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Conjugal transfer of a *Sinorhizobium meliloti* cryptic plasmid evaluated during a field release and in soil microcosms

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ABSTRACT

Horizontal gene transfer (HGT) is a central evolutionary mechanism that mediates the diversification and adaptation of bacteria in general and of rhizobia in particular. The few quantitative data on the conjugal transfer of rhizobial plasmids in soil correspond to the pSym (symbiotic genes-carrying replicons), with no information available regarding transfer frequencies in soil of other (namely accessory/cryptic) plasmids that are present in several rhizobial species. Thus, we examined here the conjugal transfer in non-sterile soil of the model *Sinorhizobium meliloti* cryptic plasmid pSmeLPU88b. Under field conditions the proportion of nodules containing indigenous rhizobia that acquired the plasmid pSmeLPU88b and then nodulated the trapping plants could be estimated as <0.1% (transconjugants/nodule) over an 18-month sampling period that followed inoculation. The collected evidence showed that the release of rhizobia by means of standard seed-inoculation procedures did not result in a massive transfer of the introduced cryptic plasmid pSmeLPU88b to the indigenous bacteria that nodulate trapping alfalfa plants. Using a laboratory microcosm system performed with the same soil from the experimental field, we demonstrated that transconjugants were generated in the rhizosphere at a frequency of *ca.* 1.43×10^{-6} transconjugants/recipient, a frequency from 10^2 to 10^3 times lower than that corresponding to the transfer of the same plasmid in rich-medium agar plates. The estimation of mobilization frequencies of rhizobial plasmids in soil is a necessary step toward the development of quantitative predictive models of gene-dispersal frequencies from inoculated strains to other rhizobia and soil bacteria.

Legume inoculation was the earliest deliberate application of bacteria in agriculture, and since then has been used for over a century in an attempt to improve legume productivity by providing the leguminous plant with its specific rhizobia at the time of planting. These symbiotic bacteria induce nitrogen-fixing nodules on the roots of their host legumes. Millions of hectares of various legume crops are inoculated worldwide each year with their compatible rhizobia [1]. Within this context, advances in the knowledge of the molecular aspects of the symbiosis together with the ability to manipulate the bacteria have created the prospective possibility of engineering rhizobia into more efficient legume inoculants. Thus, the transfer of genetic information to other rhizobia and to other soil bacteria is an important issue. Horizontal gene transfer (HGT), a central phenomenon in bacterial-genome evolution and diversification, should be specifically considered if recombinant rhizobia are intended to be released for bio-fertilization. The relevance of this issue is reflected by several independent evidences that demonstrated the significance of the HGT of symbiotic genes in the processes of rhizobial speciation [2–6]. In the particular case of *Rhizobium leguminosarum* bv viciae, the plasmid transfer of a symbiotic plasmid (pSym) in bulk soil could be quantitatively estimated using microcosms systems [7]. Unfortunately, though many rhizobia carry significant amounts of non-symbiotic plasmid DNA, most of them bear yet unknown functions [8], there is no information on the conjugal transfer rates of these replicons in soil.

In the specific instance of *Sinorhizobium meliloti*, the nitrogen-fixing symbiont of alfalfa, the ubiquitous presence of mobilizable/conjugative cryptic plasmids has been shown [9]. While the conjugal transfer of the pSymA megaplasmid is usually repressed *via* a complex regulatory circuit [10], the cryptic plasmid mobilome in *S. meliloti* is a highly mobile genetic compartment [9]. Thus, the

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possibility of spreading *S. meliloti* genes from the inoculants strains to the resident bacterial community *via* transmissible plasmids certainly needs to be further evaluated. The evaluation of the mobilization frequencies of rhizobial cryptic plasmids in soil is a necessary step toward the development of predictive models of gene-dispersal frequencies (risk assessment) from inoculated strains to other rhizobia as well as unrelated soil bacteria.

