

The Plasmid Mobilome of the Model Plant-Symbiont *Sinorhizobium meliloti*: Coming up With New Questions and Answers

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ABSTRACT Rhizobia are Gram-negative Alpha- and Betaproteobacteria living in the underground that have the ability to associate with legumes for the establishment of nitrogen-fixing symbioses. *Sinorhizobium meliloti* in particular—the symbiont of *Medicago*, *Melilotus*, and *Trigonella* spp.—has for the past decades served as a model organism for investigating, at the molecular level, the biology, biochemistry, and genetics of a free-living and symbiotic soil bacterium of agricultural relevance. To date, the genomes of seven different *S. meliloti* strains have been fully sequenced and annotated, and several other draft genomic sequences are also available (<http://www.ncbi.nlm.nih.gov/genome/genomes/1004>). The vast amount of plasmid DNA that *S. meliloti* frequently bears (up to 45% of its total genome), the conjugative ability of some of those plasmids, and the extent of the plasmid diversity has provided researchers with an extraordinary system to investigate functional and structural plasmid molecular biology within the evolutionary context surrounding a plant-associated model bacterium. Current evidence indicates that the plasmid mobilome in *S. meliloti* is composed of replicons varying greatly in size and having diverse conjugative systems and properties along with different evolutionary stabilities and biological roles. While plasmids carrying symbiotic functions (pSyms) are known to have high structural stability (approaching that of chromosomes), the remaining plasmid mobilome (referred to as the non-pSym, *functionally cryptic*, or *accessory* compartment) has been shown to possess remarkable diversity and to be highly active in conjugation. In light of the modern genomic and current biochemical data on the plasmids of *S. meliloti*,

the current article revises their main structural components, their transfer and regulatory mechanisms, and their potential as vehicles in shaping the evolution of the rhizobial genome.

EXTRACHROMOSOMAL REPLICONS IN *S. MELILOTI*: PLASMID CONTENT AND DIVERSITY

Bacteria grouped within the *Rhizobiaceae*, *Phyllobacteriaceae*, and *Bradyrhizobiaceae* families, collectively known as rhizobia, inhabit the soil under free-living conditions and are associated in symbiosis with the root of legumes as nitrogen-fixing organisms. Rhizobia do not form a single taxonomic cluster. Instead, they are distributed within distantly related lineages within the

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alpha- and beta-subdivisions of the proteobacteria. The superb nitrogen-fixing capacity of the *rhizobia*-legume symbioses argues for the use of rhizobia for the introduction of nitrogen into agricultural soils, as a means of avoiding the massive use of chemical fertilizers (1, 2).

A common feature of the rhizobial genome is that, in addition to the chromosome, plasmids are usually present that carry genetic material encoding widely diverse functions (3, 4). The plasmids carrying genes involved in the symbiotic process have been named symbiotic plasmids or *pSyms* (5, 6, 7). In addition to these symbiotic elements, rhizobia may carry other plasmids—referred to as nonsymbiotic plasmids, *non-pSyms* or the *functionally cryptic* or *accessory* compartment—that are not indispensable for symbiosis or have simply not yet been assigned a specific function. That any of these plasmids may be lost, recovered, or also change their copy number or the contents of their genetic information is consistent with the plasmid's role as transient vectors of genomic structural change(s) within a given bacterial population (8).

In many rhizobia, plasmid DNA could constitute a substantially high percentage of the bacterial DNA, as in *Sinorhizobium meliloti* where the contribution of (mega)plasmid DNA is ca. 45% of the total. *S. meliloti* is a model species for studying plant-bacteria interactions and, in particular, rhizobia-legume symbiosis and symbiotic nitrogen fixation. In the type strain *S. meliloti* 1021 the genome consists of one chromosome of 3.65 Mbp and two symbiotic megaplasmids, pSymA and pSymB, that are 1.36 and 1.68 Mbp, respectively. In many other strains non-pSym (i.e., cryptic) plasmids of different sizes have also frequently been reported (9, 10, 11, 12, 13, 14) (also, see further on).

SYMBIOTIC (MEGA) PLASMIDS (pSyms)

As mentioned above, pSyms are those plasmids that bear essential genes for the establishment of a fully functional symbiotic nodule in the legume host. The complete genetic sequencing of the model strain *S. meliloti* 1021 more than 10 years ago enabled a knowledge of the gene content of both these pSym plasmids (15, 16). The availability of such information furthered progress in the development of new tools for the functional study of symbiosis, which, in turn, shed additional light on the structure and modular conformation of both megaplasmids. This novel information deepened our understanding of how a symbiosis such as this model association represents could have evolved (15, 16). pSymA codifies genes required for nodulation (*nod*), for the nitrogenase (*nif*), and for

nitrogen fixation (*fix* [15]). In addition, pSymA carries genes likely to be involved in nitrogen and carbon metabolism, transport, and stress and resistance responses that give *S. meliloti* an advantage in its specialized niches (15). The determination of the *S. meliloti* 1021 complete genomic sequence indicated that pSymA could have been acquired by horizontal gene transfer (HGT) because of the megaplasmid's lower GC content (60.4%) and its different codon usage compared with the other replicons (i.e., the bacterial chromosome and pSymB).

pSymB was found to bear genes encoding solute-uptake systems along with the enzymes involved in both polysaccharide biosynthesis and catabolic activities. pSymB also exhibited many features of a typical chromosome such as the presence of essential housekeeping genes including those encoding the arginine tRNA, *minCDE* cell division, and the asparagine-synthetic pathway. In contrast, no essential gene could be mapped on pSymA.

Giuntini et al. (17) applied comparative genomic hybridization on an oligonucleotide microarray in order to estimate genetic variations in four natural strains of *S. meliloti* compared with the type strain 1021. For the analysis, they used two strains obtained from Italian agricultural soils and two isolated from a desert soil in the Aral Sea region. The assay showed that the largest amount of genetic polymorphism—i.e., the genes that were found to be more variable in the comparison between the type strain 1021 and the field isolates—was present in pSymA (17). Gene diversity was not randomly distributed among the three replicons (pSymA, pSymB, and chromosome) in the four natural isolates. Consistent with this, pSymA showed a higher density of insertion sequence elements and phage sequences compared with pSymB (15). In general, transposable elements tend to accumulate in regions where they do not disrupt essential genes. All these data suggest that pSymA is a plasmid-like replicon, whereas pSymB has several chromosomal characteristics. This conclusion was also supported by the observation that pSymA could be cured in *S. meliloti* 2011, whereas pSymB could not (18). The pSymA-cured derivative strain showed no difference in its growth behavior compared with the wild-type strain in both complex and defined media, but otherwise was unable to use a number of substrates as a sole source of carbon on defined media (18). Slater et al. (19) proposed a common mechanism of secondary chromosome formation in *Rhizobiaceae* and other bacteria. A prerequisite for this evolution was proposed to be the intracellular presence of a second replicon capable of stably and efficiently replicating large DNA molecules.

