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

Wittleben, F.T.

Chapter 3

Horizontal gene transfer from *Agrobacterium* to *Streptomyces*

Felix T. Wittleben, Martijn Rolloos, Dennis Claessen,
Paul J.J. Hooykaas and Bert J. van der Zaal

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

Abstract

Agrobacterium-mediated transformation (AMT), is a transfer process wherein the bacterium moves a defined part of a plasmid via an inducible type-IV secretion system (T4SS) into a eukaryotic recipient cell. AMT has been developed into an important technique for the generation of transgenic plants and fungi. To expand the use of AMT, more insight into the requirements for successful transfer of DNA is essential. This study was aimed to investigate AMT to the Gram-positive bacterium *Streptomyces lividans*. In theory such AMT to a genetically-tractable bacterium might provide a high-throughput assay to address remaining questions regarding the fate of the transferred DNA, and the role of the virulence proteins involved. AMT to *Streptomyces* was compared to the transfer via the bacterial conjugative plasmid RP4, containing a similar T4SS. Unfortunately, despite the use of a range of different media and incubation conditions, no AMT of *S. lividans* was detected mediated by the Ti plasmid. However, RP4-mediated DNA transfer from *A. tumefaciens* to *S. lividans* was observed. These findings can provide a basis for a dedicated approach to compare elements of both DNA transfer systems helping to unravel the host bias of the AMT system.

Introduction

Agrobacterium tumefaciens, a Gram-negative bacterium of the rhizosphere, is naturally able to transfer DNA and proteins into plant cells. In laboratory settings transfer is also possible to other eukaryotic organisms, for example yeasts and filamentous fungi (de Groot *et al.*, 1998; Lacroix *et al.*, 2006, Soltani *et al.*, 2008). The highly specialized process of *Agrobacterium*-mediated transformation (AMT), able to cross the *trans*-kingdom barrier, has evolved from bacterial conjugation (Alvarez-Martinez & Christie, 2009; Guglielmini, de la Cruz & Rocha, 2013). The specific transfer and transformation process is mediated by a specialized inducible type-IV secretion system (T4SS) encoded on the tumor-inducing (Ti) plasmid. This T4SS, named here $T4SS_{Ti}$, is functioning during AMT and shares homology with other bacterial T4SSs. However, conjugative plasmid transfer mediated by the $T4SS_{Ti}$ to other bacteria has been shown in only a few cases (Beijersbergen *et al.*, 1992; Kelly & Kado, 2002; Kado & Kelly, 2006).

In general, the process of conjugative DNA transfer can be divided into distinctive parts (Alvarez-Martinez & Christie, 2009). The T4SS or also called the mating-pair formation (Mpf) system, creates the physical channel between the donor and the recipient cells. The T4SS consists of several protein subunits creating a transmembrane spanning structure. A coupling protein (CP), situated at the cytoplasmic side of the T4SS system, is responsible for specifying which substrate will be transferred via the T4SS. And the DNA transfer and replication (Dtr) system, which processes DNA within the donor cell and further aids with the transfer of the DNA into recipient. The to be transferred DNA is specified by a distinct *cis*-acting region that is recognized by the Dtr system, the origin of transfer (*oriT*). The Dtr system consists amongst others of a relaxase protein that makes a nick at the *oriT*, after which it remains covalently bound to the DNA. The protein-DNA complex is then recognized by the CP and transferred via the T4SS. Several Dtr auxiliary proteins aid the relaxase in its function. To study AMT to bacteria, the comparison to a related transfer system of the conjugative plasmid RP4 was made. As recipient for DNA, the Gram-positive bacterium *Streptomyces lividans* was used. Moreover, *S. lividans* is present in the same biome as *A. tumefaciens*, which could make it a natural recipient for AMT.

A. tumefaciens normally infects wounded plant tissue, causing crown gall tumors (Escobar & Dandekar, 2003). Tumors are formed after transfer and integration of genes located on the T-DNA part of the Ti plasmid, while another part of this plasmid contains the virulence (vir_{Ti}) genes encoding the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$. For transfer to occur, induction by extracellular environmental triggers (low pH) and compounds (e.g. acetosyringone) released by wounded cells is required (Sheikholeslam & Weeks, 1987). Acetosyringone activates the $VirA_{Ti}/VirG_{Ti}$ signaling pathway that regulates vir_{Ti} gene expression (Krishnamohan, Balaji & Veluthambi, 2002). Contrary to other bacterial conjugative systems, the transferred DNA sequence located on the Ti plasmid is marked by two *cis*-acting elements for recognition by the Dtr_{Ti} system (Yadav *et al.*, 1982; Waters *et al.*, 1991; Alvarez-Martinez & Christie, 2009). These two imperfect direct repeats are called the right border (RB_{Ti}) and left border (LB_{Ti}). The region defined by these borders is called transfer-DNA (T-DNA). With the aid of auxiliary Vir_{Ti} proteins $VirC1_{Ti}$, $VirC2_{Ti}$, and $VirD1_{Ti}$ the relaxase $VirD2_{Ti}$ can nick the RB_{Ti} and LB_{Ti} sequence, whereby it becomes covalently bound to the 5′-ends. The RB_{Ti} is accompanied by an overdrive sequence (OD_{Ti}) to enhance border processing (Toro *et al.*, 1988). The area between the borders is liberated as a T-strand, bound at the 5'-end by $VirD2_{Ti}$, and is transported to the recipient cell via the $CP_{Ti}/T4SS_{Ti}$ system, migrating into the nucleus of the recipient cell for subsequent genomic integration (Gelvin, 2003; Christie & Gordon, 2014).

The IncPα plasmid RP4 is a conjugative plasmid able to transfer itself and other mobilizable plasmids from one bacterial cell to another (Pansegrau *et al.*, 1994). The

conjugative transfer is mediated via the Dtr_{RP4}, CP_{RP4} and $T4SS_{RP4}$ encoded on the plasmid. The relaxase of RP4 $TraI_{RP4}$, part of the Dtr_{RP4}, can recognize the *cis*-acting $oriT_{RP4}$ sequence. $TraI_{RP4}$, with the aid of auxiliary proteins, is able to nick at the $oriT_{RP4}$, whereafter a single-strand can be transferred via the $CP_{RP4}/T4SS_{RP4}$ into the recipient cell (Pansegrau *et al.*, 1990). As normal for bacterial conjugation, only one $oriT_{RP4}$ is present on the plasmid and therefore the entire plasmid sequence is transferred via the $CP_{RP4}/T4SS_{RP4}$. The transferred single-strand is converted into a double-strand and re-circularized with the aid of endogenous DNA processing systems within the recipient cell (Lanka & Wilkins, 1995; Pansegrau & Lanka, 1996; Parker & Meyer, 2005; Fernández-López *et al.*, 2014). One big difference to the AMT system is the presence of a plasmid-encoded primase in the Dtr_{RP4}, which is also transferred by $CP_{RP4}/T4SS_{RP4}$. The primase seems to play an important role for RP4-mediated conjugation (Merryweather, Barth & Wilkins, 1986). Within the recipient cell, the primase is thought to process the single-stranded plasmid to initiate double strand formation via the recipient cell endogenous replication machinery (Lanka & Barth, 1981). The host range of AMT are primarily eukaryotic cells, but it was also demonstrated that the $CP_{Ti}/T4SS_{Ti}$ Mpf system could mobilize plasmid RSF1010 into other *A. tumefaciens* cells (Beijersbergen *et al.*, 1992) and plant cells (Buchanan-Wollaston *et al.*, 1987). RSF1010 is lacking a CP/T4SS system and RB_{Ti} or LB_{Ti} sequences but contains instead its own $oriT_{RSF1010}$ and its own $Dtr_{RSF1010}$ (Scholz *et al.*, 1989). In this manner, the RSF1010 was able to complement its own $Dtr_{RSF1010}$ functions with the $CP_{Ti}/T4SS_{Ti}$ for plasmid transfer.

RP4 has a broad host-range, enabling it to be maintained in many Gram-negative bacteria (Grahn *et al.*, 2000). It is also able to mobilize plasmids from Gram-negative to Gram-positive bacteria (Mazodier, Petter & Thompson, 1989). Interestingly, in laboratory settings RP4 can transfer DNA from Gram-negative *E. coli* bacteria to yeast cells, thus crossing the *trans*-kingdom barrier too (Sawasaki *et al.*, 1996).

To compare the Ti plasmid and RP4 plasmid transfer systems for this study, two different experimental levels needed to be considered. One should be the environmental level, where the composition of the medium for the co-cultivation could play a role. A good setting for the medium should at the on hand to sustain growth of the donor and recipient cells, while on the other hand provide compatible conditions supporting transfer to occur. In general, for AMT low nutrient concentrations and low pH will be optimal, whereas for RP4-mediated transfer no transfer-specific medium seems to be required. The other level to be considered should deal with facilitation and comparison of the transfer process. To be able to compare AMT and RP4-mediated transfer on a simplified functional level, several adjustments had to be made to the AMT system. In contrast to AMT, RP4 typically transfers itself or a complete separate mobilizable plasmid by means of only one $oriT_{RP4}$ sequence. Hence, an analogous configuration needed to be established for AMT. It was discovered that AMT would be possible if the T-DNA and vir_{Ti} were separated from the Ti plasmid and presented on a different plasmid. DNA transfer via the $CP_{Ti}/T4SS_{Ti}$ is possible when T-DNA (defined by RB_{Ti} and LB_{Ti} sequences) is supplied *in trans* on a separate shuttle vector (Hoekema *et al.*, 1983), in combination with all essential vir_{Ti} genes making up the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$ present on a separate plasmid, called the Ti helper plasmid (Hoekema *et al.*, 1983). This system was called the binary system. Additionally, it was demonstrated that a vector containing only a single border (RB_{Ti}) sequence could be processed by the Dtr_{Ti} and transferred via the $CP_{Ti}/T4SS_{Ti}$ to recipient cells (Kelly & Kado, 2002; Rolloos *et al.*, 2014). Thus, AMT can be modified to resemble the RP4-mediated transfer, by taking the RB_{Ti} sequence as a functional analog of an $oriT_{RP4}$. As a bacterial model organism to be used as recipient cells for studying any RB_{Ti}-only shuttle vector transfer via the $CP_{Ti}/T4SS_{Ti}$, *Streptomyces* could be a suitable substitute for plant cells as it was already described that AMT to *S. lividans* was possible (Kelly & Kado, 2002; Kado & Kelly, 2006). A high-throughput bacterial screening system could speed up the study of certain factors influencing AMT. As a standard method for

genetic manipulation of *Streptomyces*, a DNA transfer system based on RP4 is already widely used (Mazodier, Petter & Thompson, 1989; Paget *et al.*, 1999). With this method, a transfer-deficient RP4 plasmid lacking $oriT_{RP4}$ present in *E. coli* is used to deliver integrative vectors for genomic integration in *Streptomyces.* (Mazodier, Petter & Thompson, 1989; Paget *et al.*, 1999). If efficient, AMT might also serve as a future tool for dedicated genetic manipulation of *Streptomyces*, as they are very important organisms, being a major source of natural antibiotics (Chater, 2006; Newman & Cragg, 2007; Zhu, Sandiford & van Wezel, 2014).

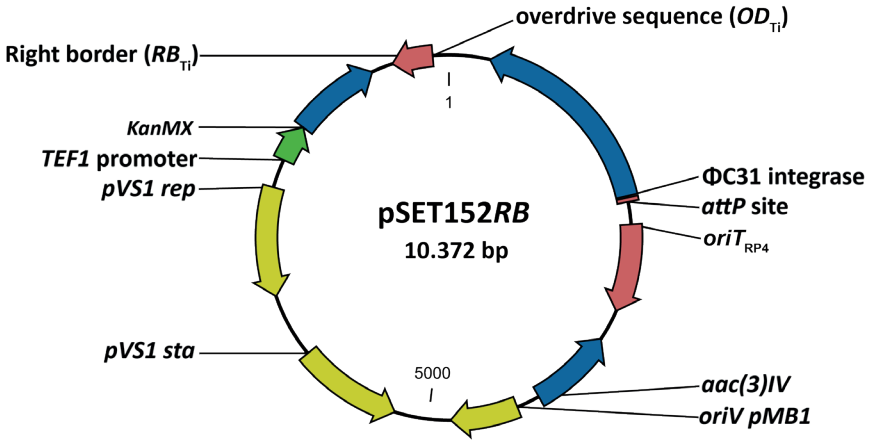

Figure 1. Shuttle vector pSET152*RB*, used for transfer between *Agrobacterium* and *Streptomyces*. The vector encodes a ΦC31 phage integrase, able to integrate the entire vector into the host genome, via site-specific recombination. The integrase recognition sequence on the shuttle vector is *attP* and in the *Streptomyces* genome *attB* (Kuhstoss & Rao, 1991). It further contains a *pVS1* minimal replicon (*sta* and *rep*) for *A. tumefaciens*, a replicon for *E. coli*, a *KanMX* resistance cassette for use in *S. cerevisiae*, an apramycin resistance cassette (*aac(3)IV*), a 25 bp octopine-type right border (RB_{Ti}) recognition sequence for $VirD2_{Ti}$ combined with an upstream adjacent 38 bp overdrive (OD_{Ti}) sequence for Dtr_{Ti} auxiliary protein recognition (Rolloos *et al.*, 2014), and a RP4 based origin of transfer ($oriT_{RP4}$) for mobilization by RP4 (Mazodier, Petter & Thompson, 1989). The shuttle vector pSET152Δ*RB* just lacks the RB_{Ti} for $VirD2_{Ti}$ recognition (vector map not shown). The shuttle vector pSET152*RB*Δ*oriT* is identical to pSET152*RB*, except for the deletion of the $oriT_{RP4}$ region (vector map not shown).

Results

Construction of a transfer system for *Agrobacterium*-mediated transfer to *Streptomyces*

Kelly and Kado (2002) previously reported AMT to *S. lividans*. The study described the transfer of two shuttle vectors, both containing a RB_{Ti} sequence, from the recombination deficient Ach5-based *A. tumefaciens* LBA4301 (Klapwijk, van Beelen & Schilperoort, 1979) to *S. lividans* 66 TK64 (Kelly & Kado, 2002), facilitated via the Dtr_{Ti}, CP_{Ti} and $T4SS_{Ti}$ encoded on the Ti helper plasmid pUCD2614. The Ti helper plasmid was created by subcloning only the vir_{Ti} regulon of the Ti plasmid pTiC58 (Rogowsky *et al.*, 1990). The first mobilizable vector contained the RB_{Ti} sequence, a hygromycin and kanamycin/gentamicin resistance cassette. The second mobilizable vector was a fusion of the plasmid pSET152 (Bierman, Logan & O'Brian, 1992) and the first vector (Zyprian & Kado, 1990). Plasmid pSET152 contained an apramycin resistance cassette as well as the gene encoding a ΦC31 phage integrase, for genomic integration of plasmid pSET152 by recombination between the *att* sites on the plasmid and the chromosome of *S. lividans* (Kuhstoss & Rao, 1991; Bierman, Logan & O'Brian, 1992). The second vector did not contain the hygromycin resistance cassette of the parental vector nor the $oriT_{RP4}$ sequence present on pSET152. At the onset of the study, it was attempted to repeat the experiments performed by the Kado lab. Unfortunately, the originally used strains and plasmids could no longer be obtained (CI Kado, personal communication). Therefore, this study used a set-up resembling the original set-up of Kelly & Kado (2002) but with different strains and plasmids. Kelly & Kado (2002) used strain *S. lividans* TK64 containing a loss of function mutation of proline synthesis and streptomycin resistance and lacking two linear plasmids. For this study the progenitor *S. lividans* wild-type 66 (1362) without genomic modifications was used. Here, the C58 based *A. tumefaciens* strain LBA1100 (Beijerbergen *et al.*, 1992) was used as donor. This strain harbors the Ti helper plasmid pAL1100 (Beijerbergen *et al.*, 1992) which is derived from the octopine Ti plasmid pTiB6, by a large deletion including the T-regions. The constructed shuttle vector pSET152*RB* (**Figure 1**), based on plasmid pSET152, contained in addition to the $oriT_{RP4}$ sequence a RB_{Ti} with an adjacent OD_{Ti} (**Figure 1**).

To verify the correct function of the pSET152*RB* shuttle vector (**Figure 1**), the plasmid was transformed via protoplast transformation directly into *S. lividans* according to Gust, Kieser & Chater (2002). The transformation resulted in apramycin resistant colonies (n = 3; data not shown). The shuttle vector was unable to replicate in *S. lividans*, and the apramycin resistance of *S. lividans* was thus only acquired after the integrase-mediated integration event of the plasmid into the recipient cell's genome (Paranthaman & Dharmalingam, 2003). The stable genomic integration of the plasmid was verified by PCR as well as by subsequent cultivation of positive colonies on medium selecting further on apramycin (n = 3; data not shown).

Next, it was tested if the shuttle vector pSET152*RB* could be processed and transferred via the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$. For this the plasmid was additionally equipped with an *ARS6/Cen4* sequence for stable low copy maintenance in yeast, resulting in plasmid pSET152Y*RB*. This was qualitatively tested by using AMT transfer to yeast following the modified protocol of Bundock *et al.* (1995). As plasmid donor *A. tumefaciens* strain LBA1100 containing pAL1100 and pSET15Y*RB* was used, and as recipient *Saccharomyces cerevisiae* strain YPH250. This co-cultivation resulted in observable kanamycin resistant yeast colonies with an estimated conjugation frequency of around 10^{-5} (n = 3; data not shown).

To exclude that free plasmid pSET152*RB* during AMT, present in the co-cultivation mix from lysed bacteria, could be responsible for transformation of *S. lividans*, the conjugation

protocol of Gust, Kieser & Chater (2002) was followed, with the change of adding only free plasmid instead of a donor strain. Around 1 × 10^7 pre-germinated spores were used and incubated with purified pSET152*RB* plasmid DNA (1 µg/1 × 10^7 pre-germinated spores). After incubation and selection, no apramycin positive colonies were observed, showing that free plasmids were not taken up under the conditions used (n = 3; data not shown).

AMT to *S. lividans* based on methodology by Kelly and Kado (2002)

To investigate the plasmid transfer between *A. tumefacien*s and *S. lividans* mediated by the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$, a co-cultivation experiment was performed. The original co-cultivation protocol of Kelly & Kado (2002) was followed. First, pre-germinated *S. lividans* spores were combined with induced *A. tumefaciens* LBA1100 cells containing pSET152*RB* and then cultivated together for 4 d. During this time *S. lividans* was able to form mycelia, which produced new spores at the end of the co-cultivation. The *S. lividans* spores found after the co-cultivation were recovered and a dilution series was plated on antibiotic selection plates. None of the transferred *S. lividans* spores exhibited growth on antibiotic selection (n = 9). As a control, part of the dilution series was plated on medium without antibiotic selection to verify growth of *S. lividans* cells, which led to bacterial growth in each experiment (qualitative measurement; no data shown).

During conjugation, cell-to-cell contact is crucial for the T4SS to create a transmembrane bridge for DNA transfer. To study the interaction between the organisms, Kelly & Kado (2002) used an aggregate formation assay. In their study, it was observed that acetosyringone induced *A. tumefaciens* LBA4301 cells to aggregate with *S. lividans* hyphae into structures visible in test tubes. To check for this aggregation, a comparable experimental set-up was used. As donor cells, two different *A. tumefaciens* strains were used: a derivative of *A. tumefaciens* LBA1100 containing the Ti helper plasmid pAL1100 as well as the shuttle vector pSET152*RB*, and a derivative of *A. tumefaciens* LBA2210 containing the plasmid RP4 as well as the shuttle vector pSET152*RB*. As recipients *S. lividans* 66 as well as the unrelated *Streptomyces venezuelae* were used. In the experiments, *A. tumefaciens* LBA1100 was tested induced as well as non-induced. Remarkably, in none of the experimental combinations and conditions, using induced or non-induced LBA1100 as well as in non-induced conditions LBA2210, any visible aggregation was detected such as reported by Kelly & Kado (2002) when using strain LBA4301 (**Table 1**).

Table 1. Aggregate formation assay between *Streptomyces* hyphae and *A. tumefaciens*. Induced (with acetosyringone; AS) or non-induced *A. tumefaciens* was cultured together with 48 h old *S. lividans* hyphae at room temperature to observe possible formation of aggregates between both organisms, visible to the eye. Observations were taken for following time points: 5, 15 and 30 min and 12 h (each n = 1).

Streptomyces	***A. tumefaciens***	***A. tumefaciens* induction**	**Observation**
S. lividans	LBA1100 (pSET152*RB*)	5.5 h induced in IM+AS	No aggregation
S. lividans	LBA1100 (pSET152*RB*)	5.5 h in LC	No aggregation
S. venezuelae	LBA1100 (pSET152*RB*)	5.5 h induced in IM+AS	No aggregation
S. lividans	LBA2210 (pSET152*RB*)	5.5 h in LC	No aggregation

IM: Induction medium; AS: Acetosyringone (200 µM)

Modifications of co-cultivation conditions for AMT to *S. lividans*

The co-cultivation, essentially replicating the published protocol for AMT transfer of *S. lividans* by Kelly & Kado (2002), did not result in transformants with the strains and plasmids used in this study. This raised the question, if the applied co-cultivation conditions allowed DNA transfer between the strains. To find compatible conditions for Dtr_{Ti}-, CP_{Ti}-, $T4SS_{Ti}$-mediated DNA transfer to occur, several variations alongside the conditions used by Kelly & Kado (2002) were tested: use of different media, a different co-cultivation method required for the use with a specific medium, as well as using a different strain of *A. tumefaciens*. The compared media were: (1) Induction medium (IM) used normally for AMT to yeast, containing minimal nutrients and specifically a low pH supporting the expression and function of the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$; (2) R2 medium, a specific growth medium for *Streptomyces* used by Kelly & Kado (2002) with a pH of around 7; and (3) R5 medium, a variation of the R2 medium, containing yeast extract for better growth of *Streptomyces* with a pH of around 7. Further, different methods of selection for recipient cells were tested. One selected method was the overlay procedure commonly used for conjugation to *Streptomyces* (Gust *et al.*, 1999), also used in the Kelly & Kado (2002) experiment. In this case only the *Streptomyces* specific media R2 and R5 could be used for co-cultivation, selection and growth of *Streptomyces*. In the overlay method after co-cultivation, an aqueous overlay containing an antibiotic for selection of the transformants and an antibiotic for selection against the donor strain was applied directly on the co-cultivation plate. Subsequently, surviving *Streptomyces* cells were grown until new spores were available for isolation and further selection on the medium that was also used for the co-cultivation, for example R2 or R5 medium as they support *Streptomyces* growth. The overlay method is optimized for *Streptomyces* growth and does not account for any requirements for *Agrobacterium* cells and induction of the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$. The other method was based on a standard AMT protocol that specifically allowed *Agrobacterium* cells to induce their $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$ (Bundock *et al.*, 1995). In this method a filter was placed on the co-cultivation medium to keep the mixture of donor and recipient cells separate from the medium. The filter still allowed transfer of nutrients and compounds. The advantage of this method was that the co-cultivation could be performed on one specific co-cultivation medium. After co-cultivation the filter could be removed and the biofilm (containing the donor and recipient cells) could be transferred to a different *Streptomyces* medium for selection and growth (R2 or R5). This method was in particular necessary, when IM medium was used for the co-cultivation, as continued presence of this medium did not support growth of *S. lividans* cells after co-cultivation. But *S. lividans* was found to stay viable when cultured on cellophane filters on IM for 4 d and then being transferred to R5 growth medium (data not shown). As a final variable, an *A. tumefaciens* strain LBA1126 was used that did not require induction of the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$, as mutations in the Ti plasmid $virA_{Ti}$ and $virG_{Ti}$ regulatory genes ensure expression of the vir_{Ti} genes.

First, the protocol for yeast transformation using IM, providing optimal condition for *A. tumefaciens* virulence, was tested (Bundock *et al.*, 1995). The donor strain was *A. tumefaciens* LBA1100 containing the shuttle vector pSET152*RB* and the recipient strain was *S. lividans*. The used cell-to-cell ratio for the co-cultivation was 1×10^8 donor cells to 1×10^8 pre-germinated recipient spores. The co-cultivation was performed for different time spans (48 h, 96 h, and 5 d) on a filter on IM at 21° C. No apramycin resistant S. *lividans* colonies were subsequently observed after plating all different co-cultivations on R5 selection medium for up to 3 weeks (n = 3, for each time point; data not shown).

To directly compare the co-cultivation on IM medium to the co-cultivation on *Streptomyces* specific media, a co-cultivation experiment was performed on IM, R2, and R5 medium, using either the overlay or the filter method. For this comparison, the co-cultivation length was set at 4 d, just as for the initial experiments based on the Kelly & Kado (2002). *A. tumefaciens* LBA1100 containing the Ti plasmid and the shuttle vector pSET152*RB* was

used again as plasmid donor strain, with *S. lividans* as recipient strain. For further comparison another recipient, *S. venezuelae* was used as well. However, none of these approaches and variations yielded any apramycin resistant *Streptomyces* colonies (**Table 2).**

Table 2. Overview of different methods for AMT co-cultivation with *Streptomyces*. The *A. tumefaciens* strain LBA1100 with Ti helper plasmid pAL1100 and shuttle vector pSET152*RB* was tested as donor in different co-cultivation conditions with two different *Streptomyces strains* as recipient. During co-cultivation, the phenolic-inducer acetosyringone (AS) was present in each medium at a concentration of 200 μM. The co-cultivation period was 4 d at 21° C. After co-cultivation on filter, the resulting biofilm was removed and plated on strain specific medium with selection for apramycin resistance. With the overlay method, both strains were placed together directly on the agar plate, and after co-cultivation the biofilm was treated directly with antibiotics to select for apramycin resistance and against *A. tumefaciens* (for *S. lividans* n = 3; and for *S. venezuelae* n = 2).

Streptomyces	**Method**	**Medium**	**Transformants**
S. lividans	Cellophane filter	IM + AS	No
S. lividans	Cellophane filter	R2 + AS	No
S. lividans	Overlay	R2 + AS	No
S. lividans	Cellophane filter	R5 + AS	No
S. lividans	Overlay	R5 + AS	No
S. venezuelae	Cellophane filter	IM + AS	No
S. venezuelae	Cellophane filter	R5 + AS	No

IM: Ti plasmid specific induction medium with minimal nutrients and specific pH; R2: *Streptomyces* specific growth medium; R5: a variation of R2 medium containing additional yeast extract; AS: Acetosyringone

To investigate whether the use of a filter (with the filter method) during AMT would have negatively affected plasmid transfer, a co-cultivation was set-up directly on the medium As *S. lividans* cells were not able to continue their life cycle on IM medium, the biofilm containing the donor and recipient strain now needed to be removed directly from the IM medium surface, in order to be transferred to a selection medium. For making direct comparisons between IM and R5 medium, the same direct plating method was applied for R5 medium as well. The co-cultivation was performed on 6-well plates as the smaller surface area of the medium allowed for a more thorough removal of the biofilm. It was found that the maximal length of the co-cultivation period was limited to a window between 2 and 3.5 h. After that time window *S. lividans* spores would start creating hyphae, which would grow into the medium making it from then onwards impossible to remove the biofilm from the surface. After removal from the surface, the co-cultivation mixture was plated on antibiotic selection plates selecting for transformants and selecting against the donor cells. In none of the experiments any apramycin positive *S. lividans* colonies could be detected (n = 3; data not shown).

As already indicated above, another strain of *A. tumefaciens* was tested in order to have a comparison to the previously used strain LBA1100, in case this would have been an unfortunate choice. *A. tumefaciens* LBA1126 has mutations in the $virA_{Ti}$ and $virG_{Ti}$ regulatory genes on the Ti plasmid (Bundock & Hooykaas, 1996), which leads to constitutive activation of the vir_{Ti} genes. Therefore, no induction of the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$ should be required for this strain. This would in principle allow for full $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$ activity even on *Streptomyces* specific media, which do not provide for the conditions triggering virulence gene expression. Hence, *A. tumefaciens* LBA1126 harboring pSET152*RB* was used for a co-cultivation with *S. lividans* on R5 medium (filter and overlay method) as well as on IM medium (filter method) for 4 d. Also, after these co-cultivation experiments no apramycin resistant colonies of *S. lividans* were observed (n = 3; data not shown). These results indicated that if any conjugative transfer via the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$ would have occurred, it would have been below the frequency of 10^{-9}, the lower detection limit of this experiment.

RP4-mediated DNA transfer to *Streptomyces*

Although the plasmid substrate for AMT could be equipped with a single RB_{Ti} sequence to functionally resemble an *oriT* as used in the conjugative process of RP4, no experimental evidence for AMT transfer to *Streptomyces* could be obtained in this study. To have a direct comparison though, RP4-mediated transfer to *Streptomyces* had to be tested using *A. tumefaciens* as donor strain. So far, RP4 had not been tested or reported for transfer of DNA from *Agrobacterium* to *Streptomyces*.

A routine method for DNA transfer to *Streptomyces* has been established using *E. coli* ET12567. The ET12567 strain harbors the non-transmissible conjugative plasmid pUZ8002 (Paget *et al.*, 1999), which is a derivative of the conjugative plasmid RP4 (Pansegrau *et al.*, 1994) lacking the $oriT_{RP4}$ region. Therefore, pUZ8002 is not able to transfer itself, but is still able to mobilize other plasmids containing an $oriT_{RP4}$. To compare the routine pUZ8002 mediated *E. coli* to *Streptomyces* conjugative plasmid transfer with transfer starting from *A. tumefaciens*, the plasmid pUZ8002 was isolated from *E. coli* ET12567 and introduced into *A. tumefaciens* LBA288 lacking a Ti plasmid. Next, a co-cultivation experiment was performed using *A. tumefaciens* LBA288 containing pUZ8002 as the donor strain for the shuttle vector pSET152*RB* and *S. lividans* as recipient, using the overlay method on R5 medium for 4 d. No apramycin resistance *S. lividans* colonies were observed on the selection plates (n= 3; data not shown).

To verify if the isolated pUZ8002 plasmid would also be functional outside of the *E. coli* ET12567 strain, pUZ8002 was also introduced into another strain *E. coli* DH10β. Following the same protocol (Paget *et al.*, 1999), *E. coli* DH10β containing pUZ8002 and pSET152*RB* was co-cultivated with *S. lividans*. The co-cultivation was performed on specific MS medium, providing optimal conditions for a fast sporulation of *Streptomyces*. In this experiment after selection, no apramycin resistant *S. lividans* colonies could be observed (n = 3; data not shown). As the threshold of detection in this assay would be at frequency of 10^{-9} for this experiment, if transformation had been successful the frequency would be below the threshold frequency. In a parallel experiment, *E. coli* ET12567 containing pUZ8002 and pSET152*RB* was used as donor strain for a co-cultivation with *S. lividans* as recipient strain on MS medium following the protocol of Paget *et al.* (1999). After this co-cultivation apramycin resistant colonies were observed, with an estimated average transformation frequency of 10^{-4} (n = 3; no data shown).

In view of these variable results with pUZ8002, it was studied whether using RP4 itself instead of pUZ8002 would allow conjugative DNA transfer from *Agrobacterium* to *Streptomyces*. To this end the *A. tumefaciens* strain LBA2210 lacking a Ti plasmid but containing the RP4 plasmid was used. Co-cultivating *A. tumefaciens* LBA2210 harboring RP4 and pSET152*RB* with *S. lividans* according to Gust, Kieser & Chater (2002) resulted in transformation of *S. lividans*. Apramycin-resistant *S. lividans* colonies were observed with an estimated frequency of 10^{-5} (**Figure 2**). The co-cultivation was performed on R5 medium. This is the first report of *A. tumefaciens* to transfer an integrative shuttle vector to *S. lividans* within 24 h via the $Dtr_{RP4}/CP_{RP4}/T4SS_{RP4}$. As control transfer to *S. lividans* was tested with *A. tumefaciens* LBA2210 containing RP4 with either pSET152*RB*Δ*oriT* lacking the $oriT_{RP4}$ sequence, or pSET152Δ*RB*Δ*oriT* lacking the border sequence as well as $oriT_{RP4}$. After use of these two shuttle vectors in the co-cultivation, no apramycin positive colonies were visible (**Figure 2**).

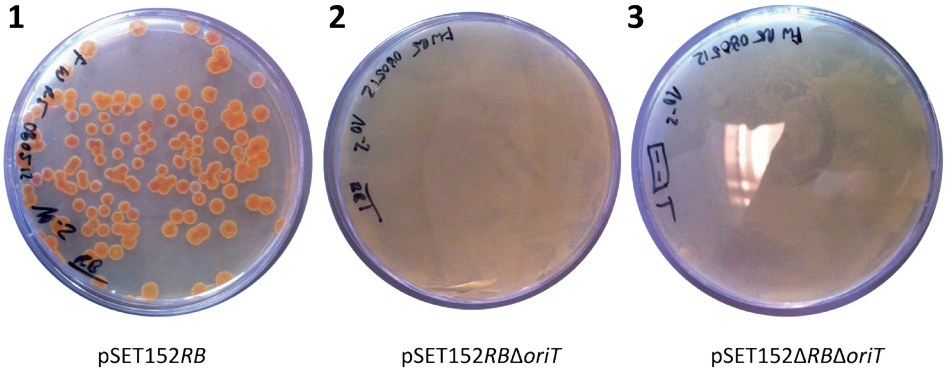

Figure 2. *A. tumefaciens* RP4-mediated transfer of shuttle vectors to *S. lividans* with or without $oriT_{RP4}$. (**1**). Apramycin resistant *S. lividans* colonies visible when plasmid pSET152*RB* was used. (**2**). No *S. lividans* colonies visible when conjugation was attempted with plasmid pSET152*RB*Δ*oriT*. (**3**). No *S. lividans* colonies visible when transfer was attempted with plasmid pSET152Δ*RB*Δ*oriT*. Each picture shown was a good representation of the other observed plates (n = 3). The screening was performed to determine only a visible qualitative result, but the transformation frequency was estimated to be around 10^{-5}. The co-cultivation (24 h) and selection was performed on R5 medium at 30° C.

Influence of co-cultivation media on DNA transfer from to *S. lividans*

As the RP4-mediated DNA transfer was possible from *Agrobacterium* to *S. lividans*, the co-cultivation conditions used for the AMT experiments were compared to assess if DNA transfer could occur during the specific AMT conditions (**Table 3**). This was a qualitative approach, and no transformation frequencies were determined. Only the occurrences of apramycin resistant colonies were noted. The media tested were IM, R2 as *Streptomyces* specific growth medium, and R5 as a more complex *Streptomyces* specific growth medium, being a derivative of R2 containing yeast extract, also already mentioned above. To emulate conditions required for AMT, the vir_{Ti} inducer acetosyringone was added to the R2 and R5 *Streptomyces* media or was left out for comparison. As RP4-mediated DNA transfer to *S. lividans* was possible, acetosyringone was added to indicate if there would be an influence on the DNA transfer from *A. tumefaciens.* In addition, either the filter method or the overlay method was used. Also the temperature was varied to be either 21° C, as optimal for AMT, or 30° C, as optimum for *S. lividans* transformation. The length of the co-cultivation was 24 h as this is an optimal time window for RP4/pUZ8002-mediated DNA transfer. Apramycin positive colonies were observed after co-cultivation on R2 and R5 media, with or without acetosyringone, at 21° C or 30° C, and with both co-cultivation methods used (n = 3; for each data point). No apramycin positive colonies were observed on any of the co-cultivations using IM (n = 3; for each data point). These data are again indicative for problems with the cultivation of *Streptomyces* on IM during co-cultivation, as also encountered during earlier performed co-cultivations.

Table 3. RP4-mediated transfer from *A. tumefaciens* LBA2210 containing pSET152*RB* to *S. lividans* in different co-cultivation conditions. Co-cultivation occurred on the medium and by the method indicated in the table. After co-cultivation only the qualitative occurrence of apramycin resistant colonies was determined (n = 3; each data point). Acetosyringone (AS) was added to the medium where indicated, at a concentration of 200 μM.

Medium	Method	Temp. in [° C]	Time in [h]	Transformants
R2	Cellophane filter	21/30	24	Yes
R2 + AS	Cellophane filter	21/30	24	Yes
R2	Overlay	21/30	24	Yes
R2 + AS	Overlay	21/30	24	Yes
R5 +AS	Cellophane filter	21/30	24	Yes
R5	Cellophane filter	21/30	24	Yes
R5 +AS	Overlay	21/30	24	Yes
R5	Overlay	21/30	24	Yes
IM + AS	Cellophane filter	21/30	24	No
IM	Cellophane filter	21/30	24	No
IM + AS	Overlay	21/30	24	No
IM	Overlay	21/30	24	No

IM: Ti plasmid specific induction medium with minimal nutrients and specific pH; R2: *Streptomyces* specific rich growth medium; R5: a variation of R2 medium containing additional yeast extract; AS: Acetosyringone

Comparison of RP4-mediated transfer from *A. tumefaciens* and *E. coli* to *S. lividans*

To gain more clarity about the RP4-mediated DNA transfer to *Streptomyces*, the transformation efficiencies of *E. coli* ET12567 harboring the pUZ8002 and the shuttle vector pSET152*RB,* and of *A. tumefaciens* LBA2210 containing the RP4 plasmid as well as pSET152*RB* were determined. The standard *Streptomyces* transformation protocol using *E. coli* uses MS medium, a rich medium specific for fast sporulation of transformed *Streptomyces* cells (Paget *et al.*, 1999). The transformation frequency of *A. tumefaciens* with *S. lividans* on MS medium could not be determined, as *A. tumefaciens* then showed an increased production of extracellular matrix; a very thick slime layer was formed on top of the bacterial lawn and no colonies of *S. lividans* could be identified. Furthermore, it is unknown if the antibiotic overlay can permeate such a barrier. Slime formation was avoided by using R5 medium, used for *S. lividans* transformants selection. No apramycin resistant *S. lividans* were observed after co-cultivation of *E. coli* ET12567 using pUZ8002 and pSET152*RB* on R5 medium. Apramycin resistant *S. lividans* colonies were only detected after a RP4-mediated transfer both with *A. tumefaciens* on R5 medium at 21° C or *E. coli* on MS medium at 30° C (**Table 4**). Also, apramycin-positive cells were only found after a co-cultivation time of 24 h. Co-cultivation for 30, 60, and 120 min did not result in any positive transformation of *S. lividans*, neither from *A. tumefaciens* at 21° C nor from *E. coli* at 30° C (**Table 4**).

Table 4. Comparison of RP4-mediated conjugation of *E. coli* and *A. tumefaciens* to *S. lividans* on different media. As plasmid donors *E. coli* ET12567 containing the plasmid pUZ8002 and *A. tumefaciens* LBA2210 containing the plasmid RP4 were used. The recipient strain was *S. lividans*. Co-cultivation was performed for 30 min, 60 min, 120 min and 24 h (n = 3; for each time point). Co-cultivation with *E. coli* was performed at 30° C and with *A. tumefaciens* at 21° C. Error shown is SEM. The conjugation efficiency was determined by dividing the output transconjugants CFU by the input recipient cells.

Donor strain (plasmids used)	Medium	Conjugation efficiency			
		30 min	60 min	120 min	24 h
E. coli ET12567 (pUZ8002, pSET152*RB*)	MS	0	0	0	$(3.3 \pm 1.0) \times 10^{-4}$
	R5	0	0	0	0
A. tumefaciens LBA2210 (RP4, pSET152*RB*)	MS	n.d.	n.d.	n.d.	n.d.
	R5	0	0	0	$(6.5 \pm 4.1) \times 10^{-5}$

MS: *Streptomyces* sporulation medium; R5: *Streptomyces* growth medium; n.d.: not determined

Discussion

This study investigated DNA transfer from *A. tumefaciens* to *S. lividans* via two functionally different but structurally related transfer systems, those of the RP4 and Ti plasmid. To compare both transfer systems, a compatible functionally similar DNA substrate was required, carrying a single RB_{Ti} site as an equivalent of an $oriT_{RP4}$, thus making the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$ transfer system a functional mimic of the conjugative plasmid transfer by the $Dtr_{RP4}/CP_{RP4}/T4SS_{RP4}$. In this study, previously published results showing AMT to *S. lividans* (Kelly & Kado, 2002) could not be corroborated. Variations in co-cultivation conditions also did not result in any detectable AMT via the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$. However, it was shown that *A. tumefaciens* containing the RP4 plasmid was able to mobilize an $oriT_{RP4}$ containing integrative vector to *S. lividans*. Comparing all data, it had to be concluded that none of the conditions regularly used for AMT transfer to yeast and plant cells was compatible with DNA transfer from *A. tumefaciens* to *S. lividans*.

In 2002, Kelly & Kado reported a method for AMT to *S. lividans*, followed by a methodological publication (Kado & Kelly, 2006). The strains and constructs originally used for this study (Kelly & Kado, 2002) were not available anymore. In the set-up of Kelly & Kado (2002), rich *Streptomyces* specific R2 medium was used for the co-cultivation of organisms (Kieser *et al.*, 2000) at a temperature of 25° C. These conditions seemed to be not optimal for $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$-mediated DNA transfer conditions. Key factors for successful AMT are specific medium, pH and temperature. According to literature, rich medium is sub-optimal for AMT (Turk *et al.*, 1991; Liu *et al.*, 1993). A minimal medium like IM is optimal for AMT, mimicking the natural environment for transformation (Beijersbergen *et al.*, 1992; Bundock *et al.*, 1995). The optimum pH for AMT is an acidic pH between 5 and 6 (Turk *et al.*, 1991; Liu *et al.*, 1993; Gelvin, 2006). The pH of R2 medium is around 7.2 (Okanishi, Suzuki & Umezawa, 1973) providing less optimal conditions for the transformation. According to literature, the window between 21°-22° C provides an optimal window for a more efficient assembly of the $T4SS_{Ti}$ pilus (Dillen *et al.*, 1997; Fullner and Nester 1996; Baron *et al.*, 2001). The 25° C co-cultivation temperature chosen by Kelly & Kado (2002) is within the upper limit of possible AMT. To investigate why no DNA transfer was observed with the published conditions, a different medium and a different method was tried. In particular IM, a specialized medium for optimized expression and function of the $Dtr_{Ti}/CP_{Ti}/T4SS_{Ti}$, was used for co-cultivation and combined with a filter method to be able to retrieve *S. lividans* for selection after the co-cultivation. This approach unfortunately also did not result in AMT of *S. lividans* (**Table 2**).

Though *S. lividans* was shown to be viable on IM, RP4-mediated DNA transfer to *S. lividans* on IM did not occur (**Table 3**). Therefore, different media, co-cultivation times and temperatures were tested subsequently. R5 rich medium (specific for *Streptomyces*) was used instead of IM medium, cultured on cellophane filter or directly on the agar surface, but this also did not generate positive results (**Table 2**). For activation of the virulence system the medium should contain preferably a lower pH (between 5 and 6), and fewer nutrient sources than present in the tested R5 and R2 media at a temperature of around 21° C. The virulence proteins $VirA_{Ti}$ and $VirG_{Ti}$ form a two-component signaling pathway, which mediates the expression of the other Ti plasmid encoded virulence proteins at low pH and room temperature in the presence of acetosyringone (Stachel, Nester & Zambryski, 1986; Shaw *et al.*, 1988; Turk *et al.*, 1991). However, previously an *A. tumefaciens* helper strain called LBA1126 was constructed that showed hypersensitivity for induction of vir_{Ti} expression even at a pH of 7 (Bundock & Hooykaas, 1996). LBA1126 contains modifications in both $virA_{Ti}$ and $virG_{Ti}$. Its $VirA_{Ti}$ protein has its periplasmic domain replaced by that of the *E. coli* TAR chemoreceptor, so that the protein is locked in a highly responsive state making it also less thermo sensitive and less pH sensitive (Melchers *et al.*, 1989; Turk *et al.*, 1993). The $VirG_{Ti}$ protein with substitution (I77V) already gives maximal vir_{Ti} gene expression in the presence of

low amounts of acetosyringone (Scheeren-Groot *et al.*, 1994). Unfortunately, also when using the strain LBA1126 no transformants of *S. lividans* could be observed after co-cultivation on R5 or even IM medium.

When tested in this study, *E. coli* ET12567 containing pUZ8002 was able to mobilize the shuttle vector pSET152*RB* containing the *oriT*$_{RP4}$ into *S. lividans* (**Table 4**). However, when plasmid pUZ8002 and pSET152*RB* were co-introduced into *A. tumefaciens* LBA288 or *E. coli* DH10β no conjugative transfer to *S. lividans* was observed. Both, *A. tumefaciens* and *E. coli* DH10β are known to methylate DNA (Gelvin, Karcher & DiRita, 1983; Kahng & Shapiro, 2001; Grant *et al.*, 1990). *Streptomyces* species in general contain a methylation restriction system for foreign DNA (González-Cerón, Miranda-Olivares & Servín-González, 2009), and for that reason the methylation deficient *E.coli* strain ET12567 is used as a donor for RP4-mediated *Streptomyces* transformation (Flett, Mersinias & Smith, 1997). But for *S. lividans* the restriction system is functioning in a low state (McNeil, 1988; González-Cerón, Miranda-Olivares & Servín-González, 2009). Therefore, methylated DNA that would come from the donor strains used during co-cultivation should only show a roughly 10% reduction in transformation efficiency as previously described (González-Cerón, Miranda-Olivares & Servín-González, 2009). In this study it was not attractive to replicate the experiment of González-Cerón, Miranda-Olivares & Servín-González (2009) to check if isolated DNA from *A. tumefaciens* would have a reduced transformation efficiency due to methylation, as high-quality plasmid DNA is difficult to obtain from *Agrobacterium*. Indeed RP4-mediated DNA transfer from *A. tumefaciens* LBA2210 to *S. lividans* turned out to be possible, although at a lower frequency than from *E. coli* ET12567 (**Table 4**). This raised questions about the original modification of pUZ8002. If pUZ8002 would only lack the *oriT*$_{RP4}$ sequence, the lack of mobilization activity was unexpected. The plasmid pUZ8002 was initially created for use in the *E. coli* strain ET12567 (McNeil *et al.*, 1992). The modification of the plasmid (except the mutation in the *oriT*$_{RP4}$ region) was not fully known and citations lead to a final reference marked as personal communication of the author (Paget *et al.*, 1999). It could be for example that the *E. coli* strain ET12567 could supply a function for pUZ8002 to mediate DNA transfer. It is also to note that ET12567 pUZ8002-transfer *to S. lividans* did not occur when co-cultivation was performed on R5 medium, where *A. tumefaciens* RP4-mediated DNA transfer was possible.

It could be assumed that the environmental impact of the used co-cultivation medium, pH and temperature would be the main parameters influencing the transformation as discussed earlier. During conjugative plasmid transfer between bacteria, the DNA-bound relaxase is transferred into the recipient as well, enabling re-circularization of the single-stranded DNA molecule (Dostal *et al.*, 2011). However, in the recipient cell endogenous DNA processing proteins are required for double-strand DNA formation. Remarkably, the RP4 plasmid encodes a primase that is transferred in the course of co-cultivation. The primase acts on the *oriT*$_{RP4}$ present on the shuttle vector, initiating priming of the single-stranded DNA for DNA polymerization by the host DNA replication machinery (Yakobson *et al.*, 1990; Miele *et al.*, 1991; Pansegrau *et al.*, 1994). The presence of a transferred primase could explain the successful RP4-mediated transfer of the shuttle vector pSET152*RB* from *A. tumefaciens* to *S. lividans*, while AMT remained undetectable. Also it is not certain if the re-circularization of the plasmid at its *RB*$_{Ti}$ could occur in *S. lividans* after AMT, although evidence has been obtained that this can happen in yeast (Bundock *et al.*, 1995; Rolloos *et al.*, 2014). If the RP4 primase is necessary for RP4-mediated transfer to *S. lividans*, adding this primase to the Ti system may lead to a more versatile T-DNA transformation with which transfer to *Streptomyces* may be possible. Another observation was made related to the speed of RP4-mediated transfer to *Streptomyces*. The successful transformation of *S. lividans* using *A. tumefaciens* with RP4 and pSET152*RB* was observed after 24 h only, but no transfer was detected in the first 2 h **(Table 4)**. This is in accordance with Mazodier, Petter & Thompson

(1989), showing that transfer occurs only with *Streptomyces* germ tubes that emerge from the spore around 2-6 h after germination, and not with vegetative mycelium (Jyothikumar *et al.*, 2008; Chater, 2011). The RP4-mediated transfer from *A. tumefaciens* occurs at a similar speed as pUZ8002-mediated transfer from *E. coli* (Gust, Kieser & Chater, 2002). This indicates that the length of co-cultivation beyond 48 h, as used in many AMT protocols (optimized for yeast transformation) would not be not necessary when transfer to *Streptomyces* would be attempted again. The sensitive period would be between 2-6 h of *Streptomyces* spore germination, reducing the effective co-cultivation time to within the 24 h window, thus making the method comparable to that with RP4 transformation methods.

In conclusion, the initial findings of Kelly & Kado (2002) could not be replicated. In different co-cultivation environments, no AMT to *S. lividans* was observed. With the collected data *S. lividans* could no longer be considered as a research objective to create a high-throughput screening platform to study AMT. But DNA transfer mediated by the RP4 plasmid was shown to be successful between *A. tumefaciens* and *S. lividans*, indicating that the continuation to study the comparison between both transfer systems would be highly interesting.

Materials and methods

Organisms and constructs

All used organisms are listed in **table 5**. All used plasmids are listed in **table 6**. *E. coli* DH10β was made competent and transformed using the protocol of Inoue *et al.* (1990). Transformation of *E. coli* ET12567 was done according to Chung *et al.* (1989). Introduction of plasmids into *A. tumefaciens* was done by electroporation (den Dulk-Ras & Hooykaas, 1995). Transformation of *S. lividans* was done according to Gust, Kieser & Chater (2002). All strains of *E. coli* and *A. tumefaciens* containing plasmids were cultured on appropriate antibiotic selection (**Tables 5 and 6**) on LB agar or in liquid LB medium (10 g/L bacto tryptone, 5 g/L bacto yeast extract, 8 g/L NaCl, pH 7.0). *E. coli* was cultured at 37° C, *A. tumefaciens* at 29° C and *Streptomyces* at 30° C.

Cloning

Molecular cloning was performed according to standard laboratory methods (Sambrook & Russell, 2001). Plasmids were contained and amplified in *E. coli* DH10β. The plasmid pUZ8002 was isolated from *E. coli* ET12567 (pUZ8002) using the plasmid isolation kit GeneJet Plasmid Midi-prep Kit (Fermentas).

Construction of pSET152 based shuttle vectors

The shuttle vectors used were constructed starting from the pSET152 vector, which was optimized for *Streptomyces* genomic integration using the integrase of bacteriophage ΦC31 (Bierman, Logan & O'Brian, 1992). The border sequence, *KanMX* resistance cassette and the *pVS1* minimal replicon (*sta* and *rep*) were isolated from pOphis vectors (Rolloos *et al.*, 2014). The plasmid pOphisΔ*RB* (original name: pOphis Borderless/BL) was digested with PvuII and the fragment containing a *KanMX* cassette and the *pVS1* replicon was inserted into the PvuII digested pSET152 backbone containing the $oriT_{RP4}$, *pMB1* replicon, and ΦC31 integrase gene. This created the vector pSET152Δ*RB* containing. Plasmid pOphis*RB* was digested with PvuII and the fragment containing the 25 bp RB_{Ti} sequence in combination with a upstream adjacent 38 bp OD_{Ti} sequence, *KanMX* cassette and *pVS1* replicon was as well ligated into the PvuII digested pSET152 backbone containing the $oriT_{RP4}$, *pMB1* replicon, and ΦC31 integrase gene. This created the following vector pSET152*RB*. The $oriT_{RP4}$ region (synonymously with $oriT_{RK2}$; Bierman, Logan & O'Brian, 1992) was removed cutting pSET152*RB* and pSET152Δ*RB* with ApaI and SphI. The 840 bp fragment containing only the $oriT_{RP4}$ was separated from the backbone fragment. The $oriT_{RP4}$ sequence on pSET152 was itself based on the 760 bp HaeII fragment of the RP4 plasmid (Guiney & Yakobson, 1983). The ApaI and SphI overhangs of the linear plasmids were blunted using T4 DNA polymerase (Thermo Scientific) and ligated with T4 DNA ligase (Thermo Scientific) creating the plasmids pSET152*RB*Δ*oriT* and pSET152Δ*RB*Δ*oriT*. The plasmids pSET152*RB* and pSET152Δ*RB*, pSET152*RB*Δ*oriT* and pSET152Δ*RB*Δ*oriT* have the blunt ended PvuII fragment inserted in the same orientation as was verified via restriction digest. To test transfer to yeast pSET152*RB* was additionally equipped with the autonomous replication and centromeric region sequence *ARS6/Cen4* to allow for stable replication in yeast cells after transfer. The *ARS6/Cen4* sequence was amplified from vector pUC34CPF with primer pair 5'-AGG TAC CCC ACC TGG GTC CTT TTC ATC ACG TG and 5'-AGG TAC CTA ATG GTT TCT TAG GAC GGA TCG CTT GC, both containing KpnI sites. The KpnI digested PCR fragment was introduced in the KpnI sites of the plasmids resulting in the plasmid pSET152Y*RB* (Y: indicative of yeast compatible shuttle or mobilizable vector).

Aggregate formation assay

The aggregate formation assay between *A. tumefaciens*, *S. lividans* or *S. venezuelae* was performed as described in Kelly & Kado (2002). *A. tumefaciens* LBA1100 (pSET152*RB*) was induced prior for 5.5 h in induction medium (IM) at 29° C, containing 200 μM acetosyringone (as described by Bundock *et al.* (2005). For the coagulation experiment, *A. tumefaciens* LBA2210 (pSET152*RB*) was prior cultured for 5.5 h with a starting OD_{600} of 0.25 to reach growth in the logarithmic phase of the culture at 29° C. The combined cultures in the test tubes were observed at room temperature for coagulation at 5, 15, and 30 min, as well as after 12 h.

Co-cultivation experiments

AMT co-cultivation of *A. tumefaciens* to *S. lividans* was essentially as performed after Kelly & Kado (2002), using R2 medium (Kieser *et al.*, 2000) as well as R5 medium (Kieser *et al.*, 2000) both containing acetosyringone as inducing agent for AMT.

Other AMT co-cultivations were performed using the protocol of Bundock *et al.* (1995). Modifications in this protocol were as follows: *A. tumefaciens* was pre-cultured over night before the co-cultivation in LB broth containing appropriate antibiotics, the culture was then diluted into a final concentration of OD_{600} of 0.25 in IM containing 200 μM acetosyringone, and further cultured whilst shaking at 29° C, 5 h prior to the co-cultivation. Sterile cellophane filters were used during *Streptomyces* co-cultivation (Kieser *et al.*, 2000). Commercially available cellophane wrapping film was used, cut into discs fitting a 90 mm petri dish, and autoclaved while submersed in H_20. Per co-cultivation, an average of 1×10^6 spores were used. Spores were heat shocked for pre-germination for 10 min at 50° C and kept for 1 h at 30° C, until mixed with *A. tumefaciens*. The length of co-cultivation varied according to the experiment, as described in the results. Co-cultivation was performed at either 21° C or 30° C, as described in the results. The biofilm was removed from the cellophane filter with a metal scraper and placed in 1 mL H_2O. The mixture was vigorously shaken until the biofilm was homogenously suspended in the solution. Selection of transformed *Streptomyces* cells was performed either on R2 medium (Kieser *et al.*, 2000), or R5 medium (Kieser *et al.*, 2000) at 30° C containing 50 μg/mL apramycin and 200 μg/mL cefotaxamine. For growth on 6-well plates, the modified protocol for optimized AMT was used **(Chapter 2)**. After 2 and 3.5 h the recovered biofilm was removed and placed directly on selective plates.

The transformation of *Streptomyces* using *E. coli* ET12567 (pUZ8002, pSET152*RB*), *E. coli* DH10β (pUZ8002, pSET152*RB*), *A. tumefaciens* LBA288 (pUZ8002, pSET152*RB*) and *A. tumefaciens* LBA2210 (RP4, pSET152*RB*) were performed according to Gust, Kieser & Chater (2002). Co-cultivation was performed at 29° C. When *S. lividans* (1×10^7 pre-germinated spores) was cultivated without an *E. coli* donor strain but just with 1 μg of isolated pSET152*RB* plasmid DNA, the co-cultivation was also performed according to Gust, Kieser & Chater (2002). Transformed *S. lividans* cells, after co-cultivation, were cultured and selected on appropriate on MS medium or R5 medium plates (Kieser *et al.*, 2000), containing 50 μg/mL apramycin and either 25 μg/mL nalidixic acid to select against *E. coli,* or 200 μg/mL cefotaxamine to select against *A. tumefaciens*. *S. venezuelae* selection was done on MYM medium (4 g/L maltose, 4 g/L yeast extract, 10 g/L malt extract and 20 g/L bacto agar) that was supplemented after autoclaving with 2.5 mL/L R2 trace element solution (Kieser *et al.*, 2000). The transformation efficiency was determined by dividing the output transconjugants CFU by the input of *Streptomyces* cells.

Verification pSET152*RB* genomic integration

To verify the genomic integration of the shuttle vector pSET152*RB*, primer Apra-INT-FW 5'-ACG CCG AGG AGA AGT ACC TG and primer Apra-INT-RV 5'-ACG CCG AGG AGA AGT ACC TG were used to detect the apramycin cassette in the *S. lividans* genome with PCR, using DreamTaq DNA polymerase (Thermo Scientific). Genome isolation was performed with the GenElute bacterial genomic DNA kit (Sigma-Aldrich), after positive cultures were isolated from co-cultivation with *E. coli* ET12567 (pUZ8002, pSET152*RB*).

Table 5. Organisms used in this study.

Strain	Description	Resistance	Reference
A. tumefaciens			
LBA288	based on WT C58, Cured of pTiC58, based on LBA201 but with spontaneous Rif and Nal; cryptic plasmid pAtC58	Rif	Hooykaas *et al.*, 1980
LBA1100	pAL1100 or octopine pTiB6 ΔT_L, ΔT_R , Δ*tra*, Δ*occ*; C58 chromosomal background; cryptic plasmid pAtC58	Rif, Spec	Beijersbergen *et al.*, 1992
LBA1126	VirG I77V, VirA-TAR, C58 chromosomal background; cryptic plasmid pAtC58	Rif, Spec	Bundock & Hooykaas, 1996
LBA2210	RP4, cryptic plasmid pAtC58, C58 chromosomal background	Rif, Km, Tc, Cb, Nal	PJJ Hooykaas (unpublished)
E. coli			
DH10β	*F- araDJ39*, *A(ara, leu)7697*, *AlacX74*, *galU*, *galK*, *rpsL*, *deoR*, *Φ80dlacZAM15*, *endAI*, *nupG*, *recA1*, *mcrA*, *A(mrr hsdRMS mcrBC)*		Grant *et al.*, 1990
ET12567	pUZ8002; *dam*$^-$, *dcm*$^-$, *hsdM*$^-$	Cm, Km	Paget *et al.*, 1999
Streptomyces			
S. lividans	66 (1326); WT		Cruz-Morales *et al.*, 2013
S. venezuelae	ATCC 10712		Pullan *et al.*, 2013

Cb: Carbenicillin; Cm: Chloramphenicol; Km: Kanamycin; Nal: Nalidixic acid; Rif: Rifampicin; Spec: Spectinomycin; Tc: Tetracycline; WT: Wild-type

Table 6. Genetic constructs used in this study.

Plasmid	*oriV*	Description	Resistance	Reference
pAL1100	*In-cRh-1*	pAL1100:ΔT_L, ΔT_R, Δ*tra*, Δ*occ*	Spec	Beijerbergen *et al.*, 1992
pSET152	*pMB1*	lacZαMCS; *ori*T_{RP4}; Int$^{\Phi C31}$	Apra	Bierman, Logan & O'Brian, 1992
pSET152*RB*	*pMB1, pVS1*	*OD*$_{Ti,Oct}$; *RB*$_{Ti,Oct}$; *ori*T_{RP4}; *KanMX*	Apra	This study
pSET152Δ*RB*	*pMB1, pVS1*	Δ*OD*$_{Ti,Oct}$; Δ*RB*$_{Ti,Oct}$; *ori*T_{RP4}; *KanMX*	Apra	This study
pSET152*RB*Δ*oriT*	*pMB1, pVS1*	*OD*$_{Ti,Oct}$; *RB*$_{Ti,Oct}$; Δ*ori*T_{RP4}; *KanMX*	Apra	This study
pSET152Δ*RB*Δ*oriT*	*pMB1, pVS1*	Δ*OD*$_{Ti,Oct}$; Δ*RB*$_{Ti,Oct}$; Δ*ori*T_{RP4}; *KanMX*	Apra	This study
pSET152Y*RB*	*pMB1, pVS1*	*OD*$_{Ti,Oct}$; *RB*$_{Ti,Oct}$; *ori*T_{RP4};*KanMX*; *ARS6/Cen4*,	Apra	This study
pUG34CFP	*pMB1*	pRUL1001; contains *ARS6/Cen4*	Cb	Sakalis, 2013
pOphis*RB*	*pVS1*	*OD*$_{Ti,Oct}$; *RB*$_{Ti,Oct}$, Δ*LB*$_{Ti,Oct}$, *KanMX*	Km	Rolloos *et al. 2014*
pOphisΔ*RB*Δ*LB*	*pVS1*	pOphis Borderless/BL; Δ*OD*$_{Ti,Oct}$; Δ*RB*$_{Ti,Oct}$; Δ*LB*$_{Ti,Oct}$; *KanMX*	Km	Rolloos *et al.*, 2014
pUZ8002	*Inc*Pα	RP4/RK2 derivative Δ*ori*T_{RP4}	Km	Paget *et al.*, 1999
RP4	*Inc*Pα	*mob*; *tra*; *ori*T_{RP4}	Km, Tc, Cb	Pansegrau et *al.*, 1994

oriV: Origin of vegetative replication (synonymously with origin of replication *oriRep* or *ori*); Apra: Apramycin; Cb: Carbenicillin; Km: Kanamycin; Spec: Spectinomycin; Tc: Tetracycline; *ori*T_{RP4} is synonymously with *ori*T_{RK2}; Oct: octopine from pTi15955; Y: *ARS6/Cen4*; *RB*$_{Ti,Oct}$: Right Border pTi$_{Oct}$; *LB*$_{Ti,Oct}$: Left Border pTi$_{Oct}$; *OD*$_{Ti,Oct}$: Overdrive pTi$_{Oct}$ (Rolloos *et al.*, 2014)

Acknowledgements

This work is part of the research programme Green Genetics with project number 828.11.001, which is (partly) financed by the Dutch Research Council (NWO).

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