During the last decade our laboratory has been studying the cryptic plasmids in *S. meliloti*, and the mobilization properties of two cryptic plasmids from the strain *S. meliloti* LPU88 were reported [11]. One of those plasmids, pSmeLPU88b (40 kb), proved to be mobilizable if conjugative helper functions were supplied by the accompanying 149-kb plasmid pSmeLPU88a (binary conjugal system). The molecular characterization of the Dtr region of plasmid pSmeLPU88b has been recently reported [12]. In order to explore the mobilization frequency of cryptic plasmids in soil, we selected pSmeLPU88b as a model plasmid to trace mobilization. To that end strain *S. meliloti* LPU88AII, a spontaneous streptomycin (Sm)-, gentamicin (Gm)-, and spectinomycin (Sp)-resistant derivative of strain LPU88, was released into an experimental field to estimate the transfer of pSmeLPU88b to the indigenous *Medicago sativa*-nodulating rhizobia. The donor strain, LPU88AII, was selected based on the well known mobilization properties of its plasmid system [11], the conjugal transfer of the resident plasmid without the need for specific inducers, and the significant transfer frequency of pSmeLPU88b under laboratory conditions. *A priori*, all these features were thought to favor the possibility of detecting transfer events from the inoculated strains to the soil community of alfalfa-nodulating rhizobia.

In the field assay, *S. meliloti* LPU88AII was introduced into the soil through seeds that had been coated with the strain at a final inoculum of $8 \times 1\ 0^5$ cfu/seed and randomly sown at a density of 3.5 g/m^2 over a parcel with an area of 6 m^2 (*i.e.*, 1 m × 6 m). The inoculation protocol was selected to emulate procedures that are commonly used in local agricultural practices. A control parcel of the same size was likewise sown at a same density with non-inoculated alfalfa seeds. The inoculated and the control parcels were separated by a 1.5-m margin of grass. In order to examine the conjugal transfer of plasmid pSmeLPU88b from the inoculated strain LPU88AII to the alfalfa-nodulating soil rhizobia, we investigated the presence of plasmid pSmeLPU88b in streptomycin-sensitive (Sm^s) rhizobia recovered from the nodulated plants in the field. Only those Sm^s indigenous alfalfa-nodulating rhizobia were examined for the presence of pSmeLPU88b. The search for plasmid pSmeLPU88b in the Sm^s isolates was performed in bacteria recovered from those nodulated plants that had been grown from seeds introduced at the beginning of the assay, as well as from new trapping plants grown from noninoculated seeds introduced at the times indicated in Fig. 1.

Of the more than 2500 root nodules from the inoculated parcel that were analyzed throughout the experiment, 1400 contained isolates that proved to be Sm^s and thus corresponded to nodulation by indigenous rhizobia. This result indicated that the rhizobia in the soil had reached the rhizosphere of the inoculated seeds, a region where plasmid transfer has been reported to be particularly active. All the Sm^s isolates recovered from the nodules were tested for acquisition of the plasmid pSmeLPU88b. The presence of pSmeLPU88b in indigenous rhizobia recovered from the surface sterilized nodules was analyzed by a multiplex PCR targeting three different plasmid markers (*cf.* the legend to Supplementary Fig. S1 for primer sequences and the size of each PCR product). No evidence of plasmid transfer was obtained over a period of a year and a half; thus indicating that, over the 18-month sampling period, the proportion of nodules containing indigenous (Sm^s) rhizobia that had acquired the plasmid pSmeLPU88b was lower than 7.2×10^{-4}