Current items of evidence taken together support the notion of a near chromosome status for pSymB (i.e., one of a so-called *chromid* [20, 21]). The genes present in pSymB were recently observed to be more widespread in different taxa than those encoded by the other *S. meliloti* replicons (20). Within such a context, pSymB was proposed to have a role in intraspecies differentiation through positive gene selection, whereas pSymA was suggested to contribute to the emergence of new functions (20). Another recent investigation compared the full genomic sequence of three strains of *S. meliloti*: 1021, AK83, and BL225C (22). The pSymB showed a high resistance to genomic rearrangements, demonstrating an almost perfect shared synteny. Only strain AK83 presented few rearrangements in certain regions of its pSymB. Within pSymA, however, a very low degree of synteny was observed, thus indicating a much greater rate of rearrangements. When the *S. meliloti* SM11 genome (23) was included in the analysis, plasmid pSmeSM11c (the equivalent to the pSymA of strain 1021) presented the most diverse information in comparison with the pSymA homologs from strains AK83 and BL225C (22). Another interesting feature of pSmeSM11c was that large gene regions proved to be closely related to regions in different plasmids from strain *Sinorhizobium medicae* WSM419. In addition, pSmeSM11c carries other novel gene regions, such as additional plasmid-survival genes; the *acdS* gene involved in modulating the level of the phytohormone ethylene; and a set of genes with predicted functions associated with degradative capabilities, stress response, and amino-acid metabolism and associated pathways (23).

The higher structural variation in pSymA is also consistent with its potential self-transmissibility via conjugation (24), compared with the pSymB, whose mobilization depends on the type IV secretion system present in the pSymA megaplasmid (25). Later on in this review, we will discuss the regulatory aspects associated with the conjugative mobilization of both of these pSyms.

NON-pSym (CRYPTIC OR ACCESSORY) PLASMID REPLICONS IN *S. MELILOTI*

The size of the non-pSym plasmids is highly variable, ranging from ca. 7 kb (26) to up to 260 kb (22). Certain regions of these plasmids may be involved in the emergence of genetic rearrangements (27, 28) owing to the presence of insertion sequences, the generation of deletions, or plasmid cointegrations. The information available, mainly stemming from sequence data, indicates

that the non-pSyms may encode phenotypic features providing the rhizobia with adaptive advantages. Non-pSym plasmids are thought to encode functions that most likely impact the environmental fitness of the rhizobia. For this reason, the observation that the phenotypic changes resulting from gene knockouts in these plasmids proved to be difficult to study under laboratory conditions was certainly logical. Stiens et al. (11) isolated the plasmid pSmeSM11a from a dominant indigenous *S. meliloti* subpopulation within the context of a long-term field-release experiment involving genetically modified *S. meliloti* strains in Germany. An analysis of the plasmid sequence revealed that approximately two-thirds of pSmeSM11a was occupied by accessory genetic modules that could be providing adaptive advantages or broadening the host bacterium's responsive spectrum. Therefore, the authors postulated that the presence of pSmeSM11a might have been responsible for the dominance of strain SM11. The finding that non-pSym plasmids may encode information for the metabolism of soil compounds or root exudates suggested that these plasmids could play a significant role in the free life cycle of the rhizobia and generated speculation on future possibilities for using the plasmid information for enhancing rhizobial capabilities to colonize rhizospheric environments. In addition, reports have indicated that the presence of non-pSym plasmids may be related to non-essential symbiotic capacities. For example, Bromfield et al. (29) found that the presence of the cryptic plasmid pTA2 stimulated competitiveness for nodulation, and genes directly involved in nodulation efficiency have been described on the plasmid pRmeGR4b of *S. meliloti* GR4 (30, 31, 32). By contrast, Velázquez et al. (33) reported that the development of fully effective root nodules in alfalfa by *S. meliloti* strain SAF22 was attenuated by the presence of the cryptic plasmid pRmSAF22c, which was interfering with the normal nodular development necessary to sustain a fully effective nitrogen-fixing symbiosis. Thus, an understanding of how these rhizobia become adapted to changing environments (soil and plant host) will clearly require a more solid characterization of the non-pSym mobilome, because those plasmids appear to be a main cellular *trans* source of new functions.

THE DIVERSITY OF NON-pSym PLASMIDS IN *S. MELILOTI*

Over the years, different investigations were aimed at examining the genomic diversity of bacteria associated with the genus *Medicago*, paying special attention to the presence of cryptic plasmids in the associated rhizobial

strains. The most convenient technique for examining large plasmids—such as those borne by *S. meliloti*—was developed by Eckhardt (34) and was based on the lysis of the bacterial cells within an agarose gel (lysis *in situ*). Since under those conditions of minimal mechanical stress (i.e., the shear forces applied) little physical damage to the plasmids occurs, they are released intact in the lysate before migration in the gel. Several modifications of the original technique have been described (7, 35, 36) and have been used accordingly to screen for the presence of large plasmids in rhizobia.

Within the context of the genomic analysis of a collection of *S. meliloti* isolates from Germany by IS fingerprinting, Kosier et al. (12) demonstrated the existence of a high diversity of plasmid profiles in Eckhardt gels. In that study, 78 *S. meliloti* field strains were first grouped into 18 different plasmid profiles with 96% of the isolates containing cryptic plasmids. The most common plasmid profile exhibited a ca. 250 kb band that was present in 18 strains (23%). In another study aimed at investigating the symbiotic characteristics of *S. meliloti* populations present in soils with different agricultural histories, Velázquez et al. (37) isolated rhizobia from three different sites in Salamanca, Spain. In each soil, 7 plasmid profiles were found with 13 different plasmid profiles being observed overall. None of these earlier studies, however, characterized whether or not the plasmid profiles obtained were representative of the existing plasmid diversity within the populations investigated. Pistorio et al. (13) gathered a collection of *S. meliloti* isolates that, while remaining manageable and practical from an experimental point of view, contained a good representation of the plasmid diversity present in the soils sampled. Those authors used *S. meliloti* isolates recovered from 25 different soils of central Argentina to investigate whether any correlation existed between the overall genomic constitution of the rhizobia and the content of cryptic plasmids present in those same bacteria (see the following section). The analysis by Eckhardt-like gels of the plasmid content of 64 isolates resulted in 22 different plasmid profiles with at least 38 discrete plasmid bands. In order to evaluate to what extent the local diversity of plasmid profiles was represented in the collection of those isolates, the cumulative Shannon-Weaver index (38) was calculated, and the resulting values for a given number of strains ($n > 49$) reached a plateau. The asymptotic behavior of the cumulative Shannon-Weaver index, for the region of higher n values, indicated that the different plasmid profiles from the *S. meliloti* collection constituted a satisfactory representation of the existing plasmid diversity (13).

REPLICATION SYSTEMS PRESENT IN THE *S. MELILOTI* pSym AND NON-pSym PLASMIDS

The best studied replication genes in soil bacteria belong to the *repABC* system. More specifically, within the *Rhizobiaceae* family these *repABC*-type replicons predominate in the plasmids whose replication regions have been investigated (39, 40). The system is named according to the characteristic genetic arrangement of *repA-repB-repC* that, as such, conforms an operon (41). The RepA and RepB have predicted amino acid sequences similar to those of proteins related to plasmid partition; the *repA* genes encode ATPases of the ParA family, while the *repB* genes encode for the *parS*-binding proteins ParB. The *parS* stretch is a centromere-like sequence and can be located at different positions within the *repABC* operon. One general model suggests that the ParA (RepA) proteins polymerize into filaments, and ParB (RepB) binds both the *parS* region and ParA, thus acting as an adaptor between the plasmids and the filaments that are responsible for the segregation process (42). An alternative mechanism suggests a diffusion-ratchet model, where ParB associates with the plasmid chases and redistributes the ParA gradient on the nucleoid, which in turn mobilizes the plasmid (43). The RepC is essential for plasmid replication as a replication-initiator protein (39, 41). The large intergenic sequence between *repB* and *repC* contains a gene encoding a small antisense RNA (ctRNA). This RNA is a *trans*-incompatibility factor, modulating the RepC levels and thereby the resulting plasmid copy number (44, 45, 46, 47). Current genomic data indicate that most *S. meliloti* plasmids described so far bear *repABC* replication cassettes. Other plasmids bear a *repC* replication cassette without partition components and are evolutionarily related to the *repABC* family, because they bear a RepC replication-initiator protein (48, 49). In this family, an antisense RNA also plays a central role as a negative regulator of the expression of *repC*, and is a *trans*-incompatibility determinant as well (49).

Other plasmid-replication systems have been found to be present in *S. meliloti*. One includes only one member, the 7.2-kb plasmid pRm1132f isolated from strain *S. meliloti* 1132. This replication system belongs to group III of the rolling-circle-replication plasmids (26). Finally, Watson and Heys (50) isolated a plasmid replication region from strain *S. meliloti* MB19 that contained genes similar to those associated with several broad-host-range plasmids. The Rep protein in this instance was related to the one present in the broad-host-range plasmid pVS1 from *Pseudomonas aeruginosa*.

CONJUGATIVE PROPERTIES OF THE NON-pSym PLASMID MOBILOME IN *S. MELILOTI*: THE FREQUENCY AT WHICH NON-pSym PLASMIDS ARE FOUND TO BE TRANSMISSIBLE—EVIDENCE IN SUPPORT OF A STRIKINGLY ACTIVE MOBILOME

HGT, homologous recombination, and gene conversion appear to be central mechanisms contributing to the shaping of genomic structures in rhizobia (13, 24, 25, 51, 52, 53, 54, 55, 56, 57, 58, 59). As mentioned above, *S. meliloti* isolates frequently bear a varying and significant amount of non-pSym-plasmid DNA (12), which in several instances has been reported to be either mobilizable or self-transmissible via conjugation (9, 14). In direct relevance to these considerations, a publication by Bailly et al. (60) aimed at investigating the diversity of bacteria associated with plants of the genus *Medicago* provided strong evidence in support of an active HGT involved in modeling the genomes of the symbionts *S. meliloti* and *S. medicae*. All examples of this type made the transferable character of the less studied non-pSym plasmids in *S. meliloti* an issue of central concern for investigation because of their possible role as vehicles of adaptation, evolution, and diversification. Thus, using a collection of *S. meliloti* isolates recovered from humic soils of Argentina, Pistorio et al. (13) studied the percentage of rhizobia that carried transmissible plasmids, the presence of helper functions, and the incidence of phenomena restricting plasmid entry. In that work, the quantitative estimation of diversity was numerically analyzed by means of the Shannon-Weaver (61) and the Simpson (62) indices through the use of different operational taxonomic units (OTUs) that were indicative of: (i) the kind of plasmid profiles present, (ii) the type of PCR fingerprints (i.e., genomic variants), and (iii) possible variants with respect to the combinations of plasmid profiles and genomic fingerprints within the collection of isolates. Interestingly, no clear evidence of association (linkage) was obtained between particular genomic backgrounds and their accompanying plasmids. This result thus argued for the existence of an active horizontal exchange of plasmids, the occurrence of active recombinational events, or both. In order to investigate how active the plasmid transfer within the collected diversity was, a systematic search for transmissible plasmids was performed by screening representative *S. meliloti* isolates from each of the 22 different plasmid profiles (i.e., the plasmid OTUs) identified. Transmissible plasmids could be found in 14% of the isolates representing the different plasmid OTUs, indicating the presence of either self-transmissible or mobi-

lizable plasmids (13). Of the isolates representing one plasmid OTU each, 29% were demonstrated to be capable of mobilizing the model plasmid pSmeLPU88b (9) to a third bacterial strain, thus providing strong evidence for the ubiquitousness of compatible helper functions within the *S. meliloti* isolates (13). Remarkably, such conjugative system could also be found in a strain from a distant geographic origin as well, as demonstrated by the ability of the European *S. meliloti* GR4 to mobilize plasmid pSmeLPU88b (9). Strain GR4 bears the self-transmissible plasmid pSmeGR4a, and the mobilizable plasmid pSmeGR4b (14, 63). Figure 1 presents a summary of the different events observed when plasmid pSmeLPU88b was transferred to an *S. meliloti* final recipient through the use of representative isolates from the different plasmid-diversity groups as potential donors. Certain isolates (14%), although refractory to receiving the plasmid pSmeLPU88b, accepted other broad-host-range plasmids. Such behavior could be the consequence of surface exclusion and/or either plasmid-replication or recipient-strain DNA-restriction system incompatibility. Irrespective of these observations, the results taken together argued for a strikingly high proportion of the *S. meliloti* genotypes (36%) bearing either transmissible plasmids or helper functions (see Fig. 1). The available evidence indicates that the non-pSym compartment in *S. meliloti* is a highly active plasmid mobilome.

Contrasting with the information on the conjugative character of several *S. meliloti* plasmids, little research has been done to characterize transfer frequencies of pSym and non-pSym plasmids in soil samples and in the field. Studies for the *S. meliloti* cryptic plasmid pSmeLPU88b (64), and those reported by Kinkle and Schmidt for the symbiotic plasmid (pSym) pJB51 from a pea symbiont *Rhizobium leguminosarum* bv. *viciae* (65), indicate that rhizobial plasmids with detectable mobilization frequencies in the laboratory are transferred in nonsterile rhizospheric soil at rates between 10^4 and 10^6 events/recipient bacteria, irrespective of their symbiotic or nonsymbiotic character. The evaluation of plasmid transfer in nature has been considered long since a key issue to understand the relevance of the horizontal gene flow in the adaptive responses of the bacteria. Precise estimations of plasmid mobilization frequencies with analysis of the transient host bacteria (including nonrhizobia) will be required, both in soil and the rhizosphere, in support of any future attempt to develop predictive models of conjugative gene dispersion within the natural rhizobial communities.

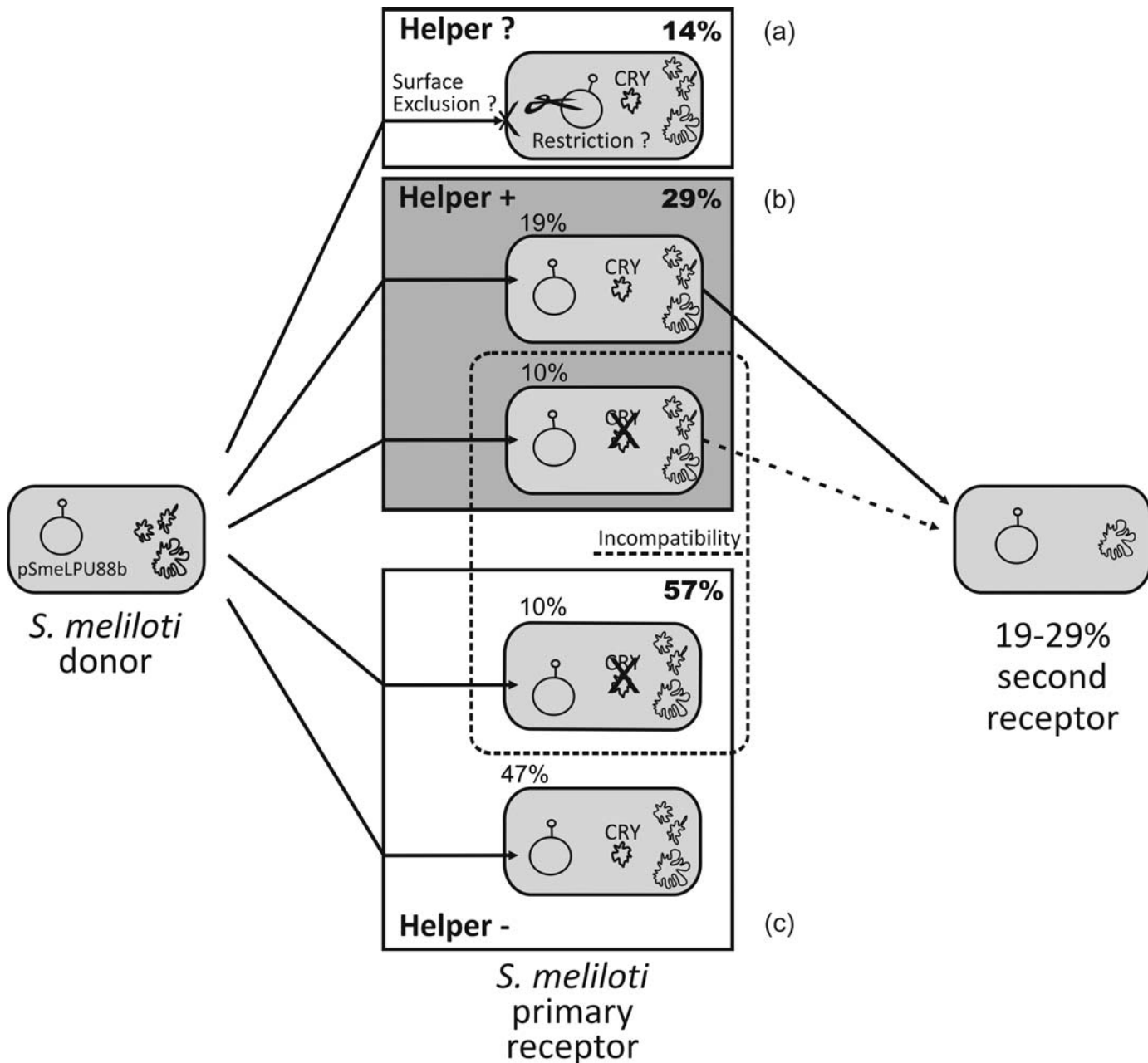


FIGURE 1 Scheme of the different possible destinies of the model plasmid pSmeLPU88b::Tn5 when transferred to *S. meliloti* from different plasmid-diversity groups (plasmid OTU types; see reference 13). The percentage assigned to each event was calculated upon consideration of an abundance of plasmid OTU types in which all are equally represented. Box A represents those OTUs that proved unable to host plasmid pSmeLPU88b::Tn5 because of either surface-exclusion phenomena or plasmid restriction within the recipient cell. Box B represents OTUs bearing helper functions able to mobilize the model plasmid. Box C represents isolates that, under our experimental conditions, were not able to mobilize plasmid pSmeLPU88b::Tn5. Some isolates belonging to the helper (+) class (Box B) or the helper (-) class (Box C) bear cryptic plasmids that display a replication incompatibility with plasmid pSmeLPU88b::Tn5 (dotted box). Cry, cryptic plasmid. The percent values on the right denote the maximal expected transfer frequency to a third bacterial strain (Reprinted from *FEMS Microbiol Ecol* [13] with permission of the publisher). doi:10.1128/microbiolspec.PLAS-0005-2013.f1

GENETICS OF CONJUGATIVE MODULES IN *S. MELILOTI*: pSym- AND NON-pSym-PLASMIDS

Considerable efforts have been made to understand the genetics of the rhizobial conjugative systems (for a comprehensive summary, see the review by Ding and Hynes [66]). The available genomic information shows that the conjugative regions in rhizobia can be divided into four Dtr (DNA transfer and replication) types (I to IV) and three Mpf (Mating pair formation) types (I, II, and IV) according to their gene structure plus their associated regulatory mechanisms (66, 67, 68). While the conjugative activities of the plasmids bearing type I Dtr/Mpf proved to be regulated by *quorum sensing* (e.g., plasmids pRetCFN42a from *Rhizobium etli* CFN42, pNGR234a from *Sinorhizobium fredii* NGR234, and pTiC58 from *Agrobacterium tumefaciens* C58), plasmids with type II Dtr/Mpf were found to have an RctA-repressed conjugative regulation (e.g., the megaplasmid pSymA in strain *S. meliloti* 1021, and plasmids pRet42d and pAtC58). Finally, a strong association has been observed between each of the Dtr types (I through IV) and a specific kind of (cognate) relaxase, a correspondence that provides a strong indication of the coevolution of the genes within these conjugative elements in rhizobia. Figure 2 shows the phylogeny of representative rhizobial relaxases and how they correlate with each of the Dtr types.

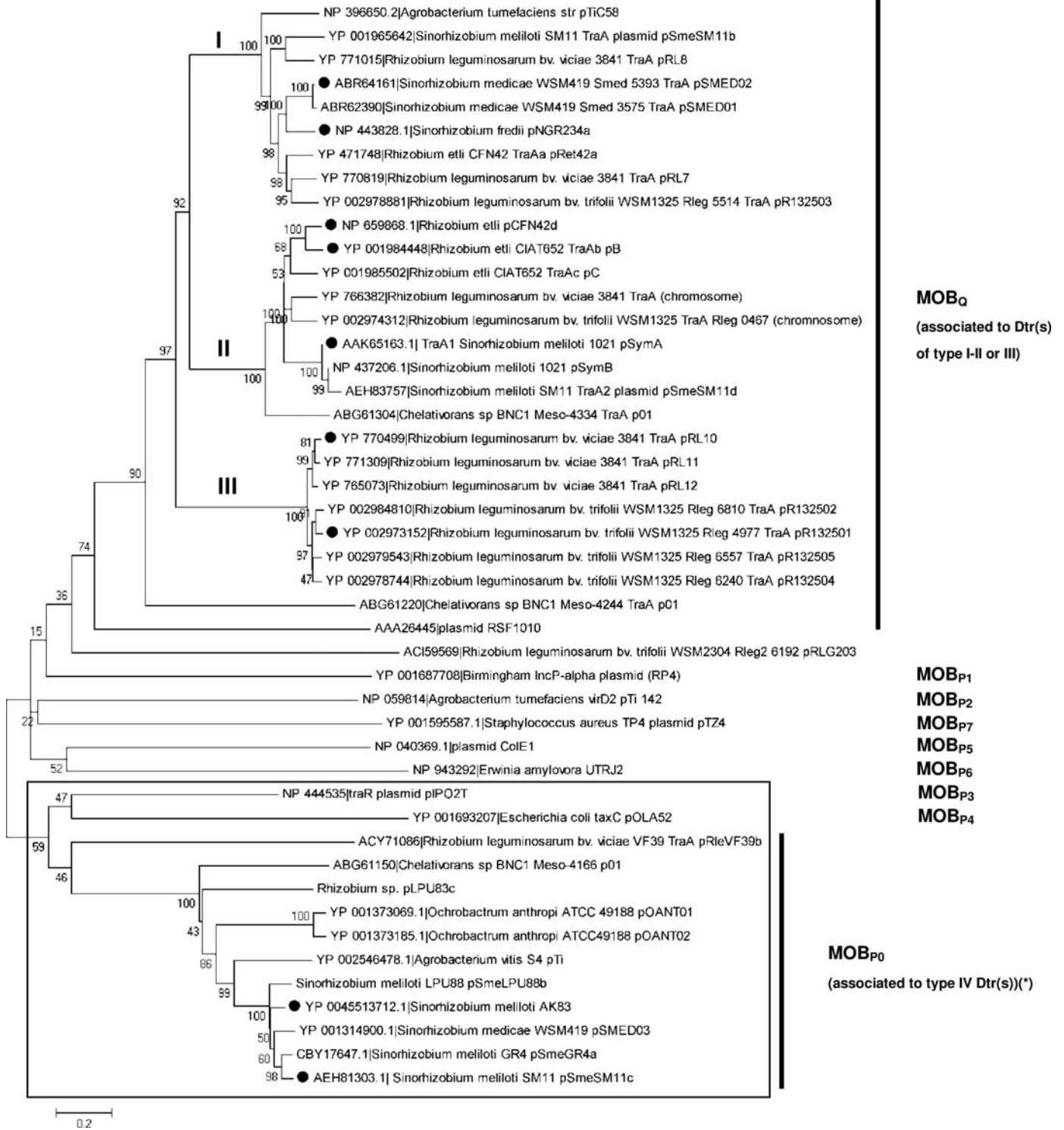
In the specific case of *S. meliloti*, plasmids may bear a Dtr/Mpf belonging to any of the four types described in rhizobia (Fig. 2). The megaplasmids pSymA and pSymB from the model strain *S. meliloti* 1021 bear a type II Dtr/Mpf (we will describe below how conjugation is regulated in these symbiotic megaplasmids). The pSymA homologs in strains *S. meliloti* SM11 and AK83 both carry, in addition to the type II, a type IV Dtr/Mpf first described for a non-pSym plasmid (67) and recently reclassified as type IVB (68). With respect to the non-pSym *S. meliloti* plasmids, most functional analyses have concentrated on the model plasmid systems pSmeLPU88a/b and pSmeGR4a/b, both of which were also found to carry type IVB Dtrs/Mpfs (Fig. 2) (67, 69). The remarkable ubiquitousness of the Mpf functions able to mobilize pSmeLPU88b (13, 67) points to the *S. meliloti* type IVB Dtr as being a key component for gene exchange within the species. Interestingly, this type IVB Dtr has also been found in plasmids from other rhizospheric and soil bacteria that include *Agrobacterium* (e.g., pATS4a), *Ochrobactrum* (e.g., pOANT01), and *Chelativorans* (e.g., pBNC-01 [67]). In all these organisms, the type IVB Dtr presented a highly con-

served synteny with a gene sequence (*parA*-like-*oriT*-*mobC*-*mobZ*) (67). The *oriT* in plasmid pSmeLPU88b could be functionally mapped on a 278-bp fragment slightly overlapping with the *mobC* locus (67).

As occurs with the Dtrs I, II, and III, the Dtrs IVB also encode cognate relaxases whose phylogenies reveal a clearly distinct branch (MOB_{P0}), close to the MOB_{P3} and MOB_{P4} enzymes and separated from all other known MOB_Q-like rhizobial relaxases (67). Sequence analysis of the type IVB relaxase from plasmid pSmeLPU88b (designed MobZ) revealed the presence of three conserved motifs (I, II, and III) as in other relaxases (70). Within Motif I (amino acids 109 to 129) of the relaxase, as expected, a tyrosine (Y115) was present consistent with the requirement of this residue for the catalytic activity of the enzyme. Upon a profile-profile analysis and fold recognition, the residue Y115 aligned—within a predicted α -helix—with the experimentally confirmed catalytic residue Y25 of the relaxase MobA from plasmid R1162 (67).

The bacteria carrying plasmids with relaxases of the MOB_{P0} group thus far reported inhabit soils, plants, or aquatic environments; with the sole exception of *Ochrobactrum anthropi*, which species (although also present in soil) has been found mainly in humans (71). The close phylogenetic relationship among the relaxases of the MOB_{P0}, MOB_{P3}, and MOB_{P4} clades suggests that their host plasmids might constitute frequent HGT vehicles among bacteria with overlapping environments (e.g., among soil- and/or plant-associated bacteria). At the moment, no evidence has been found for conjugative-transfer regulation in plasmids bearing the type IVB Dtrs/Mpfs. Recent evidence indicated that the conjugative transfer of plasmid pRleVF39b carrying a type IVA Dtr/Mpf is under repression control by a transcriptional-regulator protein (TrbR) whose sequence includes a helix-turn-helix xenobiotic-response element (66). Nevertheless, the existing genetic differences between types IVA and IVB Dtrs/Mpfs—plus the absence of a *trbR* ortholog in the fully sequenced plasmid pSmeGR4a—both suggest that this regulation is likely not to be present in the *S. meliloti* plasmids bearing type IVB Dtrs/Mpfs.

Current genomic data on the *S. meliloti* extrachromosomal replicons revealed an increasing sequence variation from pSymB homologs → pSymA homologs → non-pSym plasmids. Interestingly, the increasing genetic variation in each of these replicons parallels their likewise increasing conjugative-mobilization rates: from a strict conjugative control in the pSym plasmids to the lack of any evident conjugative regulation in some of the



non-pSym *S. meliloti* plasmids (i.e., plasmids bearing type IVB Dtrs/Mpfs). Such a gradient of replicon plasticity strongly suggests that novel genes might likely reach rhizobia through the non-pSym mobilome, with only some of those genes being incorporated (after mid- and long-term selection) into structurally more stable replicons. An adaptive strategy of this type recalls the mosaic character of the rhizobial plasmid mobilome. A comparison of the *nod*-gene locations among the plasmids (see black dots in Fig. 2) with the phylogenetic topology of the rhizobial relaxases reveals that the symbiotic genes may be found on plasmids bearing any of the four Dtr types, thus indicating that pSym plasmids arise as independent chimeras not specifically associated with any particular mobilization system. A deeper sequencing of the *S. meliloti* plasmid mobilome will be necessary to better understand how gene exchange among replicons (and their further selection) operates over an evolutionary time scale.

THE NEED FOR ADDITIONAL GENETIC DETERMINANTS OTHER THAN THE Dtr AND Mpf REGIONS AS REVEALED BY THE STRAIN-DEPENDENT REQUIREMENT FOR MOBILIZATION OF PLASMID pSmeLPU88b IN *S. MELILOTI*

The mobilization of pSmeLPU88b by plasmid pSmeLPU88a has recently been shown to require the newly identified gene *rptA* (for *Rhizobium*-plasmid transfer) in strain *S. meliloti* 2011, but not in strain LPU88 (72). A mutant carrying a disrupted copy of *rptA* in plasmid pSmeLPU88a was still able to facilitate the mobilization of plasmid pSmeLPU88b from strain *S. meliloti* LPU88, but not from strain 2011. Strikingly, no extra copies of the *rptA* gene were found by hybridization in strain LPU88. These observations raise new questions as to

(i) what the alternative active components are that complement the deficiencies in *rptA* in strain LPU88 (but not in strain 2011), and (ii) whether or not such components operate in association with the Dtr or the Mpf conjugative regions. Interestingly, syntenic regions containing *rptA* homologs could also be identified in plasmids from the strains *S. meliloti* SM11, AK83, and GR4; *S. medicae* WSM419; *S. fredii* HH103; and *Agrobacterium vitis* S4; thus suggesting a more extensive requirement of *rptA* for plasmid mobilization (72). Analysis of plasmids in these strains suggests that the presence of *rptA* is associated to the type IVB Dtr-bearing plasmids.

REGULATION OF THE CONJUGATIVE TRANSFER OF MEGAPLASMIDS pSymA AND pSymB IN THE MODEL STRAIN *S. meliloti* 1021

As mentioned in the previous sections, studies on conjugative transfer in rhizobia have so far identified at least two different regulatory transfer systems: (i) those associated with plasmids containing type I Dtr/Mpf and exhibiting transfer regulation through a *quorum sensing* that responds positively to population density via *N*-acylhomoserine lactones (73, 74, 75, 76), and (ii) systems present in plasmids bearing type II Dtr/Mpf characterized by a so-called RctA-repressed conjugative transfer that occurs only under conditions disturbing the activity of the transcriptional repressor RctA. The *rctA* gene is usually associated with a *virB*-type T4SS (type IV secretion system); and, on the basis of this feature, several symbiotic plasmids of *R. etli*, *S. fredii*, and *S. meliloti* would belong to this category, although experimental data have been obtained so far only for the 372-kb symbiotic plasmid of *R. etli* CFN42, a symbiont of *Phaseolus vulgaris* (the common bean), and the

FIGURE 2 Phylogenetic (neighbor-joining) tree showing the relationships between different relaxases of the MOB_{P/Q} cluster as inferred from their complete protein sequences. The bootstrap-consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the proteins (corresponding to the indicated taxa) analyzed (80). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with the branch lengths in proportion to the evolutionary distances used to construct the phylogenetic tree. Those distances were computed by means of the Poisson-correction method (81) with the units being the number of amino acid substitutions per site. The protein sequences used were obtained from GenBank under the accession numbers indicated in parentheses before the name of each plasmid replicon. The box over the tree indicates the clades that include the close MOB_{P0}, MOB_{P3}, and MOB_{P4} families of relaxases. The black dots denote those plasmid replicons that bear nodulation (*nod*) genes (Reprinted from *Plasmid* [67] with permission of the publisher). [doi:10.1128/microbiolspec.PLAS-0005-2013.f2](https://doi.org/10.1128/microbiolspec.PLAS-0005-2013.f2)

1,354-Kb pSymA of *S. meliloti* 1021 (24). Thus far, no clues have been discerned concerning the conditions governing the conjugative transfer of plasmids either with type III Dtrs—which group includes mainly *Rhizobium leguminosarum* plasmids that do not contain an Mpf system and therefore are not self-transmissible (66)—or with type IVB Dtrs/Mpfs, except for the cryptic plasmid pRmeGR4a of *S. meliloti* GR4, where conjugation efficiency was shown to be modulated by the nitrogen source (63). Care should be taken, however, when extrapolating phylogenetic data to mechanistic hypotheses. For instance, the plasmid pSmeSM11c—the pSymA homolog from *S. meliloti* SM11—was initially found to bear a type IVB Dtr/Mpf based on the phylogeny of the putative relaxase gene SM11_pC0230. However, this plasmid was also found to contain another putative relaxase (SM11_pC1037) with an associated coupling protein (SM11_pC1041) and a typical *rctA* gene (SM11_pC0937) linked to a *virB*-like Mpf operon. Thus, plasmid pSmeSM11c likely carries two different conjugative systems.

Among the pSyms, only the conjugative ability of pSymA (type II Dtr/Mpf) of *S. meliloti* 1021 has been established; whereas, as stated above, the pSymB behaves like a typical mobilizable element that depends on Mpf functions encoded in pSymA (25). Conjugation is regulated through the interplay of the pSymA transcriptional repressor RctA and the antirepressor gene *rctB*, similar to the control that operates in the *R. etli* CFN42 pSym (24). Under conditions nonoptimal for conjugation, RctA maintains the Dtr and Mpf genes silenced. When conditions are favorable for plasmid conjugation, a transcriptional activation of *rctB* occurs and RctA becomes inhibited, thereby allowing expression of the conjugative genes. The mechanism of action of *rctB* is unknown and its predicted product shows no significant homology to proteins in existing databases. In strain *S. meliloti* 1021, activation of *rctB* has recently been found to require the prior inhibition of another transcriptional repressor, RctR, that belongs to the GntR family of one-component regulatory systems (59). The DNA-binding activity of this family of regulators is usually modulated through interaction with a chemical ligand (77). According to available data, RctR does not directly repress *rctB* transcription. Instead, RctR represses the transcription of two adjacent gene operons, Sma0950/53, encoding an ABC transporter, and Sma0956/61, which encodes for several enzymes plus the transcriptional activator *rctC* (Sma0961; Fig. 3). The RctC gene product, in turn, appears to activate transcription of the antirepressor *rctB* (59). It has been

suggested that an as-yet-unknown compound would be imported by the *rctR*-adjacent ABC transporter, and that that signal compound or a metabolic derivative would bind the repressor RctR. That binding of the compound to RctR would thus relieve repression of the adjacent operons Sma0950/53 and Sma0956/61, including the activator gene *rctC*. Once produced, the RctC protein activates transcription at the antirepressor locus *rctB* and the RctA repressor becomes inhibited, thus allowing plasmid conjugation to proceed. Whether or not phosphorylation of the response-regulator protein RctC is needed for conjugative transfer or whether the active form of RctC binds directly or not to the promoter region of *rctB* have not yet been investigated. The nature and origin of the signal compound(s) derepressing conjugative functions are still unknown. The RctR-RctC system is also present in other RctA plasmids—i.e., the pSym of strain *S. meliloti* GR4, the symbiotic plasmids of different *S. fredii* strains (e.g., NGR234, HH103, and USDA257), in addition to the nontumorigenic plasmid pAtC58 of *A. tumefaciens*. This system, however, is not conserved in other RctA plasmids such as the pSym of *R. etli* CFN42 or several others of *S. meliloti*. Therefore, in these other plasmids, *rctB* activation must proceed through a different regulatory cascade.

Although no experimental evidence has been found that *quorum-sensing* mechanisms might be controlling the conjugation of RctA-containing plasmids, some indications exist of a putative relationship between the *rctA/B* system and population density. First, all the pSymA conjugative-transfer genes of *S. meliloti* strain 8530 (an *ExpR*⁺ derivative of 1021) appear overexpressed in a *mucR* mutant (78). MucR has accordingly arisen as a global regulator involved in the repression at low population densities of a number of energy-consuming processes, at least some of which are also activated by *quorum sensing through* the *ExpR/SinI* system (78). In addition, the pSyms of several *S. meliloti* strains (e.g., SM11, AK83, BL225C, and Rm41)—those also likely harboring RctA-type plasmids but not containing the above described RctR-RctC pathway to activate *rctB*—instead possess *luxI*-like and/or *luxR*-like genes adjacent to a putative *rctB* (J. Sanjuán, unpublished observations). The conservation of putative *quorum-sensing*-like genes in the vicinity of the *rctB* locus suggests a relationship between *rctB* activation and population density that certainly deserves to be investigated. What seems clear is that the primary signal(s) that drive the conjugation of the RctA-containing plasmids may be different for several of these. This

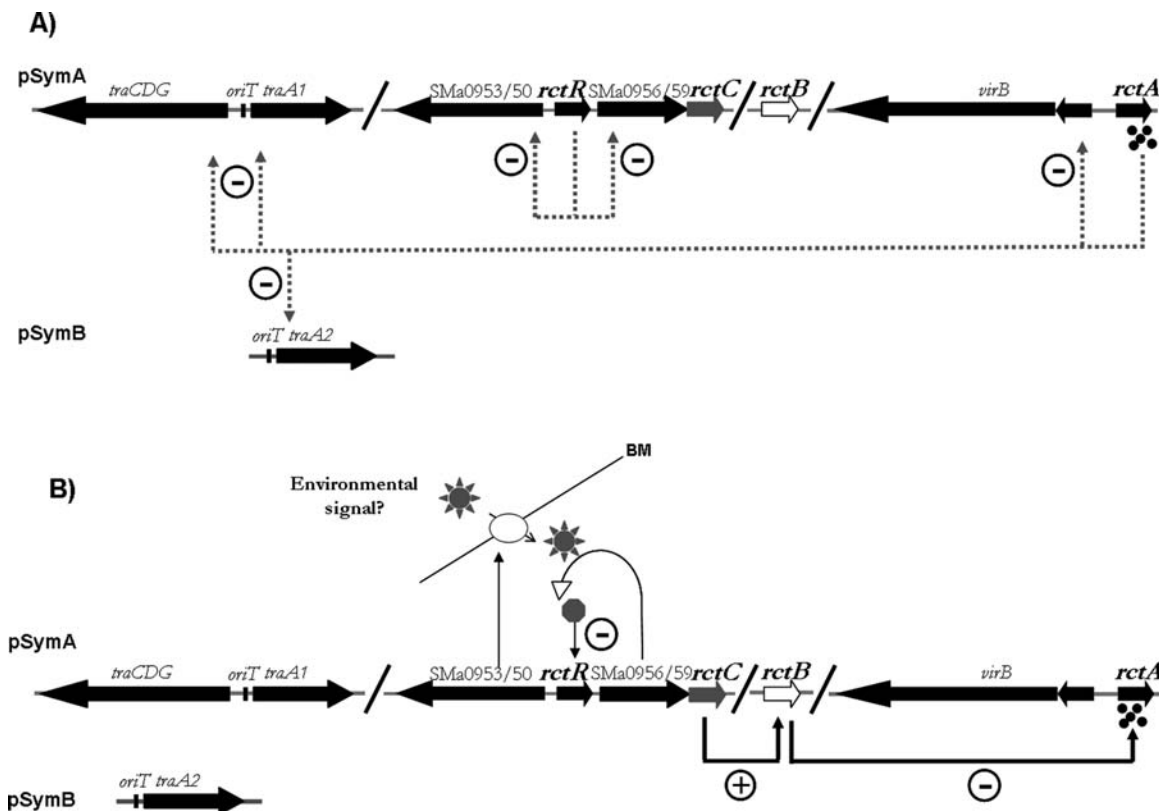


FIGURE 3 Proposed model for the regulation of conjugative transfer of the *S. meliloti* symbiotic plasmids. (A) Under nonfavorable conditions (i.e., standard laboratory media), RctR silences the Sma0953/50- and Sma0956-58-59-*rctC* operons, so that *rctB* remains inactive and RctA represses conjugative transfer in both pSyms. (B) Conjugative transfer is achieved after an unknown compound—putatively imported by the ABC transporter encoded by Sma0953-50 and modified by the Sma0956-58-59 gene products—binds RctR, thereby relieving transcription repression of *rctC*. The resulting *rctC* transcription ends up with the activation of *rctB* to counteract RctA, the conjugative-transfer operons become expressed, and plasmid conjugation is able to proceed. (+) activation; (–) repression or inhibition; BM, bacterial membrane (Reprinted from *Environ Microbiol* [59] with permission of the publisher). doi: 10.1128/microbiolspec.PLAS-0005-2013.f3

qualification together with a possible connection with population-density cues suggests a complexity of conditions that likely need to be satisfied for activating the conjugative transfer of these plasmids.

DIVING INTO THE NON-pSym PLASMID COMPARTMENT AT AN OMIC SCALE

In a recent communication, plasmids of up to 200 to 300 kb from 18 *S. meliloti* strains (18) were separated from the chromosomal DNA and ca. 1.5 Mb of nonredundant sequence could be collected by means of high-throughput technologies (López et al., presented at the International Society for Plasmid Biology Conference, Santander, Spain, 2012). The work constituted the first

deep-sequencing approach aimed at the massive characterization of the non-pSym information in *S. meliloti*. The sequence analysis of plasmid replication and mobilization elements evidenced an intricate phylogenetic distribution when compared with homologous elements from other rhizobia, thus pointing to the existence of an interspecific horizontal plasmid exchange. Interestingly, the mobilome sequence revealed a significant diversity of relaxases, in addition to the MOB_{P0} subfamily described previously. As to the kind of open reading frames present in the non-pSym mobilome, the proportion of COG classes showed clear differences compared with those from the chromosome, pSymA, and pSymB (A. Lagares and M. Pistorio, unpublished results); indicating clear functional differentiations among all four

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of these genetic compartments. The experimental approach used proved to be feasible, with deep penetration down to the scale of single-gene analysis. The thousands of genes collected other than those associated with the basic plasmid biology (replication/maintenance/transfer) represent, certainly, the most valuable portion of the new information available.

CONCLUDING REMARKS

The split configuration of the bacterial genome qualitatively into chromosome(s) along with plasmids as intercellular mobile genetic vectors is a cogent physical indication of an efficient and extensive ancestral mechanism of communal evolution and adaptation in prokaryotes. The separation of the essential housekeeping functions resident in the chromosomes from those associated with adaptive responses encoded in plasmid enables bacteria to access and manage, at the population level, an enormous and diverse amount of (horizontally) shareable and accessible information (i.e., the mobilome) that could not otherwise be handled by single bacterial cells, with the final result being that the community as a whole evolves (79). Rhizobia, in general, and *S. meliloti*, in particular, are soil bacteria where their plasmid replicons are known to provide the genetic basis both for speciation events (i.e., HGT of symbiotic traits) and for environmental adaptation. The available information demonstrates that the *S. meliloti* plasmid compartment includes replicons with a wide range of sizes, diverse gene contents, and different degrees of conservation. Among the different strains, diverse kinds of conjugative genes along with their associated regulatory mechanisms were described, with clear evidences of coevolution. A solid understanding of the conjugative mechanisms that mediate both transient and long-term genomic changes in *S. meliloti* will be necessary to help unravel fundamental questions such as how these rhizobia were born as plant symbionts, how their symbioses and specificities evolved, how active the exchange of genetic information with other rhizobia is, and—related to this last question—how ubiquitous the *S. meliloti* conjugative systems are in other plant-associated sympatric rhizospheric-soil bacteria. Upcoming research is expected to focus with much more intensity on the analysis of the (still mostly hidden) genes and functions encoded in the *S. meliloti* non-pSym-plasmid pool. Information of that sort is expected to further our understanding of the diversity of processes that contribute to the symbiotic and free-living lifestyles of these rhizobia.

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Author Queries

Q1: For Lopez et al., please provide all author names and initials, and the range of dates for the meeting in Santander.