IncP plasmids, and the Ti plasmid T pilus are composed of cyclic subunits. *J. Biol. Chem.* **274**: 22548–22555
- Escudero J, den Dulk-Ras AD, Regensburg-Tuïnk TJG & Hooykaas PJJ (2003) VirD4-independent transformation by CloDF13 evidences an unknown factor required for the genetic colonization of plants via *Agrobacterium*. *Mol. Microbiol.* **47:** 891–901
- Farrand S, Hwang I & Cook D (1996) The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. *J. Bacteriol.* **178:** 4233–4247

- Fernández-López R, Garcillán-Barcia MP, Revilla C, Lázaro M, Vielva L & de la Cruz F (2006) Dynamics of the IncW genetic backbone imply general trends in conjugative plasmid evolution. *FEMS Microbiol. Rev.* **30**: 942–966
- Fernandez D, Spudich GM, Zhou XR & Christie PJ (1996) The Agrobacterium tumefaciens VirB7 lipoprotein is required for stabilization of VirB proteins during assembly of the T-complex transport apparatus. J. Bacteriol. 178: 3168–3176
- Fronzes R, Christie PJ & Waksman G (2009) The structural biology of type IV secretion systems. *Nat. Rev. Microbiol.* 7: 703–714
- Fronzes R, Schäfer E, Wang L, Saibil HR, Orlova E V & Waksman G (2009) Structure of a type IV secretion system core complex. *Science* **323**: 266–268
- Frost LS, Leplae R, Summers AO & Toussaint A (2005) Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* **3:** 722–732
- Fuqua C & Winans SC (1996) Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. *Journals Bacteriol.* **178:** 435–440
- Fürste JP, Pansegrau W, Ziegelin G, Kröger M & Lanka E (1989) Conjugative transfer of promiscuous IncP plasmids: interaction of plasmid-encoded products with the transfer origin. Proc. Natl. Acad. Sci. U.S.A. 86: 1771–1775
- Gao R & Lynn DG (2005) Environmental pH sensing: resolving the VirA/VirG two-component system inputs for Agrobacterium pathogenesis. J. Bacteriol. 187: 2182–2189
- Garcillán-Barcia MP, Alvarado A & De la Cruz F (2011) Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiol. Rev.* **35**: 936–956
- Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao Y, Askenazi M, Halling C, Mullin L, Houmiel K, Gordon J, Vaudin M, Iartchouk O, Epp A, Liu F, Wollam C, Allinger M, Doughty D, et al. (2001) Genome sequence of the plant pathogen and biotechnology agent Agrobacterium tumefaciens C58. Science 294: 2323–2328
- Grahn AM, Haase J, Bamford DH & Lanka E (2000) Components of the RP4 conjugative transfer apparatus form an envelope structure bridging inner and outer membranes of donor cells: implications for related macromolecule transport systems. J. Bacteriol. **182**: 1564–1574
- Grohmann E, Christie PJ, Waksman G & Backert S (2018) Type IV secretion in Gram-negative and Gram-positive bacteria. *Mol. Microbiol.* **107:** 455–471
- Grohmann E, Muth G & Espinosa M (2003) Conjugative plasmid transfer in Gram-positive bacteria. *Microbiol. Mol. Biol. Rev.* **67:** 277–301
- Guglielmini J, de la Cruz F & Rocha EPC (2013) Evolution of conjugation and type IV secretion systems. *Mol. Biol. Evol.* **30**: 315–331
- Haase J, Lurz R, Grahn AM, Bamford DH & Lanka E (1995) Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific phage propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. J. Bacteriol. 177: 4779–4791
- Halary S, Leigh JW, Cheaib B, Lopez P & Bapteste E (2010) Network analyses structure genetic diversity in independent genetic worlds. *Proc. Natl. Acad. Sci. U.S.A.* **107:** 127–132
- Hamilton CM, Lee H, Li PL, Cook DM, Piper KR, Von Bodman SB, Lanka E, Ream W & Farrand SK (2000) TraG from RP4 and TraG and VirD4 from Ti plasmids confer relaxosome specificity to the conjugal transfer system of pTiC58. *J. Bacteriol.* **182:** 1541–1548