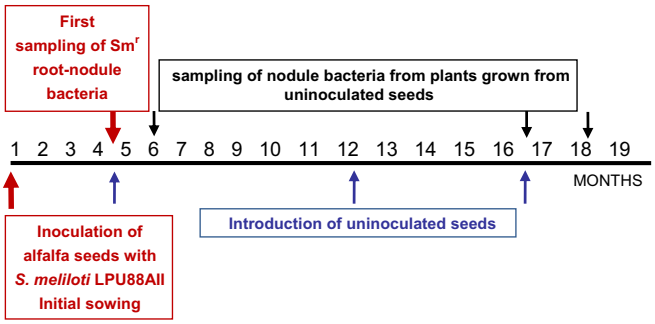

Fig. 1. Time schedule of the field-release experiment. Strain LPU88AII was released into the soil at the beginning of the experiment *via* inoculated alfalfa seeds (8×10^5 cfu per seed; *cf.* text). The introduction of alfalfa seeds to generate trapping plants at different times during the experiment is indicated below the time line (blue arrows). The arrows above the time line indicate the sampling of root-nodule bacteria recovered from either: a) plants originating from the initially inoculated seeds (red arrow), or b) plants derived from noninoculated seeds (black arrows) introduced at different times throughout the assay. A control experiment in the same soil was set up with noninoculated seeds at the beginning of the assay. The field experiment was conducted at an agricultural and experimental field at BIAGRO S.A., General Las Heras, Buenos Aires, Argentina (34° 55′ S, 58° 59′ W) that had the following physicochemical characteristics: pH = 6.9, organic carbon 1.59%, nitrogen 0.17%, and soluble phosphate 31.1 ppm. The indigenous *S. meliloti* population in the experimental field was present at 35 cells/g as determined by the most-probable-number analysis [26]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transconjugants/nodule (<0.1%). The generation of rhizobial transconjugants over the middle- and long-term time periods would depend on both the persistence of the originally inoculated donor rhizobia and the way in which the plasmid in question had been first hosted by the soil community and then transmitted to the soil rhizobia. The results of our field assay employing conventional inoculation practices indicated that a massive propagation of the plasmid introduced in this way to the indigenous rhizobia present did not occur during the first year after release.

While experiments in the field operate under fully natural conditions, this approach presents several practical restrictions associated with the need to preserve biosafety and bioregulation. The use of recombinant strains is not always appropriate, thus imposing severe limitations on the tracking of specific rhizobial genotypes within the natural soil. A useful and attractive alternative to experiments in the open field is microcosms in small contained systems [13] that recreate many of the features of natural habitats with no restrictions on the use of recombinant plasmids and/or bacteria. In the experiments presented here we used a microcosm system containing 30 g of nonsterile soil extracted from the experimental field, which we then inoculated with donor and recipient bacteria and planted with 2-day-old alfalfa seedlings. Using such an experimental design, we evaluated the transfer of plasmid pSmeLPU88b::Tn*5* (neomycin resistant, Nm^r) [11] from the donor strain *S. meliloti* LPU88TN (Nm^r; streptomycin resistant, Sm^r), to the traceable plasmid-free recipient strain *Agrobacterium tumefaciens* UBAPF2 (rifampicin resistant, Rif^r) [14]. We then monitored the evolution of donor and recipient bacteria and screened for *A. tumefaciens* (pSmeLPU88b::Tn*5*) Rif^r − Nm^r transconjugants for 5 weeks following the soil inoculation. The experiment started with a titer of donor and recipient bacteria at close to 10^6 cfu/g of soil (*cf.* the legend to Fig. 2). The progression in the numbers of *S. meliloti* donors and *A. tumefaciens* recipients in the bulk soil throughout the experiment is shown in Fig. 2. The numbers of each inoculated strain remained nearly constant (at about 10^6 cfu/g) for *ca.* 10 days and then started to decrease, as had been previously observed in studies from our laboratory and another [15,16]. Although the concentration of the *A. tumefaciens* UBAPF2 recipient strain

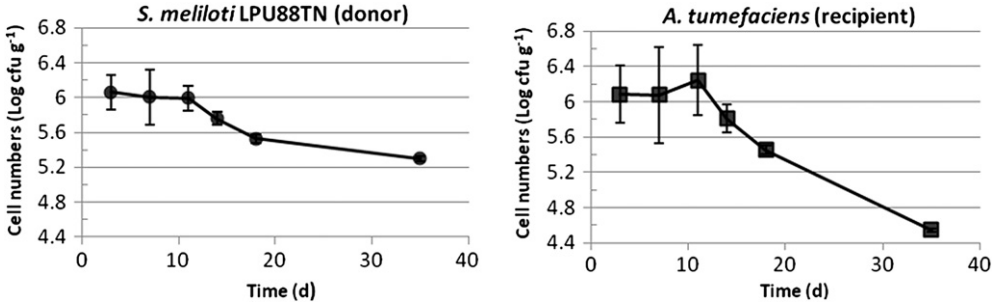

Fig. 2. Analysis of the conjugal transfer of plasmid pSmeLPU88b in microcosms systems with nonsterile soil. Time-dependent progression of the donor *S. meliloti* LPU88TN and the recipient *A. tumefaciens* UBAPF2 in the bulk soil during the microcosms experiments. Counts were obtained at days 3, 7, 11, 14, 18, and 35 after inoculation. At each sampling time, both plants were removed from the soil before enumeration of cells in the soil. Thirty g of microcosm soil were suspended in sterile phosphate-buffered saline, PBS (8 g NaCl, 1 g Na_2HPO_4, pH 8.2), and shaken for 20 min at 120 rpm. before plating. The supernatant was serially diluted and 100 μl of each dilution plated on selective TY medium [27] supplemented with Nm, Rif, or Nm-Rif to select for donor, recipients, and transconjugants, respectively. Mean values were calculated from three independent microcosm tubes. Error bars indicate standard deviations. No transconjugants were found in noninoculated control microcosms. cfu: colony forming units.

declined steadily down to a value of 3.5×10^4 cfu/g within 4 weeks, the concentration of the *S. meliloti* LPU88TN donor remained constant and close to 2.5×10^5 cfu/g of soil during the last two weeks of the assay. We cannot rule out the possibility that the greater stabilization of *S. meliloti* at higher bacterial titers compared to *A. tumefaciens* is associated with specific positive interactions between the rhizobia and the alfalfa roots. Nevertheless, no transconjugants could be detected when bulk-soil suspensions were weekly plated in selective medium, indicating that plasmid transfer (if operative) occurred in the bulk soil at frequencies lower than 8.3×10^{-5} events per recipient *A. tumefaciens*.

In view of this last result, and considering that the rhizosphere not only supports dense bacterial communities [17–19] but also has been identified as a hot-spot for HGT [20,21], we decided to screen the rhizospheric soil for the presence of transconjugants. In a microcosm experiment inoculated with 4.7×10^7 cfu of donor bacteria/g plus 3.6×10^6 cfu of recipient bacteria/g, we analyzed the rhizospheric soil at 15 and 36 days after inoculation. Subsequently we were able to identify a putative transconjugant with the expected pattern of antibiotic resistances. Fig. 2B shows the number of donor and recipient bacteria in the rhizosphere along with the estimated frequency of plasmid transfer. As also happened in the bulk soil, the number of *S. meliloti* LPU88TN in the rhizosphere declined more slowly than did the population of *A. tumefaciens* UBAPF2: Whereas the number of *S. meliloti* LPU88TN decreased by nearly 2-fold from day 15 to day 36, the number of *A. tumefaciens* UBAPF2 dropped by more than 15-fold during that same period. The estimated transfer frequency of plasmid pSmeLPU88b::Tn5 within the rhizosphere was 6.9×10^{-7} transconjugants/donor, which is more than three orders of magnitude lower than the value that had been previously observed in agar plates [11]. The transfer of pSmeLPU88b::Tn5 to *A. tumefaciens* UBAPF2 was demonstrated by visualization of the mobilized plasmid in the recipient strain, and the plasmid-specific multiplex PCR assay (Supplementary Fig. S1). At day 36 post-inoculation no transconjugants could be detected in the rhizosphere, likely due to a significant decrease in the number of donor and recipient bacteria by a factor of two and fifteen, respectively (Table 1).

According to the data from the literature, the extent of the conjugal exchange of genetic elements among environmental bacteria depends on various biotic and abiotic factors [19,22]. Intra- and intergenic gene transfer among soil bacteria have been both demonstrated by population studies and directly shown in microcosm experiments as well as within the field environment [23]. In the present work we characterized the horizontal transfer of an *S. meliloti* cryptic plasmid in nonsterile soil both in the field and in microcosms. With standard seed-inoculation procedures to introduce the plasmid-donor strain into the field, no indigenous rhizobia carrying the plasmid could be detected either in early nodules that appeared in the emerging roots of the inoculated seeds or in nodules collected from trapping (noninoculated) plants subsequently introduced into the assay. The putative transfer events that could have occurred in bulk soil and/or in the rhizosphere impacted (if any) at a frequency lower than 0.1% transconjugants/nodule induced by indigenous rhizobia. In the soil microcosms, where high numbers of donor and recipient bacteria could be used, a transfer of the cryptic plasmid pSmeLPU88b::Tn5 was detected only in the rhizosphere at a frequency 10^2–10^3 times lower than the observed in laboratory media. The remarkable lower transfer frequencies observed in experiments with soil microcosms compared to assays in the laboratory (Pistorio et al., 2003) are most likely not due to a single factor but to a group of different conditions that limit conjugation in natural environments. Restrictive conditions may include the usual oligotrophic environments in soil (plasmid transfer has been shown to be highly dependent on the availability of energy sources), the presence of a number predators for both donor and recipient bacteria, and different physicochemical limitations derived from the soil structure and composition. In addition to the limitation that the soil environment imposes to plasmid transfer, HGT will be dependent on the kind of donor and receptor genotypes present in each soil. It is difficult to estimate in our case (and in soil samples in general) the incidence of surface exclusion phenomena, and the proportion of incompatible plasmids within the population of recipient soil bacteria. Previous studies with the broad host range plasmid RP4 had previously indicated a higher conjugal transfer

Table 1
Number of donor and recipient bacteria in the alfalfa rhizosphere along with the estimated transfer frequency of pSmeLPU88b::Tn5.

Days	cfu of donors and recipients cells/root at the indicated sampling times[a]		Transfer frequency[b] (per donor/per recipient)
	S. meliloti LPU88TN	*A. tumefaciens* UBAPF2	
15	2.9×10^6	1.4×10^6	$6.9 \times 10^{-7}/1.4 \times 10^{-6}$
36	1.4×10^6	8.8×10^4	ND[c]

[a] Roots with tightly associated rhizospheric soil were immersed in 1 ml of PBS and gently vortexed for 2 min at 2500 rpm. The rhizospheric extracts obtained at day 15 and day 36 post-inoculation were each separately plated in selective media to count donor, recipient and transconjugant bacteria. In addition to the data shown here, plasmid transfer in the rhizosphere was also observed in an independent replica experiment. Agar plate was supplemented with cyclohexamide (100 μg/ml) to reduce fungal growth during isolations from soil or nodules.
[b] Frequency was expressed as conjugant per donor/per recipient strain.
[c] ND: not detected.

frequency in the rhizosphere, in comparison with the values seen in the bulk soil [24,25]. Results presented here for the *S. meliloti* cryptic plasmid pSmeLPU88b and those reported by Kinkle and Schmidt for the symbiotic plasmid (pSym) pJB5I from a pea-symbiont *R. leguminosarum* [7], indicate that rhizobial plasmids with detectable mobilization frequencies in the laboratory are transferred in nonsterile rhizospheric soil at rates between 10^{-4}–10^{-6} events/recipient bacteria, irrespective of their symbiotic or nonsymbiotic character. In agreement with such transfer frequencies for the microcosms in the rhizospheric soil, in the open field we observed no strong impact of indigenous transconjugants on the root nodules the first year after plasmid release. These results provide a quantitative view of the conjugal transfer of a model *S. meliloti* cryptic plasmid in soil. This new information will assist in all those studies focussing on prospective estimations of plasmid dispersion from commercial inoculants into the soil-rhizobial community.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejsobi.2012.11.005.

References

[1] G. Catroux, A. Hartmann, C. Revellin, Trends in rhizobial inoculant production and use, Plant Soil 230 (2001) 21–30.
[2] B.D. Eardly, F.S. Wang, T.S. Whittam, R.K. Selander, Species limits in Rhizobium populations that nodulate the common bean (Phaseolus vulgaris), Appl. Environ. Microbiol. 61 (1995) 507–512.
[3] J.T. Sullivan, H.N. Patrick, W.L. Lowther, D. Scott, C.W. Ronson, Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 8985–8989.
[4] F.G. Barcellos, P. Menna, J.S. da Silva Batista, M. Hungria, Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium (Ensifer) fredii* and *Bradyrhizobium elkanii* in a Brazilian Savannah soil, Appl. Environ. Microbiol. 73 (2007) 2635–2643.
[5] K.G. Nandasena, G.W. O'Hara, R.P. Tiwari, E. Sezmis, J.G. Howieson, In situ lateral transfer of symbiosis islands results in rapid evolution of diverse competitive strains of mesorhizobia suboptimal in symbiotic nitrogen fixation on the pasture legume Biserrula pelecinus L, Environ. Microbiol. 9 (2007) 2496–2511.
[6] G.T. Tejerizo, M.F. Del Papa, W. Draghi, M. Lozano, L. Giusti Mde, C. Martini, M.E. Salas, I. Salto, D. Wibberg, R. Szczepanowski, S. Weidner, A. Schluter, A. Lagares, M. Pistorio, First genomic analysis of the broad-host-range *Rhizobium* sp. LPU83 strain, a member of the low-genetic diversity Oregon-like *Rhizobium* sp. group, J. Biotechnol. 155 (2011) 3–10.
[7] B.K. Kinkle, E.L. Schmidt, Transfer of the pea symbiotic plasmid pJB5JI in nonsterile soil, Appl. Environ. Microbiol. 57 (1991) 3264–3269.
[8] J. Mercado-Blanco, N. Toro, Plasmids in Rhizobia: the role of nonsymbiotic plasmids, Mol. Plant Microbe Interact. 9 (1996) 535–545.
[9] M. Pistorio, M.A. Giusti, M.F. Del Papa, W.O. Draghi, M.J. Lozano, G.T. Tejerizo, A. Lagares, Conjugal properties of the *Sinorhizobium meliloti* plasmid mobilome, FEMS Microbiol. Ecol. 65 (2008) 372–382.
[10] D. Pérez-Mendoza, E. Sepúlveda, V. Pando, S. Muñoz, J. Nogales, J. Olivares, M.J. Soto, J.A. Herrera-Cervera, D. Romero, S. Brom, J. Sanjuán, Identification of the *rctA* gene which is required for repression of conjugative transfer of rhizobial symbiotic megaplasmids, J. Bacteriol. 187 (2005) 7341–7350.
[11] M. Pistorio, M.F. Del Papa, L.J. Balague, A. Lagares, Identification of a transmissible plasmid from an Argentine *Sinorhizobium meliloti* strain which can be mobilised by conjugative helper functions of the European strain *S. meliloti* GR4, FEMS Microbiol. Lett. 225 (2003) 15–21.
[12] M.d.L. Giusti, M. Pistorio, M.J. Lozano, G.A. Torres Tejerizo, M.E. Salas, M.C. Martini, J.L. Lopez, W.O. Draghi, M.F. Del Papa, D. Perez-Mendoza, J. Sanjuan, A. Lagares, Genetic and functional characterization of a yet-unclassified rhizobial Dtr (DNA-transfer-and-replication) region from a ubiquitous plasmid conjugal system present in *Sinorhizobium meliloti*, in *Sinorhizobium medicae*, and in other nonrhizobial Gram-negative bacteria, Plasmid 67 (2012) 199–210.
[13] H. Schneidereit, F.R.J. Schmidt, The use of a Sesbania rostrata microcosm for studying gene transfer among microorganisms, in: J.C. Fry, M.J. Day (Eds.), Bacterial Genetics in Natural Environments, European Meeting on Bacterial Genetics and Ecology, University of Wales, College of Cardiff, 1990, pp. 182–187.
[14] M.F. Hynes, R. Simon, A. Puhler, The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pAtC58, Plasmid 13 (1985) 99–105.
[15] M.F. Del Papa, M. Pistorio, L.J. Balagué, W.O. Draghi, C. Wegener, A. Perticari, K. Niehaus, A. Lagares, A microcosm study on the influence of pH and the host-plant on the soil persistence of two alfalfa-nodulating rhizobia with different saprophytic and symbiotic characteristics, Biol. Fertil. Soils 39 (2003) 112–116.
[16] H.S. Lowendorf, A.M. Baya, M. Alexander, Survival of *Rhizobium* in Acid soils, Appl. Environ. Microbiol. 42 (1981) 951–957.
[17] N. Kroer, T. Barkay, S. Sørensen, D. Weber, Effect of root exudates and bacterial metabolic activity on conjugal gene transfer in the rhizosphere of a marsh plant, FEMS Microbiol. Ecol. 25 (1998) 375–384.
[18] M. Dröge, A. Pühler, W. Selbitschka, Horizontal gene transfer among bacteria in terrestrial and aquatic habitats as assessed by microcosm and field studies, Biol. Fertil. Soils 29 (1999) 221–245.
[19] A.K. Lilley, J.C. Fry, M.J. Day, M.J. Bailey, In situ transfer of an exogenously isolated plasmid between *Pseudomonas spp* in the sugar beet rhizosphere, Microbiology 140 (1994) 27–33.
[20] D.A. Pearce, M.J. Bazin, J.M. Lynch, Substrate concentration and plasmid transfer frequency between bacteria in a model rhizosphere, Microb. Ecol. 40 (2000) 57–63.
[21] S.J. Sorensen, M. Bailey, L.H. Hansen, N. Kroer, S. Wuertz, Studying plasmid horizontal transfer in situ: a critical review, Nat. Rev. Microbiol. 3 (2005) 700–710.
[22] R. Pukall, H. Tschäpe, K. Smalla, Monitoring the spread of broad host and narrow host range plasmids in soil microcosms, FEMS Microbiol. Ecol. 20 (1996) 53–66.
[23] M. Droge, A. Puhler, W. Selbitschka, Horizontal gene transfer as a biosafety issue: a natural phenomenon of public concern, J. Biotechnol. 64 (1998) 75–90.
[24] J.D. van Elsas, M. Nikkel, L.S.v. Overbeek, Detection of plasmid RP4 transfer in soil and rhizosphere, and the occurrence of homology to RP4 in soil bacteria, Curr. Microbiol. 19 (1989) 375–381.
[25] J.D. van Elsas, J.T. Trevors, M.E. Starodub, L.S.v. Overbeek, Transfer of plasmid RP4 between pseudomonads after introduction into soil; influence of spatial and temporal aspects of inoculation, FEMS Microbiol. Ecol. 73 (1990) 1–12.
[26] J.M. Vincent, A Manual for the Practical Study of the Root-nodule Bacteria, Blackwell Scientific Publications, Oxford and Edinburgh, 1970.
[27] J.E. Beringer, R. factor transfer in *Rhizobium leguminosarum*, J. Gen. