Conjugal transfer of the symbiotic plasmid in *Rhizobium galegae* sv. officinalis

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Rhizobia are agriculturally important bacteria that possess the ability to fix nitrogen for their host legumes, an attribute ascribed to the presence of symbiosis-related genes usually clustered on plasmids called symbiotic plasmids (pSyms). Many pSyms have been proven self-transmissible, capable of transferring themselves to other bacteria through conjugation, thereby propagating their symbiotic features. *Rhizobium galegae* symbiovar (sv.) officinalis has a pSym, on which typical conjugation genes have been revealed. A Type IV secretion system (T4SS) functioning as a conjugation system has also been computationally predicted on a chromid, another replicon in *R. galegae* sv. officinalis. In addition, the transfer of the pSym of *R. galegae* sv. officinalis to a non-nodulating mutant strain of *R. galegae* sv. orientalis has been previously observed under laboratory conditions. Therefore, this thesis was aimed at investigating the self-transmissibility of the pSym of *R. galegae* sv. officinalis and the necessity of the T4SS' presence for the pSym transfer.

Two derivatives of the *R. galegae* sv. officinalis were generated with one strain cured of its pSym by using Tn*5-mob-sacB* transposon and the other strain excised the T4SS from the chromid by Cre-*lox* site specific recombination system. Conjugation were then performed between these two derivatives as well as between the wild-type strain and the plasmid-cured derivative, followed by the host plant nodulation tests. The tests showed no formation of a single nodule in either pair, which was unexpectedly inconsistent with the previous experimental observation. No solid explanations could be proposed at this stage. It might be due to the low transfer frequency resulted from complex associations with subtle environmental signal molecules or recipient cell recognition that presumably disabled the transmissibility of the pSym.

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1. Introduction

Conjugation is one of the most important approaches for bacteria (the recipient cells) to acquire genetic materials from other bacteria (the donor cells). Those materials are often delivered in the form of plasmids, a type of DNA molecules, which are mostly circular and replicate independently of the bacterial chromosome. Plasmids normally carry genes that are not necessarily essential for bacterial growth, but of which products benefit the host bacteria under certain circumstances, such as the genes for resistance to antibiotics and heavy metals. Plasmid conjugation contributes to bacterial genome plasticity, thereby, helping bacteria adapt to changing environmental conditions. (Snyder & Champness, 2007)

Rhizobia are a major group of soil Gram-negative bacteria that can form symbiotic associations with leguminous plants with the outcome of eliciting the formation of nodules on roots or occasionally on stems of legumes. They are the primary symbiotic fixers of nitrogen, capable of reducing atmospheric nitrogen to ammonium that is then secreted to legumes, which, in return, provide shelters as well as energy sources (Mylona et al. 1995). This biological process plays a critical role in sustainable agriculture for the great reduction of the input of exogenous nitrogen fertilizer. Rhizobia usually contain multiple replicons, consisting of a chromosome and other large-sized replicons, such as chromids (a DNA element, having distinct and consistent properties with both chromosomes and plasmids) (Harrison et al. 2010), and plasmids (Mercado-Blanco & Toro 1996). The plasmids that harbor genes needed for establishment of symbiosis (*nod, nif,* and *fix* genes) are known as symbiotic plasmids (pSyms), and other replicons provide genetic materials to enhance rhizobial chances of survival and propagation (MacLean et al. 2007).

Rhizobium galegae is one species of the genus *Rhizobium*. It has two symbiovars (sv) (orientalis and officinialis) that form effective nodules on the roots of legumes *Galega orientalis* and *Galega officinalis* respectively (Radeva et al. 2001). The genome sequencing of two representative strains from the two symbiovars of *R. galegae* was conducted by our rhizobia research group in the University of Helsinki (Janina Österman et al., unpublished). It was found that *R. galegae* sv. officinalis has three replicons, a chromosome, a chromid and a plasmid. The symbiotic region is situated on the plasmid. On the contrary, its *R. galegae* sv.

orientalis counterpart only has two replicons, a chromosome and a chromid, and the symbiotic region is resident on the chromid.

Conjugal transfer of plasmids among rhizobia have been reported extensively over the past decades and genome sequencing of many rhizobial strains has revealed the presence of typical conjugation genes (Ding & Hynes 2009). It is rather comprehensible and of significance, considering the possession of multiple replicons especially plasmids in rhizobia and their genetic needs to cope with complex soil environments. The genome sequencing of *R. galegae* sv. officinalis also manifested two genetic regions potentially involved in conjugal transfer. One is the chromid putative Type IV secretion system (T4SS) region that is homologous to the AvhB T4SS required for the self-transmissibility of the plasmid pAtC58 in *Agrobacterium fabrum* C58 (formerly *Agrobacterium tumefaciens* C58, Lassalle et al. 2011). The other region is located on the pSym, containing the *tra* operons and a *trb* operon, which were also found in other rhizobial strains specialized for their plasmids' conjugal transfer. In addition, one plasmid conjugal transfer experiment executed previously in our research group showed that the pSym of *R. galegae* sv. officinalis can be transferred to a derivative of its counterpart *R. galegae* sv. orientalis obtained by Räsänen et al. (1991) under laboratory conditions. Combination of this finding with its predicted two genetic conjugation regions evoked the interest to uncover the responsible region(s) for its pSym conjugal transfer, which distributes its symbiosis features, so that the categorization of its pSym in terms of transfer abilities could be confirmed and possible knowledge of its plasmid transfer control could be achieved in *R. galegae* sv. officinalis.

2. Literature Review

2.1. Rhizobium and rhizobial genomics

The rhizobium–legume interaction is an intensively studied model for symbiosis, evolution and differentiation in agriculture. It is the most efficient system of biological nitrogen fixation (BNF), which is the most significant biochemical process on earth besides photosynthesis and the main route for combined nitrogen to enter terrestrial ecosystems. (Lindström et al. 2010, Lindström & Mousavi 2010)

For historical reasons, the term "rhizobia" has not only been used to refer to bacteria belonging to the genus *Rhizobium* as it should be in the strictest sense (Willems 2006), but also used for genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ochrobactrum*, *Phyllobacterium*, *Shinella*, *Sinorhizobium* (syn*. Ensifer*) that are all members of alpha subclass of the Proteobacteria (Lindström & Mousavi 2010). Additionally, several symbiotic members of beta-proteobacteria are also called rhizobia (Lindström & Mousavi 2010). As more diverse range of legume plants, not only confining to the agriculturally important species, have been studied as well as dramatic improvement in molecular research methods such as DNA sequencing and typing methods, increasing number of rhizobial species are described and the classification of rhizobia is undergoing constant modifications (Suominen 2001, Williems 2006). However, the taxonomy of some rhizobia cannot always reflect their real function since the same genus or even species can contain both nitrogen-fixing and non-fixing strains but they all have "rhizobium" in their names (Masson-Boivin et al. 2009).

Rhizobia are phylogenetically, metabolically and genetically diverse, displaying a varying extent of host specificity (Masson-Boivin et al. 2009). Some of them are able to nodulate many different host plants (broad host range), while the others have a limited number of hosts (narrow host range). Nevertheless, no strict correlation between host legumes and bacterial taxonomy can be claimed in spite of some favored associations such as *Azorhizobium-Sesbania* and *Burkholderia-Mimosa* (Masson-Boivin et al. 2009). The coverage of two subclasses of the Proteobacteria and their varied biotopes such as soil and plant rhizoshpere indicate a great phylogenetic diversity of rhizobia, which is consistent with the exhibition of a wide range of metabolic features required for survival in various environments. Most of the rhizobia are heterotrophic obligate microaerophiles using rhizoshpere organic compounds as carbon and energy sources for their central metabolism (Prell & Poole 2006). A group of *Bradyrhizobium* strains can grow anaerobically by reducing nitrate and nitrite for energy conservation (Polcyn & Luciński 2003), while another group of strains in that genus can perform photosynthesis, allowing them to survive *ex planta* as well as making contributions to nitrogen fixation energetically *in planta* (Giraud & Fleischman 2004, Prell & Poole 2006). In addition, *Azorhizobium caulinodans* was reported to be a type of free-living species with the ability to fix nitrogen (Dreyfus et al. 1988, Masson-Boivin et al.

2009) and methylotrophy is another alternative metabolic option that is pertinent to symbiosis between the rhizobial species *Methylobacterium nodulans* and its host plant *Crotalaria podocarpa* (Jourand et al. 2005). The metabolic versatility of rhizobia can be reflected in their large, complex genomes, ranging from 5.4-9.2 Mb, according to the genome sequencing results of 6 representatives out of 14 known genera (Lindström & Mousavi 2010). These genomes possess a large inventory of genes for regulation, transportation and different catabolic systems needed for rhizobia to adapt to their respective environment (Masson-Boivin et al. 2009). Moreover, the distinguished ability to establish symbiosis with defined host plants indicates that more genetic requirements have to be satisfied in rhizobia, which in part explain their large genomes and extrachromosomal replicons. The differences in genome size among rhizobia can be partially accounted for by the presence of additional replicons (plasmids) carrying accessory functions and ranging in size from 100 kb to over 2Mb (MacLean et al. 2007, Ding & Hynes 2009). *B. japonicum* USDA110 that lacks plasmids has the largest chromosome among rhizobia (MacLean et al. 2007).

Symbiosis-related genes (*nod, nif* and *fix*) are often clustered on one or more large plasmids called symbiotic plasmids (pSyms) or on a replicon, recently named chromid by Harrison et al. (2010) to differentiate from plasmids and chromosomes (Ding & Hynes 2009). A chromid possesses the replication and maintenance system of plasmid style, but carries several housekeeping genes that are found on the chromosome in other species and has a nucleotide composition similar to that of the chromosome (Harrison et al. 2010). Some of the symbiotic genes are located on chromosomal regions known as symbiotic islands instead (Ding & Hynes 2009).

In addition to pSym, many rhizobia have proved to contain one or more non-symbiotic plasmids. Although they used to be called "cryptic" plasmids, they are assumed to carry the functionally important genes involved in increasing the bacteria's overall competitiveness and fitness in the rhizoshpere (Ding & Hynes 2009). Barreto et al. (2012) performed a curing of resident plasmids and generated several cured derivatives in *Rhizobium tropici* strain CIAT 899. They found that one cured non-symbiotic plasmid (which they named plasmid a) contains genes involved in the competition for nodulation in plant *Phaseolus vulgaris.* A similar function was also found for *nfe* (nodule formation efficiency) genes localized on a

large non-symbiotic plasmid (pRmeGR4b) of *Sinorhizobium meliloti* by Soto et al. (1994). They indicated that the *nfe* genes affect nodulation efficiency and competitiveness of *S. meliloti* on the roots of host alfalfa since these plants inoculated with mutant strains of *nfe* genes exhibited delayed nodulation and lower numbers of nodules. Another study on a non-symbiotic plasmid of *S. meliloti* was carried out by Stiens et al. (2006). They analyzed the whole nucleotide sequence of pSmeSM11a, a non-symbiotic plasmid from a dominant indigenous *S. meliloti* strain SM11 in the context of a long-term field release experiment with genetically modified *S. meliloti* strains. Their sequence analysis showed that pSmeSM11a carries lots of genes acquired from different sources that encode accessory functions such as broadening of its host range and catabolic capacity, making this strain outcompete the released genetically modified *S. meliloti* strains in the field release experiment. In addition, Brom et al. (2000) demonstrated other impacts that non-symbiotic plasmids of *Rhizobium etli* exert such as plasmid transfer, cellular growth and viability and utilization of different carbon sources. More functions including bacteriocin production, lipopolysaccharide production, exopolysaccharide production and synthesis of vitamins have been reported (Ding et al. 2013).

2.2. *Rhizobium galegae*

Rhizobium galegae is a legume root nodule forming species described by Lindström (1989). It nodulates goat's rue, *Galega officinalis* and fodder galega, *Galega orientalis* in a highly host-specific manner. *Rhizobium galegae* strains that form nitrogen-fixing (effective) nodules on *G. orientalis* form non-nitrogen-fixing (ineffective) nodules on *G. officinalis* and vice versa. A study conducted by Radeva et al. (2001) on a polyphasic analysis of the relationship of *R. galegae* strains from Caucasus and eight other geographic regions showed that the symbiosis-related genotypic features among *R. galegae* strains can divide this species into two groups. *R. galegae* strains establishing an effective symbiosis with *G. orientalis* were called *R. galegae* bv. orientalis whereas strains forming an effective symbiosis with *G. officinalis* were called *R. galegae* bv. officinalis correspondingly. Rogel et al. (2011) proposed the term symbiovar (sv) for rhizobia as a parallel term to pathovar in pathogenic bacteria. They believed that symbiovar is more appropriate to reflect the symbiotic capability of rhizobial strains and define their host range than the term biovar (bv). Hence, *R. galegae* bv. orientalis and *R. galegae* bv. officinalis are called *R. galegae* sv.

orientalis and *R. galegae* sv. officinalis respectively in this thesis. Neither of the *R. galegae* strains are able to infect other leguminous plants and the *Galega* plants can only occasionally be infected by other rhizobia, yet forming ineffective nodules at best (Lindström, 1989).

Legumes *G. orientalis* and *G. officinalis* originated from Caucasus and the Middle East respectively (Radeva et al. 2001, Lasseigne 2003). *G. orientalis* has a good agricultural application potential as feed and is planted as a persistent perennial forage legume (Varis 1986). It along with its symbiont bacterium is also a good candidate for phytoremediation purposes in oil-contaminated sites (Suominen et al. 2000). *G. officinalis* is not suitable as fodder, but it has medical significance (Bailey & Day 2004).

2.3. An overview of plasmid conjugal transfer

Conjugation is the one of main pathways for microorganisms to transfer their genetic materials. During conjugation, generally speaking, two strands of a circular plasmid separate, and one strand moves from the donor bacterium into the recipient bacterium, leading to a single strand in both bacteria that then serve as the templates for replication of complete double-stranded DNA molecules. Bacteria that have received DNA as a result of conjugation are called transconjugants (Snyder & Champness 2007).

Conjugation is a complex process, much more than described above, that requires a cluster of *trans-acting* genes called *tra* genes and a *cis-acting* site named *oriT* site. The *tra* genes can be divided into two components: the Mpf component involved in the mating-pair formation and the Dtr component for DNA transfer and replication. The Mpf system includes genes coding for a pilus that projects from the donor bacterium to contact with the recipient bacterium, holding them together. It also encodes a channel or mating pore through which proteins or DNAs are transferred during the mating process. Additionally, the Mpf component has a sort of protein called coupling protein that can transmit the signal that the donor cell has made contact with a recipient cell to the relaxase, a central part of the Dtr component, before DNA transfer actually occurs. The coupling protein is so called because of its role to couple the Dtr component with the Mpf component. The relaxase is then activated to nick a single strand of the plasmid to be transferred to initiate the conjugation process. The relaxase remains covalently attached to the 5′ end of that nicked

strand and drags the strand into the recipient cell along with it. It is also responsible for recyclizing the plasmid after transfer. In addition to relaxase, the Dtr component contains primase, helicase and other accessory proteins for DNA replication. The helicase separates double strands of plasmids, and the primase synthesizes RNA primers to prime the replication of the complementary strand to make double-stranded circular DNA in the recipient cell. On the contrary, in the donor cell, the 3′ OH end at the nick made by the relaxase can act as a primer for making a complementary strand of the remaining single stand. Thus, both the donor and the recipient cell end up having a double-stranded circular copy of the plasmid. The *oriT* sequence, also called *mob* site, is the origin of transfer where a single strand is nicked. It is also the site at which DNA ends rejoin after transfer (Snyder & Champness 2007).

In terms of transfer abilities, transferable plasmids can be categorized into self-transmissible plasmids and mobilizable plasmids. Self-transmissible plasmids are able to transfer on their own by utilizing their own Dtr and Mpf components. Mobilizable plasmids, however, can only be transferred with assistance of a self-transmissible plasmid. Theoretically, in the presence of a self-transmissible plasmid, any mobilizable plasmid that contains the *oriT* sequence of that self-transmissible plasmid can be mobilized. However, the simplest mobilizable plasmids carrying only the *oriT* site of a self-transmissible plasmid have not been found naturally. All mobilizable plasmids, to our current knowledge, encode their own Dtr systems including their own helicase and relaxase, but lack Mpf systems. The relaxases of mobilizable plasmids are usually able to communicate with a broader scope of coupling proteins than that of self-transmissible plasmids for the purpose of taking advantage of different Mpf systems provided by different self-transmissible plasmids. (Snyder & Champness 2007)

The essential aspect of mobilizable plasmids being transferred efficiently by another plasmid lies in if its relaxase can be specifically recognized by and interacted with the coupling protein of a Mpf system of a co-resident plasmid (Snyder & Champness 2007, Ding & Hynes 2009). Therefore, the coupling protein is a critical determinant of mobilization specificity. Szpirer et al. (2000) demonstrated that the coupling protein TraG of a self-transmissible plasmid RP4 mediates the connection between the relaxase Mob protein of a broad host range mobilizable plasmid pBHR1 and the Mpf component of RP4. They also found that the

binding of the cytoplasmic membrane TraG protein to the Mob relaxase can occur in the absence of any other conjugal protein or *oriT* site. Hamilton et al. (2000) indicated that proteins TraG and VirD4 from the plasmid pTiC58 conjugal transfer system (TraG$_{pTic58}$) and T-DNA transfer system on pTiC58, respectively, in *Agrobacterium fabrum* C58 are coupling proteins. Together with the coupling protein TraG of RP4 (TraG$_{RP4}$), these proteins confer conjugal specificity among plasmids RP4, pTiC58 and RSF1010. They found that TraG$_{pTiC58}$ cannot recognize the relaxases of plasmid RP4 or RSF1010, leading to the failure of transfer of RSF1010 and low transfer frequency of RP4 by pTiC58. TraG$_{RP4}$, on the other hand, can communicate with the relaxase of RSF1010. If TraG$_{pTiC58}$ is replaced by TraG$_{RP4}$, pTiC58 can mobilize RSF1010. Furthermore, the combined interactions of relaxase, coupling protein and Mpf component can determine the mobilization frequency (Sastre et al. 1998, Szpirer et al. 2000). It has been shown that the carboxyl terminus of the TraD coupling protein of F plasmids is engaged in efficient coupling with the F plasmid relaxase. The loss of amino acids on the carboxyl terminus broadens the range of its interaction with other relaxases at the expense of a decrease in the efficiency of F plasmid mobilization (Sastre et al. 1998).

The *mob* site and plasmid mobilization have important applications in molecular genetics. The mobilizable plasmid can be used as cloning vectors and the *mob* site can often be incorporated into the vectors. Once the foreign DNA of interest has been cloned into such cloning vectors, it can be introduced into even distantly related bacteria with the help of a promiscuous self-transmissible plasmid that provides its Mpf functions *in trans* (Snyder & Champness 2007).

2.4. Conjugal gene transfer in rhizobia

As mentioned above, symbiotically relevant genes of rhizobia are mainly located on plasmids, chromid, or chromosome in the form of symbiotic islands, which are prone to be transferable and have an accessory nature (Ochman & Moran 2001, Finan 2002). The facts that rhizobium–legume interactions involve diverse distantly related bacterial genera again support the view that the symbiotic capabilities were acquired through horizontal gene transfer. Moreover, it is evident that at the time when eukaryotic plants emerged, bacteria had already existed and diverged (Ochman & Moran 2001). Successful acquisition of symbiotic genes requires not only the transfer and integration of those genes, but more

importantly, the stable maintenance of obtained genes throughout generations of the recipient bacteria. On one hand, the persistence of symbiotic genes results from natural selection favoring traits encoded by acquired genes and gradual optimization of symbiotic performances. On the other hand, rhizobial ancestors already possessed some features that help counteract defense reactions of host plants, make up for the scarcity of certain nutrients in the host and ultimately adapt to host environment (Ochman & Moran 2001). Therefore, it is believed that rhizobial ancestors living in close proximity to legumes had latent symbiotic potential and became symbionts of legumes upon incorporation of new symbiotic genes and activation of their inherent symbiotic-related functions (Masson-Boivin et al. 2009).

Even though the conjugal transfer frequency under laboratory conditions is often relatively low or at an undetectable level (Ding & Hynes 2009), there is still mounting reported evidence for conjugal gene transfer among rhizobial strains. Sullivan & Ronson (1998) reported that a 500-kb symbiotic island in *Mesorhizobium loti* ICMP3153 could transfer itself to at least three non-symbiotic mesorhizobial strains under experimental conditions and integrate into their phenylalanine-tRNA gene, converting the original saprophytes into symbiotic counterparts. Complete genome sequencing of *M. loti* MAFF303099 (Kaneko et al. 2000) and *B. japonicum* USDA110 (Kaneko et al. 2002) also revealed the presence of a presumptive 611-kb and a 681-kb symbiotic island in these two strains with the ability to integrate into the phenylalanine-tRNA gene and valine-tRNA gene respectively.

In contrast, some rhizobia that have plasmid borne symbiotic genes were found to have the potential to transfer their pSyms, or already have attained symbiotic genes via conjugation. Pérez-Mendoza et al. (2004) developed an approach to identify functional *mob* regions in the *Rhizobium etli* genome. They found the presence of a functional *mob* region including a putative *oriT* site and a typical conjugal transfer gene (*tra*) cluster in the pSym (pRetCFN42d) of *R. etli* which suggests that this plasmid might be self-transmissible. In particular, a gene *yp028* located downstream of *traA* experimentally showed a role that it played in promoting the transfer of pSym. Overexpression of this gene can activate pSym conjugal transfer in response to unknown environmental signals, contradicting the previous thought that pRetCFN42d transfers itself through co-integration with a co-resident self-transmissible plasmid pRetCFN42a (Tun-Garrido 2003). The *yp028* gene was renamed later by Pérez-

Mendoza et al. (2005) as *rctB* (see section 2.5.2). Brom et al. (2002) carried out restriction fragment length polymorphism (RFLP) analysis of Southern-blotted total DNAs probed with several pSym-borne genes of *R. etli* CFN42. Those total DNAs are from different bean-nodulating species (*R. etli, R. gallicum, R. giardinii, R. leguminosarum, S. fredii*). The cluster analysis of RFLP results showed that strains belonging to *R. gallicum, R. giardinii, R. leguminosarum* and *R. etli* formed one group, implying that these strains shared the same pSym, and that conjugal plasmid transfer has taken place among those strains. In *R. galegae*, Suominen et al. (2001) investigated the common nodulation genes (*nodDABCIJ*) of *R. galegae* sv. orientalis by DNA sequence analysis and subsequent phylogenetic analysis. They found that the topology of phylogenetic trees constructed based on nodulation genes is incongruent with that based on the 16S rRNA genes. In addition, the presence of non-symbiotic bacteria mingled with rhizobia in the phylogenetic tree of α-proteobacteria made them believe that the symbiotic flair of *R. galegae* is not derived vertically from their primordial ancestors but rather from horizontal gene transfer, after which the symbiotic facilities evolved in a way following its host plant. All these studies unanimously proved the acquired nature of symbiosis through conjugal gene transfer in rhizobia, which exerts essential roles in evolution since the new ability enables rhizobia to exploit new ecological niches.

In agriculture practice, field trials have been performed using genetically modified rhizobial strains. Those trials revealed that the applications of rhizobial inoculants in agriculture are still problematic. For one thing, introduced rhizobia often cannot persist for a long time after being released to leguminous seeds or soil in the field due to the unsuccessful competition with other indigenous bacteria and the inadaptability of rhizobial inoculants themselves to the local environmental conditions such as weather and soil type (Amarger 2002, Hirsch 2004). For another, the potential of conjugal gene transfer has to be taken into account for the sake of biological safety control especially for those genetically modified rhizobia. If the experimentally manipulated transgenes have transferred to native bacteria before the disappearance of their genetically modified rhizobial host in the field, the transgenes could survive and sustain. Evidence has been provided that under certain circumstances, plasmids from indigenous bacteria can also transfer to rhizobial inoculants, leading to inoculants' changed phenotype (Amarger 2002, Hirsch 2004).

As a whole, more studies on conjugal transfer of rhizobial plasmids need to be carried out (Ding & Hynes 2009) in order to achieve a further understanding of the mechanisms and the regulations of conjugation, thereby allowing better monitoring and prediction of genetically modified plasmids in rhizobial inoculants.

2.5. Regulation of conjugal transfer of rhizobial plasmids

Rhizobia cannot conjugate their plasmids all the time since DNA processing and mating-pair formation is energetically expensive (Lanka & Wilkins 1995). This process should be carried out under stringent regulation systems so that plasmids only transfer under appropriate conditions. It has been summarized in a review by Ding and Hynes (2009) that there are two types of well-known rhizobial conjugation systems in terms of the different regulatory mechanisms: the quorum-sensing regulated conjugation system (type I) and the RctA-repressed conjugation system (type II). Additionally, two more types of conjugation system (type III and type IV) have also been proposed on the basis of sequence analysis.

2.5.1. Quorum-sensing regulated conjugation system

Quorum-sensing (QS) is utilized by some bacteria to regulate their cellular functions including symbiosis, pathogenesis, bioluminescence, biofilm formation, growth rate control, conjugation and antibiotic production, correlated with their population densities (He & Clay 2006). Several groups of molecular signals synthesized by bacteria are called autoinducers. They can diffuse across cell envelope and only when the cell density of bacteria making the same autoinducer is high enough, can the autoinducer reach a threshold level to interact with a transcription activator that induces the expression of target genes (Madigan et al. 2012).

There are different groups of autoinducers. The best studied group is the *N*-acyl homoserine lactones (AHLs) produced mainly by Gram-negative bacteria. Varied length and saturation of acyl side chains define the signaling specificity. Another type of autoinducer made by many Gram-negative bacteria is termed autoinducer-2 (AI-2), a cyclic furan derivative. Gram-positive bacteria generally use certain oligopeptides as autoinducers (Madigan et al. 2012).

Many species of *Rhizobium* and closely related plant pathogenic species of *Agrobacterium* have been found to use AHL-mediated QS systems. More than one regulatory circuit is

present in some reported species and in each case, a unique interactional network was displayed in each species (He & Clay 2006). The paradigm for QS regulation of plasmid transfer was established based on the tumor-inducing plasmid (pTiC58) of *Agrobacterium fabrum* (He & Clay 2006, Sanchez-Contreras et al. 2007). The transfer and integration of an oncogenic DNA segment (T-DNA) from pTiC58 into susceptible host plant cells and subsequent expression of genes on the integrated T-DNA can cause crown gall disease. The transfer of that segment relies on a *traI/traR/traM* QS system encoded by the pTiC58. When the threshold concentration of an AHL cognate, the 3-oxo-C8-HSL, whose synthesis is catalyzed by the AHL synthase TraI, is reached due to a proper bacterial density, it will bind to the transcription activator TraR that subsequently induces the expression of conjugal transfer relevant operons (*traAFBH* and *traCDG*). TraM is the anti-activator of TraR by binding to the TraR-AHL complex and thereby inhibiting the occurrence of conjugation. Additionally, the QS system in *A. fabrum* also needs the presence of plant-secreted signal molecule opines to be active. (He & Clay 2006, Sanchez-Contreras et al. 2007)

Another well-understood example is pRL1JI, the pSym of *R. leguminosarum* bv. *viciae*. The overall regulation process is shown in Figure 1. Different from the stereotypical *traI/traR/traM* QS circuit, one more regulatory gene *bisR (bi*functional *s*ignaling *r*egulator*)* was found to situate upstream of the *traR* gene, forming a *traI/bisR/traR/traM* circuit on pRL1JI. An additional chromosome-encoded QS *cinR/cinI* circuit is also involved in the regulation of plasmid transfer. A long-chain AHL cognate, 3-OH-C14:1-HSL is synthesized by the CinI AHL synthase under the control of the CinR protein which activates the expression of *cinI* in response to the concentration of 3-OH-C14:1-HSL. The BisR protein can recognize this long-chain AHL molecule and induce *traR* expression, whose product acts as the transcription activator for conjugal transfer genes. Meanwhile, BisR can repress the expression of *cinI* and diminish the amount of 3-OH-C14:1-HSL. Therefore, a strain that has pRL1JI produces only a little of 3-OH-C14:1-HSL, resulting in minor induction of *traR* by BisR. Even if there is a small amount of TraR, its activities can be inhibited by TraM analogous to TraM in *A. fabrum,* regardless of the TraI-made C8-HSL and 3-oxo-C8-HSL AHL molecules. When a strain carrying pRL1JI as a donor comes close to a strain lacking it, an appropriate amount of 3-OH-C14:1-HSL harbored by the potential recipient strain is detected by BisR in

the donor followed by induction of *traR* expression which induces the initiation of pRL1JI conjugal transfer. (He & Clay 2006, Sanchez-Contreras et al. 2007)
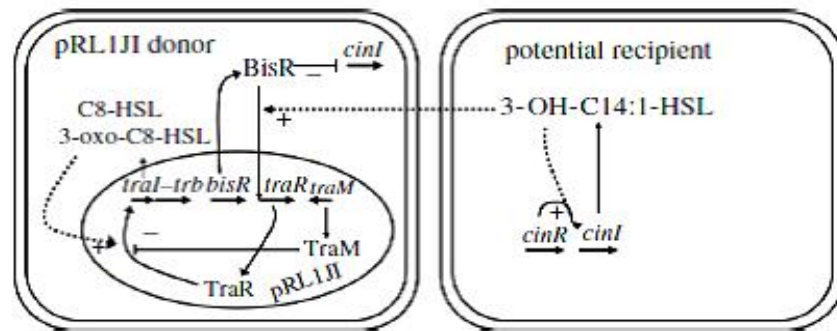

Figure 1. The model for the regulation of pRL1JI conjugal transfer in *R. leguminosarum* sv. *viciae*. The effects of AHL molecules on the regulators are shown as dashed lines. The inducing and repressing effects of regulators are shown as solid lines with "+" and "-" respectively. This figure is from Sanchez-Contreras et al. (2007) with a small modification.

Other rhizobial plasmids that have been demonstrated to use a QS-mediated conjugation system primarily include a non-symbiotic plasmid pRetCFN42a in *R. etli* CFN42 (Tun-Garrido et al. 2003), symbiotic plasmid pNGR234a in *Rhizobium* sp. NGR234 (He et al. 2003), which is now named pSfrNGR234a in *Sinorhizobium fredii* strain NGR234, and a non-symbiotic plasmid pRm41a in *S. meliloti* Rm41 (Marketon & González 2002).

2.5.2. RctA-repressed conjugation system

Another type of conjugation regulatory network different from the QS-dependent system was found on the symbiotic plasmid pRetCFN42d of *R. etli* CFN42, which is supported by the absence of QS gene homologs and two novel functional genes *rctA* and *rctB* (*r*egulation of *c*onjugal *t*ransfer) involved in conjugal transfer (Pérez-Mendoza et al. 2005). It was proved by Pérez-Mendoza et al. (2005) that the *rctA* gene represses the transcription of pRetCFN42d conjugal transfer genes in standard laboratory media, which are assumed to be unfavorable conditions for conjugation. Therefore, the high level of *rctA* gene expression sustains the low frequency of plasmid transfer. Moreover, *rctA* homologs SMa1323 and Atu5160 were computationally found to be present on the symbiotic plasmid pSymA of *S. meliloti* 1021 and the cryptic plasmid pAtC58 of *A. fabrum* C58 , respectively, and these homologs turned out to be functional substitutes for *R. etli* RctA in suppressing pRetCFN42d conjugal transfer. SMa1323 can influence pSymA conjugal transfer, using a similar mechanism as pRetCFN42d does, whereas Atu5160 does not seem to be effective for

pATC58 since pAtC58 was able to conjugate at high frequency under laboratory conditions in the presence of Atu5160. It may result from incomplete suppression activity of the Atu5160 protein or failure of expression of the Atu5160 gene. (Pérez-Mendoza et al. 2005)

A second gene that plays a role in regulation of conjugation in *R. etli* is the *rctB* gene previously named *yp028* (Pérez-Mendoza et al. 2004). It is located downstream of the *traA* gene and serves as an activator of the expression of pRetCFN42d conjugal transfer genes and its overexpression under yet-to-be-known environmental conditions can counteract the effect of the RctA protein or interfere with *rctA* gene expression, letting the transfer of plasmid proceed. However, no *rctB* homologs have been identified in *S. meliloti* or *A. fabrum* C58 as *rctA* homologs do, which suggests they use a different derepression system for conjugal transfer. (Pérez-Mendoza et al. 2005)

Sepúlveda et al. (2008) carried out a study to demonstrate the specific mechanism that the RctA protein uses to repress conjugal transfer of pReCFN42d. They found out that RctA binds to a specific region on the promoter of the *virB* operon which is divergently transcribed from the *rctA* gene and is one type of operon coding for the Mpf component needed for conjugation (see section 2.6), thus hindering the access of RNA polymerase to the *virB* promoter and *virB* transcription. The binding region is from nucleotides -26 to +5 relative to the transcription starting site, including a conserved 9-bp sequence, termed RBM (*rctA* binding motif) box (nucleotides -17 to -25). It was also found that transcription of the *virB* operon interferes with *rctA* expression. Hence, combined with *rctB* gene effects, the scenario for the regulation of pReCFN42d conjugal transfer in *R. etli* CFN42 could be as follows: under limiting conjugal transfer conditions, expression of the *virB* operon is inhibited by binding of RctA to the *virB* promoter and conjugation is restrained. When favorable yet unknown conditions for conjugation are met, the *rctB* gene can be expressed and the RctB protein interferes with *rctA* gene expression or blocks the binding of RctA to the *virB* promoter. Meanwhile, the reduced amount of bound RctA protein can activate the expression of the *virB* operon, which can further dampen the expression of the *rctA* gene. Therefore, conjugation occurs. (Pérez-Mendoza et al. 2005, Sepúlveda et al. 2008) This RctA-dependent repression mechanism was also found to be employed by *S. meliloti* for the conjugal transfer of its pSymA but with two additional regulatory elements (Nogales et al. 2013).

2.5.3. Potential third and fourth types of conjugation systems

As more genome sequences are available, a phylogenetic analysis based on TraA protein revealed the potential existence of a type III conjugation system in plasmids pRL10JI, pRL11JI, and pRL12JI of *R. leguminosarum* sv. viciae 3841 and pRleVF39d, pRleVF39e, pRleVF39f of *R. leguminosarum* sv. viciae VF39SM (Ding & Hynes 2009). Conjugation experiment showed that none of these plasmids can be self-transmissible owing to the lack of an Mpf component (Young et al. 2006, Ding & Hynes 2009). Nonetheless, pRleVF39d, pRleVF39e and pRleVF39f can be mobilized in the presence of a self-transmissible plasmid pRleVF39c yet not by the presence of well-known self-transmissible plasmids pRL1JI, pRetCFN42a and pRetCFN42d (Ding & Hynes 2009). More experimental evidence would be required to identify the specific mechanism.

Ding et al. (2013) characterized a novel rhizobial plasmid conjugation system in *R. leguminosarum* sv. viciae VF39SM. They indicated that a *trb* operon encoding an Mpf component, a *traA* relaxase gene and a coupling protein *traG* gene are required for the self-transfer of the plasmid pRleVF39b. In addition, a product of the *trbR* gene located in the region between *traA* and *traG* functions as a repressor of both *trb* operon expression and pRleVF39b transfer. Phylogenetic analysis of relaxase, coupling protein and TrbE/VirB4 (TrbE/VirB4 was chosen as a representative of Mpf component in rhizobial conjugation systems) all grouped the analyzed plasmids into four categories except that the TrbE/VirB4-based tree has three branches since rhizobial plasmids of the Type III conjugation system do not have any Mpf component (see above). The fourth plasmid group mainly contains pRleVF39b, pRLG203 from *R. leguminosarum* bv. trifolii WSM2304, pAtS4a from *Agrobacterium vitis* S4, plasmid 1 from *Chelativorans* sp. BNC1 and pSmed03 from *Ensifer medicae* WSM419. Within this group, pRleVF39b shares highest similarity to pRLG203, forming a distinct branch from the rest of type IV plasmids in the three phylogenetic trees. Hence, Ding et al. (2013) classified pRleVF39b and pRLG203 as type IVA conjugation plasmids and the remainder as type IVB conjugation plasmids.

2.6. Comparison between the conjugation system in rhizobia and other Type IV secretion systems (T4SSs)

Conjugation systems mediate the transfer of DNA molecules among bacterial populations. Proteins constituting the Mpf component of conjugation systems share extremely high similarity to the proteins of some secretion systems which are responsible for delivering effector molecules, often virulent factors of some pathogens, to eukaryotic target cells. Representatives of the pathogens include *Helicobacter pylori*, *Legionella pneumophila* and *Bordetella pertussis* (Table 1). Therefore, Christie (2001) combined conjugation systems and ancestrally related effector translocation systems into one macromolecular (DNA, proteins or DNA-protein complexes) transfer system which is referred to as the Type IV secretion system (T4SS). A review by Cascales and Christie (2003) added one more group to this superfamily, the DNA uptake and release system, which also functions as a DNA exchange system without the requirement of cell contact (Chen & Dubnau 2003, Table 1). Hence, generally speaking, the bacterial T4SSs can be used either for exchange of genetic material or delivery of effector molecules to eukaryotic cells.

A phylogenetic analysis was conducted by Frank et al. (2005) based on divergence patterns of bacterial species having a complete set of T4SS components, sequence similarity to one of the T4SS proteins, VirB4, and sequence similarity of seven concatenated sequences of the T4SS *virB* operon (*virB*3-5, *virB*8-11). Their analysis indicated that horizontal gene transfer is a major impetus to the evolution of T4SSs due to the incongruence between the species tree and the T4SS operon tree. They also carried out a parsimony analysis, which suggested that the conjugation system is the ancestral state of T4SS and effector translocation systems evolved from conjugation systems. Furthermore, there is a high chance that chromosomally encoded T4SS genes have been acquired from plasmids via horizontal gene transfer.

Table 1. Representatives of T4SSs (modified based on Cascale and Christie 2003).

Bacterial species (involved DNA elements or exported effectors)	T4SS	Consequences
Conjugation		
Agrobacterium fabrum (pTiC58)	Trb	Genetic exchange
Agrobacterium fabrum (T-DNA)	VirB	Crown gall/ Genetic exchange
Agrobacterium fabrum (pAtC58)	AvhB	Genetic exchange
Escherichia coli (plasmid F, IncF)	Tra	Genetic exchange
Escherichia coli (plasmid RP4, IncP)	Trb	Genetic exchange
Escherichia coli (plasmid R388, IncW)	TrW	Genetic exchange
Legionella pneumophila	Dot/Icm	Genetic exchange
DNA uptake and release		
Campylobacter jejuni	Cjp/VirB	DNA uptake
Helicobacter pylori	ComB	DNA uptake
Neisseria gonorrhoeae	Tra	DNA release
Effector translocation		
Agrobacterium fabrum (VirE2, VirE3 and VirF)	VirB	Crown gall
Mesorhizobium loti R7A (Msi059 and Msi061)	VirB	Symbiosis
Bordetella pertussis (Pertussis toxin)	Ptl	Whooping cough
Helicobacter pylori (CagA)	Cag	Gastritis, peptic ulcer
Legionella pneumophila (DotA, LidA, RalF)	Dot/Icm	Legionnaire's pneumonia

The T4SS in *A. fabrum* is a prototypical example, encompassing both a conjugation system and an effector translocation system. The T4SS enables *A. fabrum* to transfer oncogenic T-

DNA located on the Ti plasmid and virulent effectors VirE2, VirE3, VirF to infect plant cells, giving rise to a plant tumor called crown gall. In the T4SS involved in T-DNA transfer, VirB1-VirB11 assemble the Mpf component and VirD4 is the coupling protein (Cascales & Christie, 2003). The Ti plasmid itself is self-transmissible using another conjugation system called the TrB system (from *trbB* to *trbL*), mainly composing the Mpf component. Meanwhile, the *virC* and *virD* operons and the *tra* operon (*traAFBH* and *traCDG* operons) encode the Dtr component for T-DNA and Ti plasmid respectively (Hamilton et al. 2000; Ding & Hynes 2009). In addition, Chen et al. (2002) discovered a third T4SS in *A. fabrum* C58, the AvhB (for *Agrobacterium* virulence homologue *virB;* from AvhB2-AvhB11) system required for the self-transmissibility of the cryptic plasmid pAtC58. A putative Dtr system resembling the *tra* operon on the Ti plasmid and an *oriT*-like site were also identified. They conclusively manifested some characteristics of the three T4SS systems such as functional non-redundancy, specialized substrates to be transferred for each system, distinct inducing environmental signals and interchangeability among some components of the three systems.

Hubber et al. (2004) reported that the symbiotic island of *M. loti* R7A possesses a T4SS system that highly resembles the VirB/D4 system of *A. fabrum* judged from amino acids and *virB* operon structure similarity. Instead of transferring DNA, this system translocates effector proteins, presumably Msi059 and Msi061, to plant cells, thereby specifying symbiosis between *M. loti* R7A and its compatible host plant *Lotus corniculatus* and incompatible plant *Leucaean leucocephala*. The activation of T4SS and translocation of Mis059 and possibly Mis061 determine the host range of *M. loti* R7A, ensuring it to nodulate *L. corniculatus* but not *L. Leucocephala*, the process of which is subject to a VirA/VirG two-component regulatory system (Hubber et al. 2007).

In *S. meliloti* 1021, Jones et al. (2007) described a different role that its T4SS plays. The T4SS is encoded by a *virB* operon (*virB1-virB11*) and *virD4* on plasmid pSymA, and shows highest similarity to the AvhB system of pAtC58 in *A. fabrum* C58 (see above). Their experiment characterized the conjugal transfer functions that its T4SS has instead of determination of symbiotic host range borne by T4SS of *M. loti* R7A. It is believed that in both cases of *S. meliloti* 1021 and *M. loti* R7A, T4SSs are dispensable for intracellular symbiosis contrary to their intracellular animal pathogen relatives such as *Brucella* spp. and *Bartonella* spp. that

require a complete T4SS for animal host infections and stable maintenance within their hosts.

The pSym of *R. etli* CFN42, pRetCFN42d was also found to harbor a full set of T4SS genes: the *virB1-virB11* operon, a *traA* gene and a *traCDG* operon (González et al. 2003). These plasmid-encoded Vir proteins are highly similar to the Vir proteins in *S. meliloti* 1021 and the Avh proteins in *A. fabrum* C58 (Frank et al. 2005), whereas the conjugation system of its co-resident pRetCFN42a bears closer relatedness to the TrB system of the Ti plasmid in *A. fabrum*.

Sequence analysis has identified a complete *virB* operon (*virB1-virB11*) and a *tra* operon (*traA-traG*) in a megaplasmid pSfrNGR234b (pNGR234b) of *S. fredii* strain NGR234 (*Rhizobium* sp. NGR234) (Schmeisser et al. 2009), and a *trb* operon (*trbB* to *trbL*) and a *tra* operon (*traA-traG*) in the symbiotic plasmid pNGR234a (Freiberg et al. 1997), making both plasmids capable of being transferred to bacteria through conjugation. This T4SS together with other secretion systems including a twin arginine translocase secretion system, six type I transporter genes, one functional and one putative type III secretion system probably play the major roles to make this species outcompete other known rhizobia in the number of legumes that it can nodulate (Schmeisser et al. 2009).

2.7. Plasmid curing in rhizobia

Plasmid curing is a process of completely removing a plasmid from its host bacterium. It is often desirable to obtain a plasmid-cured derivative, which allows a direct comparison between the plasmid-containing and plasmid-cured strains. In this way, functions of lots of rhizobial plasmids have been identified (Hynes et al. 1989, Hynes & McGregor 1990, Baldani et al. 1992, Brom et al. 1992, 2000, Barreto et al. 2012).

2.7.1. Plasmid curing methods

Conventional methods of plasmid curing that mainly focused on using curing chemical agents such as acridine orange, ethidium bromide and sodium dodecyl sulphate (SDS) are not quite suitable and convenient in rhizobia, because those chemical agents are toxic or mutagenic to humans as well (Amábile-Cuevas & Heinemann 2004). Meanwhile, they are of low efficacy to cure such large and stable plasmids as pSyms in rhizobia (Hynes et al. 1989).

Therefore, other methods have been applied in different situations. Hynes et al. (1985) succeeded in removing the plasmid pAtC58 from its host strains *A. fabrum* LBA275 and LBA290 by introducing the *Rhizobium meliloti* plasmid pRme41a, which is incompatible with pAtC58. The resident pTiC58 was also cured simply by incubating those strains at higher temperature, generating the plasmid-free *A. fabrum* UBAPF1 and UBAPF2 strains that were shown to be extremely useful for subsequent studies on rhizobial plasmids. Sharma and Laxminarayana (1989) investigated the impacts of elevated temperature on the symbiotic properties of *Rhizobium* spp. (*Cajanus*). They found that pSym can be cured at 40-45 °C, resulting in the loss of its symbiotic properties, which can explain the low nitrogen fixation efficacy in the host plant pigeonpea in the semi-arid region in India.

2.7.2.   Background information of the plasmid curing method used in the thesis

Relevant background knowledge for curing of plasmids in this thesis includes Tn*5-mob-sacB* transposon, triparental mating and suicide vectors which are introduced individually as follows.

Transposons are DNA elements that can hop from one site of DNA to another site. They encode their own transposases that promote their jumping. One type of transposons cut themselves out from their original DNA and paste into another DNA (cut-and-paste), whereas the other type of transposons copy themselves and then insert elsewhere in the cell (cut-and-copy). (Snyder & Champness 2007)

Tn*5* is a type of composite transposon, which serves as an excellent model system to investigate DNA transposition (Reznikoff 2003). It carries kanamycin, bleomycin and streptomycin resistance genes flanked by insertion elements (IS) which are the smallest bacterial transposons. When Tn*5* transposes, it follows the cut-and-paste pattern. One of the most common applications of transposons is in transposon mutagenesis. Tn*5* is ideal for the purpose of creating random insertion mutation because it transposes at a fairly high frequency with almost no target site specificity and has a broad host range, which means that it can transpose in any Gram-negative bacterium. Additionally, it carries a kanamycin resistance gene as a selection marker. (Snyder & Champness 2007)

As for the usefulness of both a *mob* site (see section 2.3) and Tn*5* at molecular level, Simon (1984) constructed the Tn*5-mob,* a transposon that carries the *mob* site of a self-transmissible plasmid RP4 within it. RP4 is a broad host range plasmid that can transfer itself or mobilize other plasmids from *E. coli* into many gram-negative bacteria. The introduction of a *cis*-acting *mob* site into a transposon like Tn*5* allows the labeling of any plasmid that it has jumped into with antibiotic selection markers as well as an increase in frequency of plasmid mobilization mediated by RP4. The kanamycin resistance gene in Tn*5* has often been utilized as a marker to monitor the plasmid transfer. Hynes et al. (1986) employed this Tn*5-mob* system to mobilize the megaplasmids in *S. meliloti* to *A. fabrum*, *R. leguminosarum* and *R. trifolii* strains. One of the transferred megaplasmids was identified as the pSym by hybridization and by the fact that the recipient strains can form ineffective nodules on alfalfa which is the host plant of *S. meliloti*. The other megaplasmid was believed to play a role in extracellular polysaccharide (EPS) production since this transferred megaplasmid complemented EPS$^-$ mutants. Moreover, both megaplasmids were thought to be involved in the formation of functional nodules on alfalfa.

After the construction and convenient applications of the Tn*5-mob* transposon, a further development was devised by Hynes et al. (1989). They incorporated the *sacB* gene from *Bacillus subtilis* into this construct to make the Tn*5-mob-sacB* transposon for elimination of several cryptic plasmids in strains of *R. leguminosarum* and *S. meliloti*. When induced by sucrose, the *sacB* gene encodes levansucrase that can catalyze hydrolysis of sucrose and synthesis of levans. It was shown that expression of the *sacB* gene is lethal for many gram-negative bacteria when grown in the presence of sucrose (Pelicic et al. 1995). This new Tn*5* derivative (Tn*5-mob-sacB*), therefore, allows direct selection of the loss of plasmids or generation of deletions in plasmids (Hynes et al. 1989). After the work of Hynes et al. (1989), many more studies on plasmid curing have been performed by applying this new construct (Hynes & McGregor 1990, Gigova et al. 1997, Oresnik et al. 2000, Barreto et al. 2012) or a similar construct Tn*5*-GDYN1 (Brom et al. 1992, 2000) developed by Romero et al. (1991). Quandt et al. (2004) constructed new Tn*5-mob* derivatives and an additional Tn*5-mob-sacB* derivative to enlarge the range of plasmid mobilization and plasmid curing systems in gram-negative bacteria.

When a plasmid mobilization is conducted, occasionally, a mobilizable plasmid to be transferred and the self-transmissible plasmid belong to the same incompatibility group so that they cannot stably coexist in the same strain. Also, it is possible that the self-transmissible plasmid provides its Mpf functions only for a short time after entering a recipient strain. Triparental mating provides an option for solving those problems. As the name suggests, triparental mating needs three bacterial strains to participate in this process. The three strains are designated as a self-transmissible plasmid carrier strain, a mobilizable plasmid carrier strain and the ultimate recipient respectively. When the three strains are mixed, some of the self-transmissible plasmids from the first strains will firstly transfer themselves into the second strains, propagating through the second strain, followed by quick mobilization of the plasmids in the second strains to the final recipient with a high efficiency. (Snyder & Champness 2007)

Any DNA that cannot replicate in a particular host can be used as a suicide vector. The most commonly utilized DNA elements as suicide vectors include plasmids or phage DNAs. It can be used for transposition assays where a transposon is introduced into a suicide vector together with an antibiotic resistance gene and the suicide vector is then transferred into an incompatible host in which it remains unreplicated and is eventually lost. The only way in which the transposon can avoid being lost with the vector and confer antibiotic resistance in the host cells is by hopping to another replicable DNA molecule such as a chromosome or a plasmid, implying the occurrence of transposition. (Snyder & Champness 2007)

2.8. Cre-*loxP*-based technique for genetic region excision

2.8.1. Mechanism of Cre-*loxP* recombination system

Cre-*loxP* is a site-specific recombination system discovered in the P1 bacteriophage which uses this system to circularize and promote replication of its genomic DNA. The Cre protein is a site-specific DNA recombinase encoded by the c*re* (*cyclization recombination*) gene of the P1 bacteriophage. It is a 38 kD protein that can recognize a sequence called *loxP* (*locus of X*-over of *P*1), and catalyze the recombination between two of these sites. The *loxP* site is 34 base pairs (bp) long, consisting of an 8-bp core sequence where recombination takes place and two flanking inverted repeats of 13 bp (Figure 2). (Sauer & Henderson 1988, Pechisker, 2004) The core sequence determines the orientation of the *loxP* site, which

together with its location defines the results of recombination (Nagy 2000). The *loxP* sites can be located *cis* (on the same side) or *trans* (on the other side), namely on the same molecule or on different molecules. The recombination between the *cis*-located *loxP* sites can generate deletion or inversion of a DNA region depending on if the two *loxP* sites are oriented in the same or in the opposite direction respectively (Figure 3. a, b). In the case of *trans* arrangement of *loxP* sites, Cre recombinase mediates a translocation between the two molecules (Figure 3. c) (Nagy 2000). The Cre-*loxP* system has mainly been utilized to create deletions of gene regions.

loxP	ATAACTTCGTATA	ATGTATGC	TATACGAAGTTAT
lox66	TACCGTTCGTATA	ATGTATGC	TATACGAAGTTAT
lox71	ATAACTTCGTATA	ATGTATGC	TATACGAACGGTA
lox72	TACCGTTCGTATA	ATGTATGC	TATACGAACGGTA
lox511	ATAACTTCGTATA	ATGTATAC	TATACGAAGTTAT
lox2272	ATAACTTCGTATA	AAGTATCC	TATACGAAGTTAT

Figure 2. The sequences of different *lox* sites. One strand of all sequences are shown in a 5′-to-3′ direction. *loxP* is the wild-type sequence and the rest are mutant sites. Boxed regions are the core sequences and the base pair changes in the flanking repeats or in the core region of mutant sites are underlined.

This Cre-*loxP* system works at high efficiency and accuracy. As the *loxP* site originates from the P1 bacteriophage and is 34 base pairs long, the random occurrence of this exact site on the genomes apart from P1 is extremely low, which makes it precise to carry out the deletion of gene regions from any organism regardless of the length of fragments between the two *loxP* sites as long as the flanking *loxP* sites have been artificially incorporated followed by subsequent expression of Cre recombinase (Nagy 2000, Ullrich & Schüler 2010). Furthermore, this system does not require any additional host factor or sequence to facilitate its recombination (Nagy 2000).
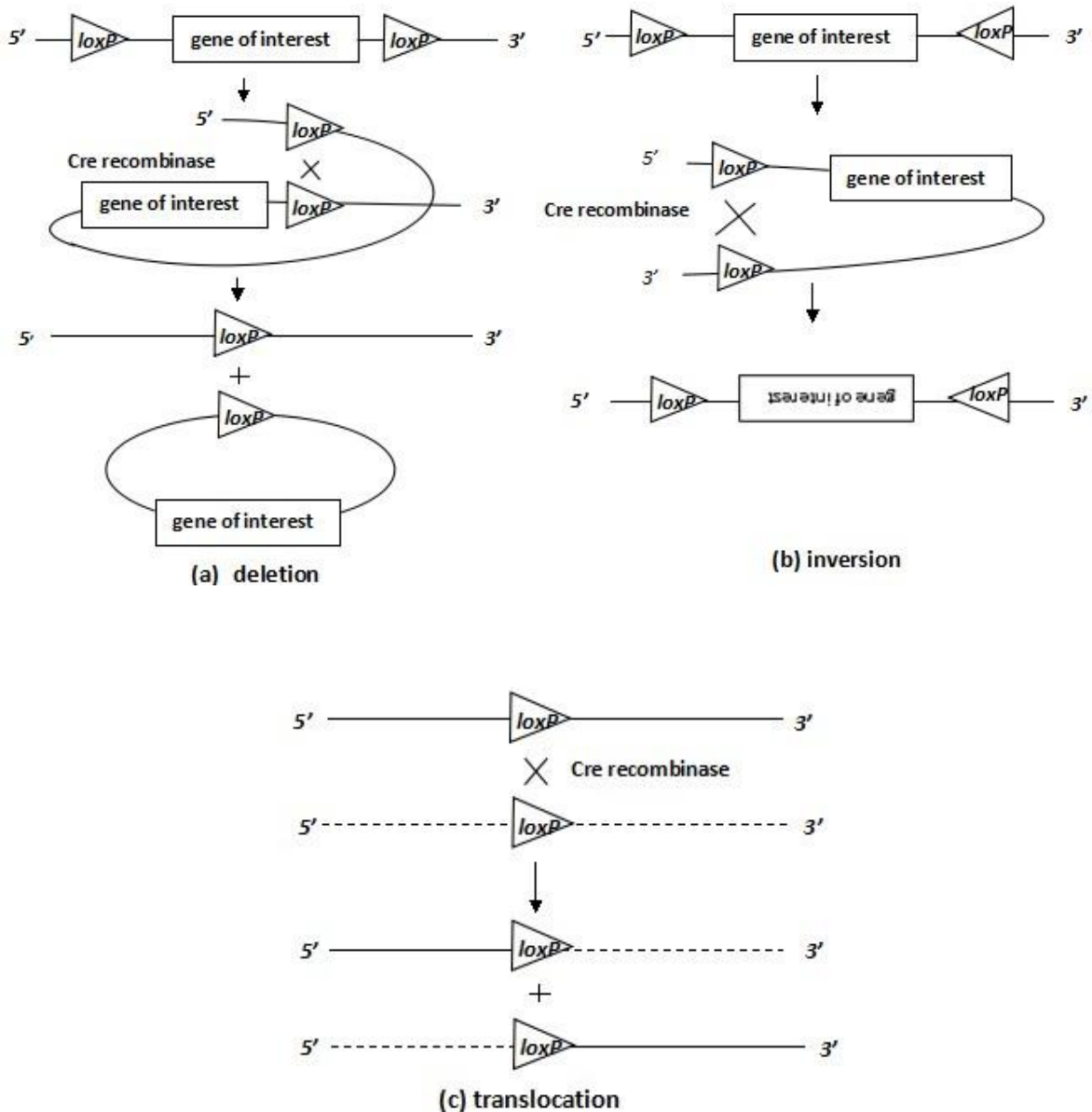
Figure 3. Schematic representation of Cre–*loxP* mediated recombination. (a) Recombination between two *cis loxP* sites orientated in the same direction creates a linear product containing one *loxP* site and a circular DNA product containing the other *loxP* site and the excised segment originally located between the two *loxP* sites. (b) Recombination between the opposite orientated *cis loxP* sites inverts the gene of interest between the two sites. (c) Recombination between two *loxP* sites from two different DNA molecules translocates the two DNA molecules.

2.8.2. Applications of Cre-*loxP* recombination system

This system has been widely used as a powerful genetic tool in a variety of eukaryotic and prokaryotic studies due to the merits mentioned above. Ullrich and Schüler (2010) have demonstrated the utility of the Cre-*loxP*-based method by successfully creating the deletion of 16.3-kb, 61-kb and 67.3-kb fragments within a Magnetosome Island (MAI) from a magnetotactic bacterium (MTB) *Magnetospirillum gryphiswaldense*, allowing functional

analysis of the MAI to be implemented. Antibiotic resistance markers always accompany artificial gene manipulations in molecular genetics studies, yet these markers could to some extent influence bacterial fitness such as energy waste from the expression of resistance genes and polar effects on the expression of downstream and upstream located genes. Leibig et al. (2008) set up this Cre-*loxP* system and removed the erythromycin and kanamycin resistance cassettes from the genomes of *Staphylococcus carnosus* and *S. aureus*. Lambert et al. (2007) devised a system to combine the gene replacement strategy with Cre-*lox* system to obtain marker-free multiple gene deletion strains in *Lactobacillus plantarum*.

As the *loxP* site is composed of two inverted repeats and the core region is the place where recombination actually takes place, after recombination, the *lox* site on the product is indistinguishable from the initial site, making another recombination event between the *lox* sites on the products possible (Albert et al. 1995). A strategy has been made to introduce base pair changes in the flanking repeats to generate different mutant *lox* sites, among which *lox66* and *lox71* are particularly efficient. One of the produced sites from recombination between *lox66* and *lox71*, the *lox72*, is a poor substrate site for Cre enzyme, so that it cannot act as a functional site for a next round of recombination (Figure 2) (Albert et al. 1995, Leibig et al. 2008). Another strategy has been to develop heterospecific *lox* sites such as *lox511* and *lox2272* that have base pair changes in the 8 bp core region (Figure 2) (Araki et al. 2002). In principle, recombination only occurs between homologous regions, i.e., identical regions. Therefore, the heterospecific *lox* sites can recombine between themselves but rarely or at least at low frequency with the wild-type *loxP* or other mutant sites, reducing the chance of re-recombination (Araki et al. 2002).

Apart from creating deletions, the Cre-*loxP* system, or systems with mutated *lox* sites, also provide an option for the integration of exogenous DNA into target DNA molecules even though deletion events are more kinetically favored (Thomson et al. 2003). Thomson et al. (2003) tested the efficiency of Cre-*loxP* mediated integration event by using lambda integrase to insert one single mutant *loxP* site into the *attB* site of the genome of *E. coli*, followed by introduction of a plasmid carrying another single mutant *loxP* site. Different single mutant *loxP* pairs were combined and the two most efficient mutant *loxP* combinations stand out, outshining wild-type *loxP* in terms of integration rate. It is plausible that the *cre* gene cannot easily be integrated into target DNA by the Cre-*loxP* system since

the expression of the *cre* gene can excise itself. Yet the integration of the *cre* gene in mouse embryonic stem (ES) cells was achieved by Araki et al. (2002) by using mutant *loxP* sites that were proved to possess high recombination efficiency and stability.

Another application of Cre-*loxP* system is to rearrange genomic segments such as inversions that disrupt the organization of genomic DNA followed by the study of phenotypic consequences. Campo et al. (2002) carried out three chromosomal inversions in *Lactococcus lactis* to different extents based on this system to study the dynamics of the *L. lactis* chromosome. All of the inverted strains showed a decreased growth rate in comparison with that of their isogenic parental strains. Meanwhile, they proved the feasibility of this system for the generation of genome rearrangements in bacteria with high recombination efficiency as well as independency of length of DNA fragments to be inverted.

3. Research objective

The transfer of pSym of *R. galegae* sv. officinalis has been previously observed. Two putative genetic regions for conjugal transfer, an AvhB-like T4SS and a region with two clusters of transfer genes (*tra* operons and a *trb* operon) have also been identified *in silico* on the chromid and pSym separately in *R. galegae* sv. officinalis. The main objective for this thesis was to investigate whether its pSym is self-transmissible or require extra genetic functions from the chromid for its conjugal transfer. The first subgoal was to cure the pSym from one strain of *R. galegae* sv. officinalis by using Tn*5-mob-sacB* transposon. The second subgoal was to excise the putative T4SS region on the chromid in another strain of *R. galegae* sv. officinalis by the Cre-l*oxP* recombination system. The third subgoal was to test if the pSym of the T4SS excised strain attained from the second subgoal is able to actively conjugate into the plasmid-cured strain obtained from the first subgoal. The plasmid transfer result can be examined by nodulation tests on the host plant.

4. Materials and methods

4.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in the work are listed in the Table 2.

Table 2. Bacterial strains and plasmids used.

Bacterial strains	Description	Sources or references
Rhizobium galegae sv. officinalis		
HAMBI[a] 1141	Wild type	Lindström (1989)
HAMBI 1207	[b]Sm^r derivative of HAMBI 1141	Lindström (1989)
Agrobacterium fabrum		
UBAPF2	Rf^r	Hynes et al. (1985)
Escherichia coli		
ST18	Δ*hemA*, donor strain	Thoma et al. (2009)
J-53	RP4-4 carrier, helper strain	Simon (1984)
Plasmids		
RP4-4	Nm^s, Ap^r, Tc^r, helper plasmid for mobilizations	Simon (1984)
pMH1701	Suicide vector, carries Tn*5*-B12S, Km^r, Nm^r	Hynes et al. (1989)
pCM157	Tc^r, Cre expression vector	Marx et al. (2002) Lohße et al. (2011)
pAL01_MCS1	Carries *gusA* gene and *lox 71* site, Km^r	Lohße et al. (2011)
pAL02/2_MCS2	Carries *lox 66* site, Gm^r	Lohße et al. (2011)

[a]HAMBI: the culture collection of the University of *H*elsinki, Faculty of *A*griculture and forestry, Division of *Microbi*ology. [b]antibiotics: Ap^r, Gm^r, Km^r, Nm^r, Rf^r, Sm^r, and Tc^r represent resistance to ampicillin, gentamicin, kanamycin, neomycin, rifampicin, streptomycin and tetracycline respectively; Nm^s represents neomycin sensitive.

Escherichia coli strains were grown at 37 °C on LB medium (Maniatis et al. 1982). *Rhizobium galegae* sv. officinalis strains and *Agrobacterium fabrum* were grown at 28 °C on tryptone yeast (TY) medium (Beringer 1974). All the strains that were destined for plasmid gel electrophoresis were grown at 28 °C on HP medium (Hynes et al. 1985). Culture media were

supplemented as required with appropriate antibiotics at the following concentrations: 100 μg/ml ampicillin (Ap), 25 μg/ml gentamicin (Gm), 50 μg/ml kanamycin (Km), 50 μg/ml neomycin (Nm), 150 μg/ml rifampicin (Rf), 1000 μg/ml streptomycin (Sm), 10 μg/ml tetracycline (Tc). For *R. galegae* sv. officinalis, neomycin was used in the place of kanamycin. The plasmid pAL01_MCS1 carries a *gusA* gene which codes for the β-glucoronidase enzyme. The concentration for its substrate X-Gluc (5-bromo-4-chloro-3-indoxyl-β-D-glucuronidase) was 50 μg/ml. As *E. coli* ST18 has a deletion mutation in its *hemA* gene, whose product is required to synthesize tetrapyrroles for growth, the media for *E. coli* ST18 was provided with 50 μg/ml of 5-aminolevulinic acid (ALA) to complement this mutation. Media were solidified by addition of 1.4 % (w/v) agar.

4.2.   Cre-*lox*-based method for excision of target region on the chromid of HAMBI 1141

4.2.1.   Construction of plasmids for integration of lox sites

To incorporate *lox* sites flanking the HAMBI1141 deletion target, the T4SS on the chromid, an upstream sequence of 1.331 kb and a downstream sequence of 1.658 kb were amplified using designed primers pairs (ZZ01-ZZ08 for upstream fragment and ZZ03-ZZ04 for downstream fragment) with the recognition sequence of the BamHI restriction endonuclease incorporated in the forward primers and that of NotI attached in the reverse primers (Table 3). A PCR master mix for 50 μl reaction of amplification of each fragment contains 37.5 μl of water, 1 x Phusion HF buffer (Thermo Scientific), 200 μM dNTP mixture, 0.5 μM forward primer, 0.5 μM reverse primer and 0.05 U of Phusion Polymerase (Thermo Scientific). Half-microliter (ca. 40 ng/ μl) of DNA template from genomic DNA of HAMBI 1141 was used in each reaction. The PCR program for the upstream sequence was set as follows: initial activation at 98 °C for 30 s followed by 34 cycles of denaturation at 98 °C for 10 s, annealing at 66.5 °C for 30 s, extension at 72 °C for 42 s, and final extension at 72 °C for 5 min. The program for the downstream sequence was almost the same with the exception of an annealing temperature at 68°C and extension time 50 s. The result of the PCR was examined by gel electrophoresis. A volume of 5 μl of amplified product mixed with 1 μl of 6 x DNA Loading Dye (Fermentas) was subjected to electrophoresis on a 1% (w/v) (Seakem® LE Agarose,Lonza) gel at 120 V for 1 h. The obtained PCR products were then purified with a QIAquick® PCR Purification kit (QIAGEN) and quantified with a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Table 3. Primers used in this work

Primers	Sequences 5'-3'
ZZ01	TTTAAAGGATCCGGTTCTCGGTCAGGTTGGAG
ZZ08	AAATTTGCGGCCGCACCGGATTCGAGGTTCGCTC
ZZ03	TTTAAAGGATCCGATGATGGCAAGTTCGGCAC
ZZ04	AAATTTGCGGCCGCCAATCACCAATGCGCTCCTG
AL064	CAGATTACGGTGACGATCCC
ZZ10	CGTGGAGATTTCGGCCTATG
ZZ09	CAGCCATCGCGTAACGTTG
AL062	CGCAACGCAATTAATGTGAG
RP-48	AGCGGATAACAATTTCACACAGGA
ZZ11	CGATCGGTGTAGAAGGTCTG
ZZ12	AGTGAGAAGTCCAGCTTCAG
ZZ13	CCGAAGCACGTGACATCCTG

Purified PCR products were cloned into pJET1.2/blunt vector using the Clone JET™ Cloning kit (Fermentas) according to the manufacturer's instructions and sequenced using primers provided by the kit to verify the sequences. All sequencing was performed by the DNA sequencing and Genomics Laboratory, Institute of Biotechnology, University of Helsinki, Finland.

Forty-five microliters (ca. 1.4 µg) of verified upstream and downstream PCR products were both digested with 3 µl of BamHI (FastDigest®, Fermentas) and 3 µl of NotI (FastDigest®, Fermentas) restriction endonucleases together with 1 × FastDigest® buffer (Thermo Scientific) and water to make 60 µl reactions at 37 °C for 30 min. After purification by a GeneJET™ PCR Purification kit (Fermentas), the digestion of PCR products were confirmed by agarose gel electrophoresis as described above.

Approximately 2 µg of plasmids pAL01_MCS1 and pAL02/2_MCS2 carrying the *lox71* site and *lox66* site respectively as well as multiple cloning sites, isolated using the GeneJET™ Plasmid Miniprep kit (Fermentas), were first digested with either BamHI or NotI restriction

enzyme individually in 20 µl reaction. The single enzyme digested vectors were then precipitated by adding 2 µl of Novagen® Pellet Paint® Co-Precipitant (EMD Chemicals, Inc.), 1/10 sample volumes of 3 M Na-acetate and 2 sample volumes of 100% ethanol sequentially. After gentle shaking and 2 min of incubation at room temperature, the mixture was centrifuged for 15 min and the supernatant was removed quickly followed by adding 200 µl of 70% ethanol and 5 min of centrifuge. The supernatant was discarded again and the pellet was air dried and eventually dissolved in 10 µl of water. Afterwards, all of the precipitated linear plasmids were digested with 2 µl of the other restriction enzyme (NotI or BamHI) and dephosphorylated with 2 U of FastAP^{TM} Thermosensitive Alkaline Phosphatase (Thermo Scientific) along with 1 × FastDigest® buffer and water to make 40 µl reactions at 37 °C for 30 min. Due to the different size between the original recognition sequences of NotI and BamHI on the two vectors, after digestion with BamHI and NotI, vector pAL01_MCS1 combined with 1/5 sample volumes of 6 × Loading Dye & SDS solution (Fermentas) was loaded on a freshly made 1% agarose gel and run at 90 V for 2 h after 10 min of inactivation at 65 °C. The vector with desired size was extracted from the gel using the GeneJET Gel Extraction kit (Thermo Scientific), whereas pure BamHI-NotI digested vector pAL02/2_MCS2 was acquired with the simple use of GeneJET^{TM} PCR Purification kit (Fermentas).

The prepared upstream and downstream PCR inserts (ca. 20 ng) were ligated into compatible digested vector pAL01_MCS1 and pAL02/2_MCS2 (ca. 20 ng) respectively, resulting in two new vectors named pZZlox71 and pZZlox66. The ligations were performed in 20 µl reactions made up of 10 U of T4 DNA ligase (Fermentas), 1 × T4 DNA ligase buffer (Fermentas) and water at room temperature for 1 h followed by enzyme inactivation at 65 °C for 10 min. The digested and dephosphorylated vectors were also ligated as controls without adding PCR inserts to examine the effects of dephosphorylation. The ligation mixtures were also precipitated as carried out above and dissolved in 5 µl of water.

4.2.2. Bacterial transformation by electroporation

The constructed pZZlox71 and pZZlox66 plasmids were transformed to *E. coli* ST18 by electroporation using a BIO-RAD Gene Pulser^{TM} electroporator. Electrocompetent *E. coli* cells were prepared as follows. Forty milliliters of early exponential-phase *E. coli* cells were collected by centrifugation at 4300 rpm for 15 min. The pellets were then resuspended in 1

ml of ice-chilled sterile MilliQ water followed by centrifugation again at 11000 rpm for 5 min at 4 °C. Afterwards, the cell pellets were washed 5 times with sterile MilliQ. After resuspension in 80 μl of 10 % (v/v) glycerol, the cells were divided into 40 μl aliquots and stored immediately at -80 °C. For electroporation, 2 μl of plasmid constructs or self-ligation controls were mixed with 40 μl of ice-thawed competent *E. coli* cells and then transferred to pre-chilled electroporation cuvettes. An electric pulse of 2.5 kV, a capacitance of 25 μF and a resistance of 250 Ω were applied, and cells were immediately suspended in 960 μl of LB with ALA$_{50}$ and transferred to test tubes for 1 h of incubation at 37 °C. The transformed cells were plated on selective media (LB with ALA and Km for pZZlox71 and pAL01_MCS1 self-ligation control; LB with ALA and Gm for pZZlox66 and pAL02/2_MCS2 self-ligation control).

Colonies containing the plasmid constructs arisen on the selective media were picked and subcultivated on new selective plates. Meanwhile, plasmids from these colonies were isolated and 200 ng-450 ng of each plasmid was subjected to BamHI and NotI single digestion as well as BamHI-NotI double digestion verification analysis. In the digestion reactions, 1 μl of restriction enzyme was used and 1 × FastDigest® buffer was replaced by 1 x FastDigest® Green buffer (Thermo Scientific) in 20 μl reaction for the convenience of subsequent gel electrophoresis.

4.2.3. Biparental mating for the integration of *lox* sites

To integrate *lox* site-carrying plasmids into the chromid of HAMBI 1141, verified *E. coli* ST18 clones containing pZZlox66 or pZZlox71 as donors were mated with HAMBI 1141 as recipient sequentially. First of all, *E. coli* ST18 pZZlox66 was grown for one day and the optical density at a wavelength of 600 nm (OD$_{600}$) measured by UV-1800 Spectrophotometer (SHIMADZU) was 0.35 (much lower than that of normal *E.coli*). The HAMBI 1141 was grown for two days and its OD$_{600}$ was 1.20. Four hundred microliters of the donor strain was washed to remove antibiotics and mixed with 1 ml of the recipient strain. The mating mixture was then suspended in 50 μl of sterile MilliQ water, spotted on a TY with ALA plate and incubated at 28 °C overnight. The cells were collected from the mating spot and resuspended in 1 ml of MilliQ water followed by a ten-fold serial dilution starting from 10^{-1} to 10^{-3}. Aliquots of 0.1 ml from each dilution were evenly spread over the surface of selective plates (TY with Gm) and incubated for 3 days until colonies appeared. The integration of pZZlox66 into the

chromid was verified by PCR and the product was expected to be a 2.2-kb fragment composing of a partial region of pZZlox66 and of the chromid. The PCR was conducted as described in section 3.2.1 except for the primers used (AL064-ZZ10, Table 3), the annealing temperature (66 °C) and extension time (66 s).

After pZZlox66 integration had been confirmed by PCR, the HAMBI 1141 single plasmid insertion mutant was grown for 3 days (OD_{600}=1.35) under selective conditions and mated with *E. coli* ST18 pZZlox71 which was grown for 8 h (OD_{600}=1.10) at a 1:5 ratio of donor to recipient. The resulting mating cells were treated as described above and transconjugants were selected on TY plates containing Nm, Gm and Gluc. A blue colony that arose on a selective plate after three days was picked and its genomic DNA was isolated, followed by PCR amplification of a 1.7-kb fragment consisting of genetic regions from both pZZlox71 and the chromid. Primers for the PCR were ZZ09-AL062 (Table 3) and the program was set as above with the exception of the annealing temperature (65 °C) and extension time (50 s). With the same genomic template, PCR verification of the first integration was performed again to ensure that both plasmids have integrated into the chromid of the same strain. The PCR products were then sent to be sequenced using the primer pair AL064-ZZ10 for the first integration and ZZ09 with a universal primer RP-48 for the second integration (Table 3).

4.2.4. Biparental mating for the induction of target excision

A three-day culture of the HAMBI 1141 co-integrate strain grown under antibiotic pressure (Nm, Gm, OD_{600}= 1.34) was then mated with 8 h grown *E. coli* ST18 carrying Cre recombinase expression vector pCM157 (OD_{600}=0.93) at a ratio of 5:2. Transconjugants were selected on Tc-containing TY plates. Colonies that appeared were subcultivated on new TY with Tc plates and tested for excision of the targeted region by verifying loss of their Nm and Gm resistance. Precise excision was validated by PCR amplification of a 1.3 kb fragment spanning a part of both upstream region and downstream region with primer pair ZZ11-ZZ12. The PCR program was the same as mentioned in section 3.2.1 with the modifications of annealing temperature and extension time which were 59 °C and 50 s respectively. The resulting PCR product was then sent to be sequenced using primer ZZ13 (Table 3). Ultimately, the deletant strain was cultivated on plain TY plates to eliminate pCM157.

4.3. Symbiotic plasmid curing in HAMBI 1207

4.3.1. Creation of random transposon insertion

Transposon Tn*5*-B12S carried on pMH1701 was used to randomly label the replicons of HAMBI 1207 through mating between an *E. coli* strain that harbors pMH1701 and HAMBI 1207. Two hundred microliters of one day grown *E. coli* at an OD_{600} of 0.82 was mixed with 1 ml of three days grown HAMBI 1207 at an OD_{600} of 1.0. HAMBI 1207 transposon insertion mutants were selected on TY agar plates containing Nm and Sm and colonies were picked at random after three days of incubation and purified for the following triparental mating.

4.3.2. Triparental mating for mobilization of the symbiotic plasmid

The triparental mating was carried out among the donor strain HAMBI 1207 insertion mutants, the plasmid free recipient strain *Agrobacterium fabrum* UBAPF2 and a helper strain *E.coli* J-53 carrying RP4-4 at a ratio of 2:2:1 (40 µl: 40 µl :20 µl). The *E.coli* was grown for one day and HAMBI 1207 mutants and *A. fabrum* UBAPF2 were grown for two to three days. The mating procedures were the same as conducted in biparental mating. *Agrobacterium* transconjugants were identified by growing on the selective TY with Nm and Rf plates. After colony purification, *Agrobacterium* transconjugants were run directly on a plasmid agarose gel (described in 4.3.4) to detect the presence of plasmids.

4.3.3. Curing of symbiotic plasmid

HAMBI 1207 insertion mutants which were defined to carry the transposon inserts on their pSym were grown for 2 days (OD_{600}= 0.69) and plated on TY plates containing 5 % sucrose after appropriate dilutions. These mutants were first incubated at 37 °C for two days and then at 28 °C for a further three days. Colonies found on sucrose-containing plates accompanied by their loss of neomycin resistance were picked and subjected to agarose gel electrophoresis of plasmids (described in 4.3.4) to further confirm the change of their plasmid pattern.

4.3.4. Agarose gel electrophoresis of plasmids

A modified Eckhardt (1978) technique based on Hynes et al. (1985) and Hynes & McGregor (1990) was applied. The bacterial strains obtained as described in 4.3.2 and in 4.3.3 as well as their respective reference strains were grown overnight in HP medium to an OD_{600} of

approximately 0.3. Bacterial cultures of 250 µl for each strain were added to 500 µl of a 0.3 % (w/v) Sarkosyl solution. Each sample was vortex-mixed, pelleted by centrifugation for 4 min and resuspended in 25 µl of lysis solution containing 10 % (w/v) sucrose, 100 µg/ml lysozyme (Sigma-Aldrich, Inc.), 10 µg/ml RNase (Roche) and Tris-EDTA (TE) buffer. The resuspensions mixed with 5 µl of 6 × DNA Loading Dye were immediately loaded into a sodium dodecyl sulfate (SDS)-agarose gel made up of 1 % (w/v) SDS, 0.7 % (w/v) agarose in 1 × Tris-Borate-EDTA (TBE) buffer. Each sample remained in the well for 2 min after loading, followed by electrophoresis at 60 V (ca. 20 mA) at 4 °C overnight. The gel was stained for 30 min in a 0.1 µg/ml ethidium bromide (EtBr) solution prior to imaging, and then exposed to ultraviolet light in Molecular Imager® Gel DocTM XR system (Bio-Rad Laboratories Inc.). The gel images were exported from QUANTITY ONE® Version 4.6.9 analysis software (Bio-Rad Laboratories Inc.).

4.4.   Bacterial mating between manipulated HAMBI 1207 and HAMBI 1141 strains

Two groups of conjugations were performed. In the first group, equal volumes (1ml) of the T4SS-deleted HAMBI 1141 strain as the donor (OD_{600}= 1.307) and the plasmid-cured HAMBI1207 strain as the recipient (OD_{600}= 1.274), which were both grown for two days were mixed. Mating cells after spot-incubation overnight on a TY plate were serially diluted up to 10^{-5}. Aliquots of 0.1 ml from 10^{-3} to 10^{-5} dilutions were spread onto TY with Sm selective plates and incubated for three days. The second group of conjugation was carried out between the two-day grown wild-type HAMBI 1141 donor strain (OD_{600}= 1.287) and the plasmid-cured HAMBI 1207 recipient strain under the same conditions as for group one.

4.5.   Nodulation tests of *G. officinalis*

4.5.1.   Sterilization of *G. officinalis* seeds

Seeds of *Galega officinalis* were surface sterilized as follows. Seeds were first submerged in concentrated sulphuric acid (95%-97%) for 15 min with intermittent shaking. Afterwards, seeds were washed with sterile MilliQ water 6 times with one minute for each time. In particular, the first washing needs to be quick to avoid overheating that may harm the seeds. After washing with 96 % ethanol for 1 min, seeds were finally washed with sterile MilliQ water again 5 times with 2 min for each time. Sterilized seeds were placed on TY plates for 3 days at room temperature for germination.

4.5.2. Preparation of bacterial inoculants

The two groups of bacterial cells described in section 4.4 were flushed from the surface of the 10^{-4} TY with Sm plates with TY broth. Their OD_{600} were about 1.8 after proper dilutions prior to inoculation.

4.5.3. Inoculation of *G. officinalis*

Two glass jars that were utilized for the growth of *G. officinalis* were filled with leca gravel (4-10 mm), sand (0.5-1.2 mm) and vermiculite (1-2 mm) mixed at a ratio of 3: 5: 5. Jars were then sterilized at 160 °C for 24 h. Germinated seeds were transferred to jars aseptically with 3 seeds per jar. Jars had been moistened beforehand with 125 ml of quarter-strength nitrogen-free Jensen nutrient solution (Vincent 1970) that contains (per liter) 1.3 g of $CaHPO_4 \times 2\,H_2O$, 0.26 g of $K_2HPO_4 \times 3\,H_2O$, 0.2 g of $MgSO_4 \times 7H_2O$, 0.2 g of NaCl, 0.17 g of $FeCl_3 \times 6H_2O$ and 1 ml of a trace element solution containing B, Cu, Mn and Mo elements. Each seed was then inoculated with 1 ml of flushed bacterial cells either from the first conjugation group or from the second group with one jar for each group. These seeds were properly covered and jars were watered with 50 ml of sterile MilliQ water. Meanwhile, *G. officinalis* seedlings were also grown in 8 sterilized glass test tubes with each tube containing a mesh covered with about 10 ml of quarter-strength Jensen nutrient solution. The germinated seeds were carefully placed onto the mesh one seed per tube so that their roots were vertically oriented. Each seed was then inoculated with 50 µl of bacterial cells (from the first group or the second group) or sterile MilliQ water as negative control with 3 tubes for the first group, 3 tubes for the second group and 2 tubes for the negative control. Inoculated jars and test tubes were maintained in a growth chamber (Sanyo Electric Co., Ltd) with a program set as 20°C for 1 h, 24 °C for 16 h, 20 °C for 1h and 16°C for 6h. Plants were allowed to grow for four weeks. Over the course of the growth, plants in the two jars were provided with 30-40 ml of quarter-strength Jensen's nutrient solution twice a week. Examination of nodulation was carried out after 2 weeks of inoculation for the test tubes and 4 weeks for the jars by visually inspecting the roots of plants. For the examination of plants in the jars, plants were carefully taken out and their roots were gently rinsed with sterile MilliQ water to wash away the attached sand.

5. Results

5.1. Generation of a T4SS deletion mutant of HAMBI 1141

The whole process of the generation of a deletion on the chromid of HAMBI1141 is shown in Figure 4.
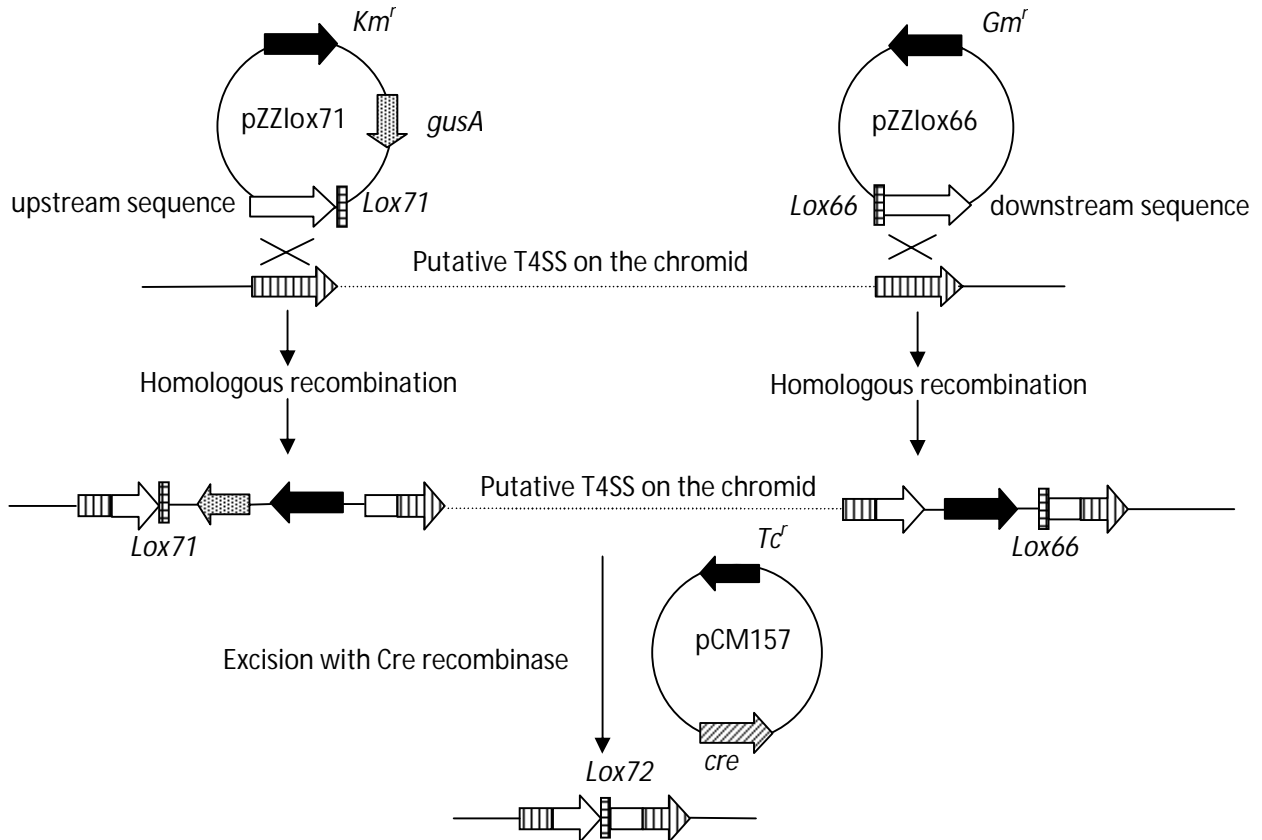

Figure 4. Cre-*lox* mediated recombination for the excision of the putative T4SS on the chromid. This figure was created based on Lohße et al. (2011) with modifications.

As mentioned in the literature review part regarding the mechanism of Cre-*lox* system, Cre-mediated recombination between two co-directional *lox* sites on the same DNA molecule excises any region between these sites. Therefore, to generate the deletion of the defined putative T4SS on the chromid, two *lox* sites designated to flank the target region were inserted relying on homologous recombination. A 1.331-kb upstream and a 1.658-kb downstream homologous flanking fragment of the target region were cloned into *lox* site-carrying plasmids pAL01_MCS1 and pAL02/2_MCS2, creating two vectors named pZZlox71 and pZZlox66, respectively. Each fragment was placed adjacent to a *lox* site with *lox71* at the 3′ end of the upstream fragment and *lox 66* at the 5′ end of the downstream fragment

(Figure 4). *E.coli* ST18 transformants that were expected to carry pZZlox71 or pZZlox66 were verified by BamHI and NotI single digestion as well as BamHI-NotI double digestion. Only two colonies (C1 and C2) of potential *E. coli* ST18 pZZlox66 were found on selective plates and they both were confirmed to carry pZZlox66 (Figure 5, a). In contrast, 3 out of 12 picked colonies (C5, C6 and C8) were discovered to carry pZZlox71 even though a total of 15 colonies of *E.coli* ST18 transformants were found (Figure 5, b).
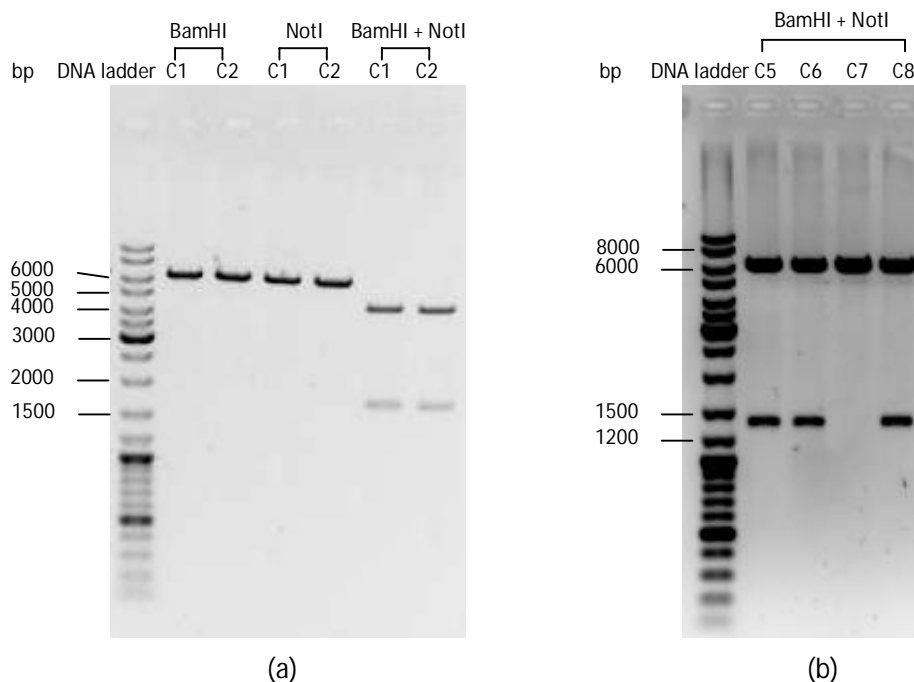

Figure 5. Restriction profiles of plasmids in *E.coli* ST18 transformants. (a) BamHI and NotI single digestion and BamHI-NotI double digestion of pZZlox66. C1 and C2 represent colony No.1 and No.2. The biggest fragments (from top to bottom) were the whole linearized pZZlox66 cloning vector (5.811 kb); the second fragments were pAL02/2_MCS2 (4.153 kb); the third fragments were the downstream sequence inserts (1.658 kb). (b) BamHI-NotI double digestion of pZZlox71. C5-C8 represent colony No.5 to No.8. The biggest fragments were pAL01_MCS1 (5.944 kb); the second fragments were upstream sequence inserts (1.331 kb). Size marker was Gene Ruler™ DNA Ladder mix (Fermentas).

The C2 was picked and mated with HAMBI 1141. Homologous recombination of the downstream sequence on the vector pZZlox66 and on the chromid integrated the entire vector including *lox66* into the chromid. A 2.179 kb PCR verification product was obtained and the 5′ end of this product started from the inside of gentamicin resistance gene toward a short region on the chromid downstream of the homologous downstream sequence (Figure 4). The C5 was then chosen to mate with the verified HAMBI 1141 single vector insertion mutant. The vector pZZlox71 carrying *lox71* was integrated into the chromid of the

mutant through homologous recombination of the upstream sequence. The acquisition of a 1.671 kb PCR product that ranged from a short chromid region upstream of the homologous upstream sequence through the *lox71* to a region upstream of the *lox71* on pZZlox71 (Figure 4) confirmed the integration. The formation of a co-integrate was ascertained by another round of PCR verification of the pZZlox66 integration with the same chromid template. The sequencing of both PCR products showed that homologous recombination had occurred as anticipated when aligned with corresponding sequences on the chromid and vectors. The two *lox* sites were orientated in the same direction, flanking the target T4SS as well as the two integrated vectors (Figure 4).

The plasmid pCM157 was then conjugated into the HAMBI 1141 co-integrate strain. Two transconjugants were found on selective plates (TY+Tc). After transferring the two transconjugants onto a new selective plate once, the target region between *lox* sites was excised upon the induction of Cre recombinase expressed from pCM157, judged from their sensitivity to neomycin and gentamicin (Figure 4). The excision was further confirmed by PCR and sequencing of the obtained PCR products. The 1.354-kb PCR product was from the inside of homologous upstream sequence over the *lox72,* a recombinant *lox* site (Figure 4), to the inside of homologous downstream sequence as expected. One of the verified deletion mutants was picked and grown on TY without tetracycline to eliminate pCM157, since this plasmid cannot stably maintain when bacteria were grown without selective pressure. After the deletion mutant was consecutively transferred in the absence of tetracycline twice, it finally lost the pCM157.

5.2. Symbiotic plasmid curing in HAMBI 1207

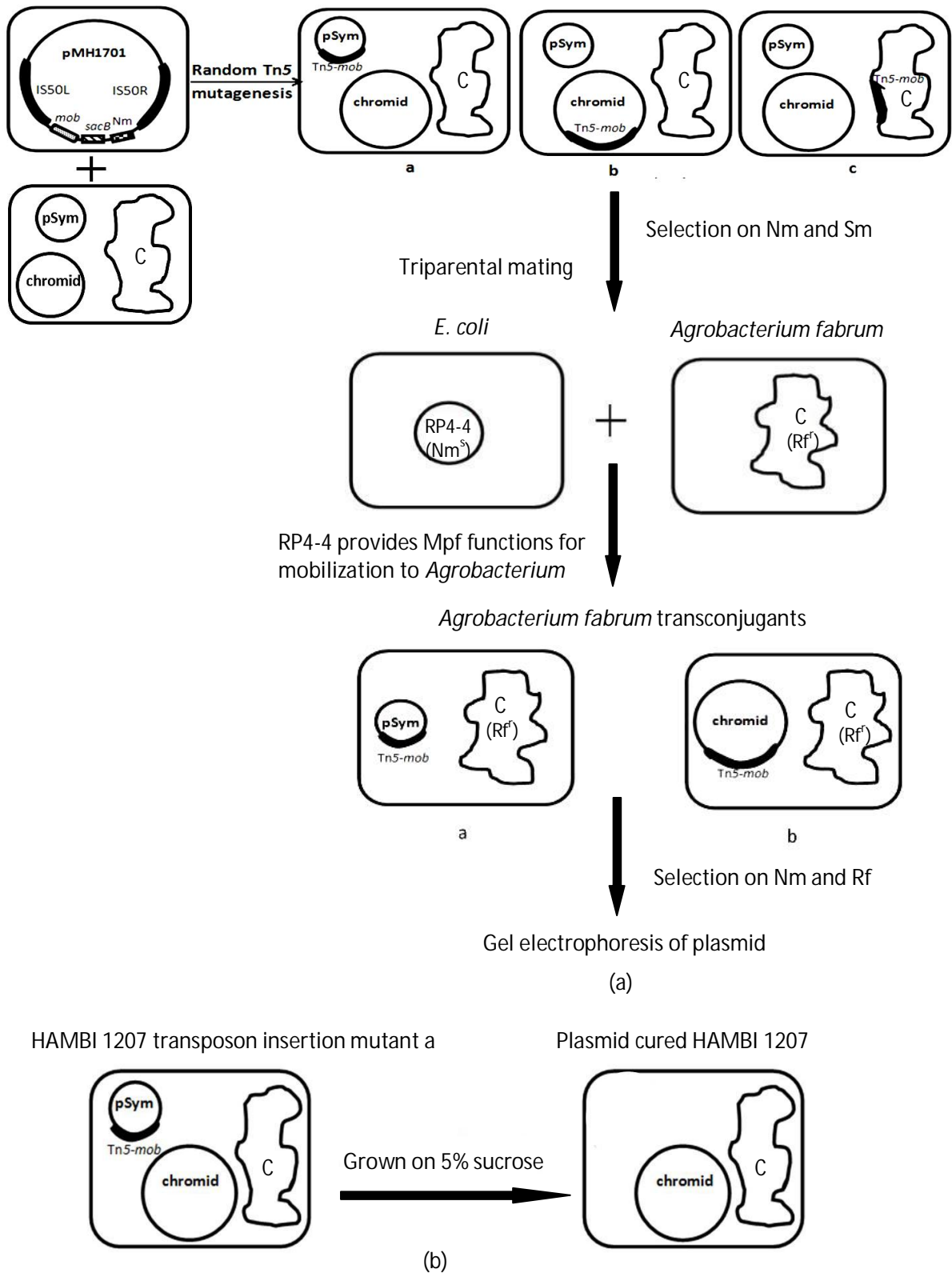The overall steps for the curing of the pSym can be seen in Figure 6.

Figure 6. Curing of the pSym in HAMBI 1207. The drawings are not to scale. The plasmid pMH1701 in *E. coli* was magnified to represent the used DNA elements on it. The Tn*5* construct mainly consists of the insertion elements (IS50L and IS50R), a *mob* site, a *sacB* cassette and a neomycin resistance gene (Nm). C stands for bacterial chromosomes. (a) Screening of HAMBI 1207 pSym inserted mutants. (b) Curing of pSym.

Plasmid pMH1701 was used to carry out the random transposon mutagenesis of HAMBI 1207. It carries a transposon Tn*5,* which has two insertion elements (IS50L and IS50R) flanking the *mob* site (*oriT*) of broad host range plasmid RP4 and a *nptI-sacB-sacR* cassette, all together called Tn*5*-B12S (shown as blackened segments called Tn*5-mob* in Figure 6). The plasmid pMH1701 is a suicide vector, unable to replicate in HAMBI 1207. Therefore, Tn5-B12S on pMH1701 remained intact only by randomly hopping onto any of the replicons in HAMBI 1207, conferring neomycin resistance to the recipient. Plenty of neomycin-resistant HAMBI 1207 transposon insertion mutants were found. Streptomycin was used to counterselect the *E. coli* donor strain.

Twelve HAMBI 1207 transposon insertion mutants were picked at random and mated with an *E. coli* helper strain carrying RP4-4 and the plasmid free recipient strain *A. fabrum* UBAPF2. RP4-4 is a neomycin sensitive derivative of RP4 and provided *in trans* the transfer functions to mobilize the Tn*5-mob* carrying plasmid or chromid to *Agrobacterium* recipient. *A. fabrum* transconjugants were obtained on selective TY with Rf and Nm plates from all of the HAMBI 1207 mutants. The outcome of the mobilization in 9 out of 12 transconjugants were examined on agarose gel (Figure 7).
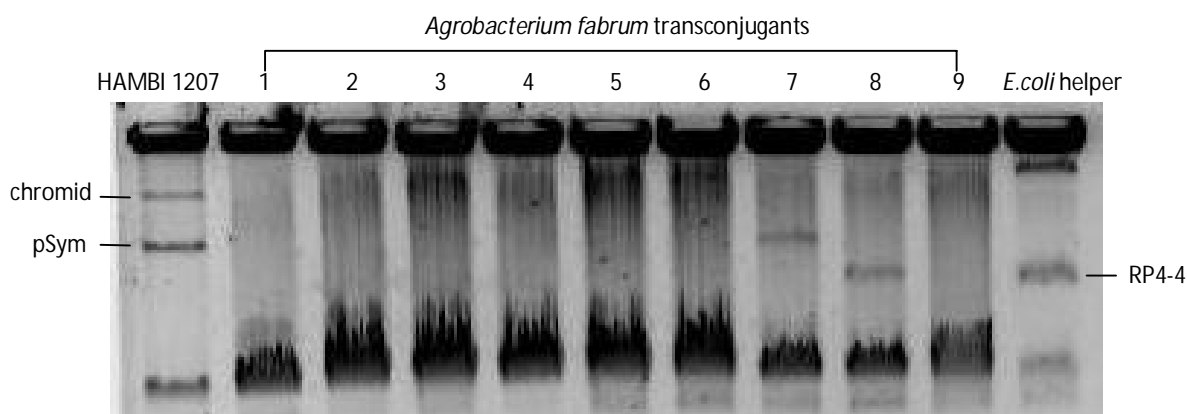

Figure 7. Plasmid profiles of *A.fabrum* transconjugants (1-9). HAMBI 1207 and *E.coli* helper strain were plasmid reference strains. Transconjugant 1 had no transferred replicons. Transconjugant 7 received the pSym from HAMBI 1207 according to the size of the fragment. Transconjugant 8 obtained the mobilizing plasmid RP4-4 from the helper strain. The rest of transconjugants may acquire the chromid of HAMBI 1207 due to the presence of indistinct fragments on the top. The darkest smears at the bottom of each lane were degraded chromosomal pieces.

The pSym of HAMBI 1207 had been mobilized into the *A. fabrum* transconjugant 7, implying that the Tn5-B12S had jumped into the pSym during the transposon mutagenesis. Accordingly, the corresponding HAMBI 1207 transposon insertion mutant which had been

used for triparental mating was picked and plated on TY containing 5 % sucrose. The *sacB* cassette on Tn5-B12S can generate the curing of the pSym when grown on media containing sucrose. Ninety colonies growing on sucrose media were screened for loss of Nm resistance since the sucrose resistance resulted from the curing of pSym would be concurrent with the loss of Nm resistance gene. Four colonies that were shown Nm sensitive were again examined on agarose gel to identify their plasmid profile changes, and one colony was found cured of its pSym (Figure 8).
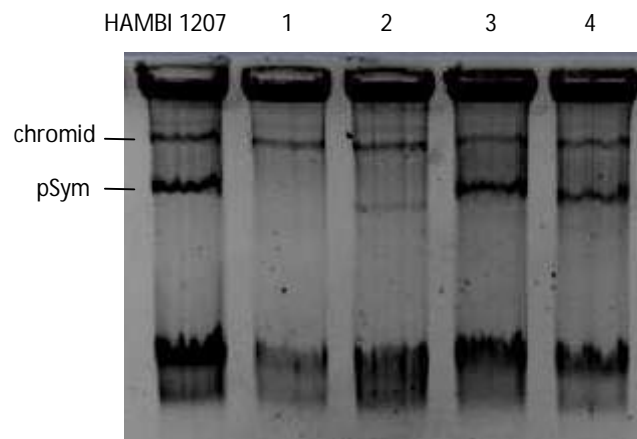

Figure 8. Curing of the pSym in HAMBI 1207 mutants (1-4) that had transposon insertion in their pSym and were grown on TY containing 5 % sucrose. HAMBI 1207 was used as the plasmid reference strain. HAMBI 1207 mutant 1 had lost its pSym, while HAMBI 1207 mutant 2 might have a deletion in its pSym since the second fragment migrated a bit faster than that of the reference strain. HAMBI 1207 mutant 3 and 4 had no plasmid profile change compared with the reference strain. The darkest smears at the bottom of each lane were degraded chromosomal pieces.

5.3. Conjugation between the HAMBI 1141 and HAMBI 1207 derivatives

The HAMBI 1207 pSym-cured derivative was conjugated with the HAMBI 1141 T4SS deletion mutant (the first group in section 4.4) and the wild-type HAMBI 1141 (the second group) separately to investigate the transmissibility of the pSym without the presence of the T4SS region on the chromid. Plenty of colonies were found on all of the streptomycin selective plates, which were used for the counterselection of the two types of HAMBI 1141 donors, that are streptomycin sensitive, contrary to their streptomycin resistant derivative, HAMBI 1207.

5.4. Nodulation test on *G. officinalis*

All of the colonies on the 10^{-4} plates (section 4.4) were collected and inoculated on *G. officinalis* to determine if the conjugal transfer of pSym had taken place, by monitoring the formation of nodules on its host plant. After 2 weeks post-inoculation, the two plants grown in two test tubes as negative controls did not have any nodules, as expected. However, the six plants in test tubes inoculated with bacterial cells either from the first conjugation group or from the second group did not form nodules either, which was consistent with the result of the five plants grown in jars, 4 weeks after inoculation.

6. Discussion

Rhizobia are renowned for their ability to establish symbiosis with legumes and fix nitrogen for them, a property which is associated with the presence of extrachromosomal DNAs, plasmids or chromids, a general feature for most rhizobial species (Mercado-Blanco & Toro 1996). The symbiotic genes found on these plasmids (pSyms) or chromids are the determinants for the establishment of successful symbiosis with legumes, whereas non-symbiotic plasmids or so-called cryptic plasmids may also encode some traits indirectly involved in symbiosis such as nodulation competitiveness and nodulation efficiency (Mercado-Blanco & Toro 1996). Conjugal transfer of these plasmids can distribute their genetic traits for symbiosis (He & Clay 2006) as evidenced by increasing reports (Brom et al. 2002, He et al. 2003, Pérez-Mendoza et al. 2004). Meanwhile, conjugal transfer genes have also been identified on many pSyms (Pérez Mendoza et al. 2005) as in the case of *R. galegae* sv. officinalis (discussed below).

In this work, curing of the pSym of *R. galegae* sv. officinalis and excision of an AvhB-like T4SS, potentially imparting the conjugal transfer functions, were performed in two different strains of *R. galegae* sv. officinalis. Conjugation was then conducted between these two strains to test if the pSym is self-transmissible using the plasmid-located clusters of transfer genes or whether the pSym needs extra, unknown help provided by the chromid-borne T4SS to be transferred.

In the plasmid curing experiment, a modified Tn*5* construct was applied and a plasmid-cured derivative was obtained. Meanwhile, a possible plasmid deletion derivative was also yielded as achieved by Hynes et al. (1989) and Hynes & McGregor (1990). The transposon Tn*5* or the

position of the Tn*5* inserted on the plasmid may be related to the deletion on the plasmid since transposable elements like Tn*5* could give rise to various mutations such as insertion, deletion, inversion and chromosomal fusion (Reznikoff 2003). Tn*5* is a composite transposon, composed of two insertion elements (IS*50*R and IS*50*L) flanking its antibiotic resistance genes. Each insertion element is also flanked by two DNA sequences that are called the outside end (OE) and the inside end (IS), with the inside end closer to the antibiotic resistance genes. The transposases interact specifically with these DNA ends and catalyze the transposition (Reznikoff 2003). Jilk et al. (1993) reported that some single base pair changes in one OE of the Tn*5* could lead to the formation of adjacent deletions starting immediately next to the other OE (wild type). The mutation in the OE might result in the inability of recognition by transposases, therefore, the failed attempts of transposition event. In addition, for the type of composite transposons, deletion could arise from their inside-end transposition to a neighboring target on the same DNA. This event causes the deletion of sequence between the original site of the transposon insertion and the target site into which it is trying to transpose along with the sequence between the two IS elements in the transposon. (Snyder & Champness 2007) However, neither of the above mentioned cases seems to be able to account for the plasmid deletion in *R. galegae* sv. *officinalis*. Since these rhizobial cells could survive on sucrose medium and were neomycin sensitive, the sequence between the two IS elements in Tn*5* where the *sacB* and *Nm* resistance genes are located might have been lost rather than the sequence adjacent to the Tn*5*. Meanwhile, Tn5 has an option of methylation of adenines in its inside ends to make them less well recognizable by the transposase, therefore, equipping itself with low possibility of inside-end transposition (Snyder & Champness, 2007). Although no clear mechanism behind the deletion can be proposed, the possible plasmid deletion is intriguing and at any event requires further confirmation and elucidation.

There were also sucrose-resistant colonies without loss of the Nm resistance. It was probably because those colonies still retained the pSym carrying the transposon construct including the Nm resistance gene, while the *sacB* gene had lost its activity, a phenomenon called spontaneous sucrose resistance that has also been reported in other gram negative bacteria with high frequency (Wu & Kaiser 1996, Copass et al. 1997, Pavelka & Jacobs 1999, Parish & Stoker 2002). This problem may mainly result from mutations in the *sacB* gene,

such as the transposition of the endogenous mobile IS elements into this gene (Hynes et al. 1989). Other mutations that decrease the bacteria's sucrose intake would give rise to similar effects (Wu & Kaiser 1996). In spite of the inherent aforementioned problem, the *sacB* cassette is still an effective genetic tool for such applications as plasmid curing and gene replacement since screening less than 100 colonies sufficed to identify one plasmid-cured colony in this case.

A Nm sensitive derivative of plasmid RP4, RP4-4 was utilized as the mobilization helper plasmid instead of RP4 in triparental mating. RP4 can easily transfer itself to the recipient and stably maintain in them. The presence of RP4 in certain rhizobial strains was found to affect the symbiotic interactions between these strains and some of their host plants (O' Connell et al. 1998, Quandt et al. 2004). Therefore, if any rhizobial recipient strain after bacterial mating is needed for symbiosis investigations, it would be more recommendable to use RP4 derivatives that can be more easily cured. However, this was not an issue in this thesis since the *A. fabrum* transconjugants from the triparental mating were only needed for the examination of mobilization of plasmids by plasmid gel electrophoresis.

On the other hand, the generation of a deletion of the chromid-borne putative T4SS by the site-specific Cre-*lox* recombination system was quite efficient. The incorporation of two *lox* sites into the chromid required two single crossovers, which normally occur at much higher frequencies than the problematic double crossovers (Schefflel et al. 2008, Ullrich & Schüler 2010). The subsequent Cre-mediated target excision was achieved after only one-time transfer on the medium with the presence of an easily curable Cre-expressing plasmid and tetracycline. That plasmid was then eliminated, by simply relieving the tetracycline pressure, to avoid any interference its presence may cause for the following experiment. The whole process was accomplished within two weeks, more efficacious and much faster than the classic allelic exchange approach for genetic deletions (Ullrich & Schüler 2010). In addition, the resulting mutant *lox* site, *lox72,* from the recombination between *lox66* and *lox71* has low binding affinity for the Cre enzyme, ensuring the stability of the chromid, in this case, if repeated gene deletions are carried out (Lambert et al. 2007).

The result that neither of the conjugation groups elicited the formation of nodules on the host plant *G. officinalis* was fairly unexpected. It was believed that at least bacteria from the

second group in which the HAMBI 1207 pSym-cured strain was conjugated with the wild-type HAMBI 1141 could induce the formation of effective nodules considering the previous observation that a non-nodulating mutant strain of *R. galegae* sv. orientalis which had been conjugated with wild-type HAMBI 1207 succeeded in forming effective nodules on *G. officinalis*. The previous result explicitly indicated that HAMBI 1207 could transfer its pSym into its symbiovar counterpart *R. galegae* sv. orientalis, thereby endowing them with the ability to fix nitrogen on *G. officinalis.* The perplexing result in this thesis, however, may be due to multiple reasons.

One possible reason might be that the HAMBI 1141 had actually transferred its pSym into the HAMBI 1207 recipient, but at a relatively low frequency under laboratory conditions so that collecting bacterial cells solely from a 10^{-4} dilution plate could not guarantee to pick out the right candidates for the inoculation test. Low rates of plasmid transfer have previously been observed in some defined self-transmissible rhizobial plasmids such as pSfrNGR234a in *S. fredii* strain NGR234 (around 10^{-9} transconjugant per donor) (He et al. 2003) and pRme41a in *S. meliloti* strain Rm41 at the frequency of 10^{-7} (González & Marketon 2003).

The sequencing of the pSym in *R. galegae* sv. officinalis conducted previously in our research group has revealed a pair of divergently transcribed operons (*traAFBH* and *traCDG*) putatively encoding DNA transfer and replication (Dtr) component proteins and a separate region containing the *trb* operon (*trbB* to *trbL*) encoding mating pair formation (Mpf) component proteins (unpublished data). In addition, it has also been found to possess the quorum-sensing (QS) regulatory genes *traI/traR/traM.* QS is one prevalent regulatory mechanism recruited by many tested rhizobia to control the occurrence of conjugation. In brief, plasmid transfer is implemented in a bacterial cell density dependent manner. Only when a certain signal molecule synthesized by the product of the *traI* gene reaches a proper concentration responsive to population density, will the transcription factor TraR be activated, which then induces the expression of conjugal transfer relevant operons. The TraM is the anti-activator of TraR, preventing the conjugal transfer genes from being expressed. (He & Fuqua, 2006) Studies have characterized numerous rhizobial plasmids such as the Ti plasmid, pRetCFN42a and pSfrNGR234a as QS-regulated plasmids, to which the arrangement of QS regulatory genes on the pSym of *R. galegae* sv. officinalis is highly similar (Fuqua et al. 1994, Marketon & González 2002, He et al. 2003). However, the expression of

the Ti plasmid *traR* gene requires extra presence of a specific subset of opines, compounds secreted from the infected plants (Piper et al. 1999). An analogous additional signal molecule perhaps from an environmental cue e.g. rhizopine was also assumed to regulate pSfrNGR234a *traR* expression and was proposed to be one reason for the low transfer frequency of pSfrNGR234a in its absence, although this proposition has not been identified (He et al. 2003). The co-existence of both components (Dtr and Mpf) on the pSym and the presence of the potential QS regulatory genes on the *trb* operon region made it logical to speculate about the possibility that the pSym of *R. galegae* sv. officinalis could indeed transfer upon reaching a certain quorum. Nonetheless, an unknown exogenous factor, probably from surrounding strains' communication apart from the *traI*-encoded signal molecule in this case, may determine the eventual efficiency of its transfer system.

Another implication that was not easily apprehensible could be that the transmissibility of the pSym was decapitated when the pSym cured derivative of the same rhizobial symbiovar as the donor was chosen as the recipient as well. In plasmid transfer, the donor cells are normally considered to play a positive role, while the recipient cells play a negative role, restricting the entry or establishment of the incoming DNA (Thomas & Nielsen 2005). Their negative role can be evidenced by their development of different conjugation inhibitive mechanisms such as restriction systems (Thomas & Nielsen 2005), entry exclusion systems (Garcillán-Barciaand & de la Cruz 2008) and incompatibility (Snyder & Champness 2007). However, these delicately devised systems are mainly applied to the scenarios that either the recipient cells already carry a closely related plasmid or the incoming DNA is recognized as foreign because of its different sequence-specific chemical signatures. Since the inhibition of the pSym transfer from *R. galegae* sv. officinalis to the *R. galegae* sv. officinalis plasmid-cured strain occurred, but not when an *R. galegae* sv. orientalis strain was used as recipient, a certain factor involved in recipient cells may account for the distinct conjugation results. Several studies made attempts to define the recipient specificity in certain plasmid groups. It was found that the interaction between the mating-pair formation (Mpf) apparatus of the donor with the lipopolysaccharide (LPS) molecules acting as a major component of the outer membrane of recipient cells mediated the recipient specificity in *E. coli* (Pérez-Mendoza & de la Cruz 2009) and *Salmonella typhimurium* (Sanderson et al. 1981). Utilizing *E.coli* LPS mutants as the recipient could reduce the transfer frequency of IncW plasmid R388 or

strongly inhibit the conjugation of the F-plasmid (Pérez-Mendoza & de la Cruz 2009). IncI1 plasmid R64 was reported to rely on the PilV adhesins, which are constituents of its encoded Type IV pilus and locate on the tip of the pilus, to determine its recipient specificity by recognizing and binding to respective LPSs on the surface of recipient cells (Ishiwa & Komano 2004). In rhizobia, Herrera-Cervera et al. (1996) observed suppressed effects on the conjugal transfer of a cryptic plasmid pRmeGR4a between *S.meliloti* strains. The effect was thought to be generated by ammonium provided in the media and influence only the rhizobial recipient. They reckoned that ammonium might modify one component such as LPS or protein OmpA of the *S. meliloti* recipient cell surface important for mating pair formation or stabilization, which then affected the plasmid transfer.

In general, LPS in rhizobia is more often considered to exert essential effects on different stages of symbiosis such as initial recognition of symbiotic partner and infection initiation (Becker et al. 2005). The genes involved in LPS synthesis were found localized on plasmids of *R. leguminosarum* bv. *viciae* (Hynes & McGregor 1990), *R. leguminosarum* bv. *trifolii* (Baldani et al. 1992), and *R. etli* (Brom et al. 1992), whereas LPS synthesis genes were also found clustered on chromosomal regions in *S. meliloti* (Clover et al. 1989). There is no published data concerning the exact location of the whole LPS-synthesizing genes in *R. galegae* sv. officinalis and the information regarding the connection between rhizobial plasmid transfer and rhizobial LPS is scarcely available. Nevertheless, it is reasonable to speculate that genes for LPS production are on the pSym or scatter through the genome of *R. galegae* sv. officinalis so that the curing of its pSym made its LPS incomplete, leading to the inability of the donor to recognize the LPS-altered recipient or the disabled proper cell contact, followed by the silence of subsequent plasmid transfer.

The above explanations for the unanticipated conjugation result are untenable until some experimental evidences are provided, which may still be practically difficult to obtain. However, at least in the plasmid-cured derivative, phenotypic changes such as utilization of compounds and synthesization of different enzymes compared with wild-type strain are worth investigating so that comprehensive functions harbored on this plasmid apart from its symbiotic activities could be determined. The inter-replicon functional interactions might also be revealed by those changed phenotypic effects. Meanwhile, the HAMBI 1141 T4SS mutant strain could be conjugated with a nodulation mutant of *R. galegae* sv. orientalis to

achieve the original goal of defining the impacts of the chromid-borne T4SS on pSym conjugal transfer.

7. Conclusion

Taken together, the usefulness of the transposon Tn*5*-B12S construct carrying an *oriT* site and a *sacB* cassette, and the Cre-*lox* site specific recombination system for plasmid curing and generation of genetic region excision respectively, were well demonstrated in this thesis. The pSym of *R. galegae* sv. officinalis was found transferred when *R. galegae* sv. orientalis acted as the recipient, whereas the same conclusion cannot be drawn when the pSym-cured derivative of the same symbiovar as the donor was used as the recipient, despite repeated trials, which may be associated with some complications such as subtle environmental signal molecules and recipient cell recognition that are far from thoroughly understood.

8. Acknowledgments

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