- Harrington LC & Rogerson AC (1990) The F pilus of *Escherichia coli* appears to support stable DNA transfer in the absence of wall-to-wall contact between cells. *J. Bacteriol.* **172:** 7263–7264
- Harrison E & Brockhurst MA (2012) Plasmid-mediated horizontal gene transfer is a coevolutionary process. *Trends Microbiol.* **20**: 262–267
- Hedges RW & Jacob AE (1974) Transposition of ampicillin resistance from RP4 to other replicons. *Mol. Gen. Genet.* **132:** 31–40
- Hermansson M & Linberg C (1994) Gene transfer in the marine environment. FEMS Microbiol. Ecol. 15: 47-54

Hinnebusch J & Tilly K (1993) Linear plasmids and chromosomes in bacteria. Mol. Microbiol. 10: 917-922

- Hoekema A, Hirsch PR, Hooykaas PJJ & Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir-* and T-region of the *Agrobacterium tumefaciens* Ti plasmid. *Nature* **303**: 179–180
- Hooykaas PJJ & Beijersbergen AGM (1994) The virulence system of Agrobacterium tumefaciens. Annu. Rev. Phytopathol. 32: 157-181
- Hooykaas PJJ, den Dulk-Ras H, Ooms G & Schilperoort RA (1980) Interactions between octopine and nopaline plasmids in *Agrobacterium tumefaciens*. J. Bacteriol. **143**: 1295–1306
- Hu X, Zhao J, DeGrado WF & Binns AN (2013) *Agrobacterium tumefaciens* recognizes its host environment using ChvE to bind diverse plant sugars as virulence signals. *Proc. Natl. Acad. Sci. U.S.A.* **110:** 678–683
- Ingram LC, Richmond MH & Sykes RB (1973) Molecular characterization of the R factors implicated in the carbenicillin resistance of a sequence of *Pseudomonas aeruginosa* strains isolated from burns. *Antimicrob. Agents Chemother.* **3:** 279–288
- Jacob AE & Grinter NJ (1975) Plasmid RP4 as a vector replicon in genetic engineering. 255: 504-506
- Jain A & Srivastava P (2013) Broad host range plasmids. FEMS Microbiol. Lett. 348: 87-96
- Jakubowski SJ, Kerr JE, Garza I, Krishnamoorthy V, Bayliss R, Waksman G & Christie PJ (2009) Agrobacterium VirB10 domain requirements for type IV secretion and T pilus biogenesis. Mol. Microbiol. 71: 779–794
- Jin SG, Prusti RK, Roitsch T, Ankenbauer RG & Nester EW (1990) Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of VirG. J. Bacteriol. **172:** 4945–4950
- Johnson CM & Grossman AD (2015) Integrative and conjugative elements (ICEs): what they do and how they work. Annu. Rev. Genet. **49**: 577–601
- Kado CI (1998) Origin and evolution of plasmids. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 73: 117-126
- Lai EM & Kado CI (2000) The T-pilus of Agrobacterium tumefaciens. Trends Microbiol. 8: 361-369
- Lang S, Kirchberger PC, Gruber CJ, Redzej A, Raffl S, Zellnig G, Zangger K & Zechner EL (2011) An activation domain of plasmid R1 TraI protein delineates stages of gene transfer initiation. *Mol. Microbiol.* 82: 1071–1085
- Lanka E & Wilkins BM (1995) DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. 64: 141-169
- Lawley TD, Klimke WA, Gubbins MJ & Frost LS (2003) F factor conjugation is a true type IV secretion system. *FEMS Microbiol. Lett.* **224:** 1–15
- Lawley TD, Gordon GS, Wright A & Taylor DE (2002) Bacterial conjugative transfer: visualization of successful mating pairs and plasmid establishment in live *Escherichia coli*. *Mol. Microbiol*. **44**: 947–956
- Lederberg J & Tatum EL (1946) Gene recombination in Escherichia coli. Nature 158: 558
- Lessl M & Lanka E (1994) Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. *Cell* 77: 321–324
- Lessl M, Pansegrau W & Lanka E (1992) Relationship of DNA-transfer-systems: essential transfer of plasmids RP4, Ti an F share common sequences. *Nucleic Acids Res.* **20**: 6099–6100
- Levin RA, Farrand SK, Gordon MP & Nester EW (1976) Conjugation in *Agrobacterium tumefaciens* in the absence of plant tissue. *J. Bacteriol.* **127**: 1331–1336
- Liang Z & Tzfira T (2013) In vivo formation of double-stranded T-DNA molecules by T-strand priming. Nat. Commun. 4: 1–8
- Liu C-N, Steck TR, Habeck LL, Meyer JA & Gelvin SB (1993) Multiple copies of *virG* allow induction of *Agrobacterium tumefaciens vir* genes and T-DNA processing at alkaline pH. *Mol. Plant-Microbe Interact.* 6: 144–156
- Llosa M, Gomis-Rüth FX, Coll M & de la Cruz F (2002) Bacterial conjugation: a two-step mechanism for DNA transport. *Mol. Microbiol.* **45:** 1–8
- Locht C, Coutte L & Mielcarek N (2011) The ins and outs of pertussis toxin. FEBS J. 278: 4668-4682
- Lu J, den Dulk-Ras AD, Hooykaas PJJ & Glover JNM (2009) Agrobacterium tumefaciens VirC2 enhances T-DNA transfer and virulence through its C-terminal ribbon-helix-helix DNA-binding fold. Proc. Natl. Acad. Sci. U.S.A. 106: 9643–9648
- Matthysse AG (2014) Attachment of Agrobacterium to plant surfaces. Front. Plant Sci. 5: 1-8

- Melchers LS, Regensburg-Tuïnk TJ, Bourret RB, Sedee NJ, Schilperoort RA & Hooykaas PJJ (1989) Membrane topology and functional analysis of the sensory protein VirA of *Agrobacterium tumefaciens*. *EMBO J.* **8**: 1919–1925
- Mortelmans K (2006) Isolation of plasmid pKM101 in the Stocker laboratory. Mutat. Res. 612: 151-164
- Nair GR, Liu Z & Binns AN (2003) Reexamining the role of the accessory plasmid pAtC58 in the virulence of Agrobacterium tumefaciens strain C58. Plant Physiol. 133: 989–999
- Narasimhulu SB, Deng XB, Sarria R & Gelvin SB (1996) Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. *Plant Cell* 8: 873–886
- Nazarian P, Tran F & Boedicker JQ (2018) Modeling multispecies gene flow dynamics reveals the unique roles of different horizontal gene transfer mechanisms. *Front. Microbiol.* **9:** 1–11
- Novick RP (1987) Plasmid incompatibility. Microbiol. Rev. 51: 381-395
- Orlek A, Stoesser N, Anjum MF, Doumith M, Ellington MJ, Peto T, Crook D, Woodford N, Walker AS, Phan H & Sheppard AE (2017) Plasmid classification in an era of whole-genome sequencing: application in studies of antibiotic resistance epidemiology. *Front. Microbiol.* **8**: 182
- Ou JT & Anderson TF (1970) Role of pili in bacterial conjugation. J. Bacteriol. 102: 648-654
- Paget MS, Chamberlin L, Atrih A, Foster SJ & Buttner MJ (1999) Evidence that the extracytoplasmic function sigma factor sigmaE is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J. Bacteriol. 181: 204–211
- Panicker MM & Minkley EG (1985) DNA transfer occurs during a cell-surface contact stage of F-sex factor-mediated bacterial conjugation. J. Bacteriol. 162: 584–590
- Pansegrau W & Lanka E (1991) Common sequence motifs in DNA relaxases and nick regions from a variety of DNA transfer systems. *Nucleic Acids Res.* **19:** 3455
- Pansegrau W, Schoumacher F, Hohn B & Lanka E (1993) Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. *Proc. Natl. Acad.* **90:** 11538–11542
- Pansegrau W, Balzer D, Kruft V, Lurz R & Lanka E (1990) In vitro assembly of relaxosomes at the transfer origin of plasmid RP4. *Proc. Natl. Acad. Sci. U.S.A.* 87: 6555–6559
- Pansegrau W & Lanka E (1996) Mechanisms of initiation and termination reactions in conjugative DNA processing. J. Biol. Chem. 271: 13068–13076
- Pansegrau W, Lanka E, Barth PT, Figurski DH, Guiney DG, Haas D, Helinski DR, Schwab H, Stanisich VA & Thomas CM (1994) Complete nucleotide sequence of Birmingham IncPα plasmids. Compilation and comparative analysis. J. Mol. Biol. 239: 623–663
- Phillips GJ & Funnell BE eds. (2004) Plasmid biology. American Society of Microbiology
- Pohlman RF, Genetti HD & Winans SC (1994) Common ancestry between IncN conjugal transfer genes and macromolecular export systems of plant and animal pathogens. *Mol. Microbiol.* **14**: 655–668
- Porter SG, Yanofsky MF & Nester EW (1987) Molecular characterization of the *virD* operon from *Agrobacterium* tumefaciens. Nucleic Acids Res. 15: 7503–7517
- Possoz C, Ribard C, Gagnat J, Pernodet JL & Guérineau M (2001) The integrative element pSAM2 from *Streptomyces*: kinetics and mode of conjugal transfer. *Mol. Microbiol.* **42**: 159–166
- Rawlings DE & Tietze E (2001) Comparative biology of IncQ and IncQ-like plasmids. *Microbiol. Mol. Biol. Rev.* 65: 481–496
- Relić B, Andjelković M, Rossi L, Nagamine Y & Hohn B (1998) Interaction of the DNA modifying proteins VirD1 and VirD2 of *Agrobacterium tumefaciens*: analysis by subcellular localization in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 9105–9110
- Rivera-Calzada A, Fronzes R, Savva CG, Chandran V, Lian PW, Laeremans T, Pardon E, Steyaert J, Remaut H, Waksman G & Orlova E V (2013) Structure of a bacterial type IV secretion core complex at subnanometre resolution. *EMBO J.* **32:** 1195–1204
- Rogowsky PM, Close TJ, Chimera JA, Shaw JJ & Kado CI (1987) Regulation of the vir genes of Agrobacterium tumefaciens plasmid pTiC58. J. Bacteriol. 169: 5101–5112

- Rossi L, Hohn B & Tinland B (1993) The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. *Mol. Gen. Genet.* **239**: 345–353
- Sagulenko E, Sagulenko V, Chen J & Christie PJ (2001) Role of *Agrobacterium* VirB11 ATPase in T-pilus assembly and substrate selection. *J. Bacteriol.* **183:** 5813–5825
- Samuels AL, Lanka E & Davies JE (2000) Conjugative junctions in RP4-mediated mating of *Escherichia coli*. J. Bacteriol. **182**: 2709–2715
- Saunders JR & Grinsted J (1972) Properties of RP4, an R factor which originated in *Pseudomonas aeruginosa* S8. J. Bacteriol. **112:** 690-696
- Schröder G & Lanka E (2005) The mating pair formation system of conjugative plasmids a versatile secretion machinery for transfer of proteins and DNA. *Plasmid* 54: 1–25
- Segal G, Russo JJ & Shuman HA (1999) Relationships between a new type IV secretion system and the icm/dot virulence system of *Legionella pneumophila*. *Mol. Microbiol.* **34**: 799–809
- Shintani M, Sanchez ZK & Kimbara K (2015) Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. *Front. Microbiol.* **6**: 242
- Smillie C, Garcillán-Barcia MP, Francia M V., Rocha EPC & de la Cruz F (2010) Mobility of plasmids. Microbiol. Mol. Biol. Rev. 74: 434–452
- Sobecky PA & Hazen TH (2009) Horizontal gene transfer and mobile genetic elements in marine systems. In *Horizontal gene transfer: genomes in flux*, Gogarten MB Gogarten JP & Olendzenski LC (eds) pp 435–453. Totowa, NJ: Humana Press
- Souza RC, del Rosario Quispe Saji G, Costa MOC, Netto DS, Lima NCB, Klein CC, Vasconcelos ATR & Nicolás MF (2012) AtlasT4SS: a curated database for type IV secretion systems. *BMC Microbiol.* **12:** 172
- Szpirer CY, Faelen M & Couturier M (2000) Interaction between the RP4 coupling protein TraG and the pBHR1 mobilization protein Mob. *Mol. Microbiol.* **37**: 1283–1292
- Thomas CM & Nielsen KM (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat. Rev. Microbiol.* **3:** 711–721
- Thomas J & Hecht DW (2007) Interaction of *Bacteroides fragilis* pLV22a relaxase and transfer DNA with *Escherichia coli* RP4-TraG coupling protein. *Mol. Microbiol.* **66**: 948–960
- Toro N, Datta A, Yanofsky M & Nester E (1988) Role of the overdrive sequence in T-DNA border cleavage in Agrobacterium. Proc. Natl. Acad. Sci. U.S.A. 85: 8558–8562
- Toro N, Datta A, Carmi OA, Young C, Prusti RK & Nester EW (1989) The *Agrobacterium tumefaciens virC1* gene product binds to overdrive, a T-DNA transfer enhancer. J. Bacteriol. **171**: 6845–6849
- Turk SCHJ, van Lange RP, Regensburg-Tuïnk TJG & Hooykaas PJJ (1994) Localization of the VirA domain involved in acetosyringone-mediated *vir* gene induction in *Agrobacterium tumefaciens*. *Plant Mol. Biol.* **25:** 899–907
- Tzfira T, Li J, Lacroix B & Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. *Trends Genet.* **20:** 375–383
- van Kregten M, Lindhout BI, Hooykaas PJJ & van der Zaal BJ (2009) *Agrobacterium*-mediated T-DNA transfer and integration by minimal VirD2 consisting of the relaxase domain and a type IV secretion system translocation signal. *Mol. Plant. Microbe. Interact.* **22**: 1356–1365
- Velappan N, Sblattero D, Chasteen L, Pavlik P & Bradbury ARM (2007) Plasmid incompatibility: more compatible than previously thought? *Protein Eng. Des. Sel.* **20:** 309–313
- Vergunst AC, Schrammeijer B, den Dulk-Ras AD, de Vlaam CM, Regensburg-Tuïnk TJ & Hooykaas PJJ (2000) VirB/ D4-dependent protein translocation from Agrobacterium into plant cells. Science 290: 979–982
- Vogel AM & Das A (1992) Mutational analysis of Agrobacterium tumefaciens VirD2: tyrosine 29 is essential for endonuclease activity. J. Bacteriol. 174: 303–308
- Vogelmann J, Ammelburg M, Finger C, Guezguez J, Linke D, Flötenmeyer M, Stierhof Y-D, Wohlleben W & Muth G (2011) Conjugal plasmid transfer in *Streptomyces* resembles bacterial chromosome segregation by FtsK/ SpoIIIE. EMBO J. 30: 2246–2254

- Volkova VV, Lu Z, Besser T & Gröhn YT (2014) Modeling the infection dynamics of bacteriophages in enteric Escherichia coli: estimating the contribution of transduction to antimicrobial gene spread. Appl. Environ. Microbiol. 80: 4350–4362
- Waters VL, Hirata KH, Pansegrau W, Lanka E & Guiney DG (1991) Sequence identity in the nick regions of IncP plasmid transfer origins and T-DNA borders of Agrobacterium Ti plasmids. Proc. Natl. Acad. Sci. U.S.A. 88: 1456–1460
- Willetts N & Wilkins B (1984) Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. 48: 24-41
- Zatyka M & Thomas CM (1998) Control of genes for conjugative transfer of plasmids and other mobile elements. *FEMS Microbiol. Rev.* **21**: 291–319
- Ziegelin G, Fürste JP & Lanka E (1989) TraJ protein of plasmid RP4 binds to a 19-base pair invert sequence repetition within the transfer origin. *J. Biol. Chem.* **264**: 11989–11994
- Ziegelin G, Pansegrau W, Lurz R & Lanka E (1992) TraK protein of conjugative plasmid RP4 forms a specialized nucleoprotein complex with the transfer origin. *J. Biol. Chem.* **267**: 17279–17286
- Ziegelin G, Pansegrau W, Strack B, Balzer D, Kröger M, Kruft V & Lanka E (1991) Nucleotide sequence and organization of genes flanking the transfer origin of promiscuous plasmid RP4. *DNA Seq.* **1**: 303–327
- Zupan JR, Citovsky V & Zambryski P (1996) Agrobacterium VirE2 protein mediates nuclear uptake of singlestranded DNA in plant cells. Proc. Natl. Acad. Sci. U.S.A. 93: 2392–2397
- Zupan JR, Hackworth CA, Aguilar J, Ward D & Zambryski P (2007) VirB1 promotes T-pilus formation in the virtype-IV secretion system of Agrobacterium tumefaciens. J. Bacteriol. **189:** 6551–6563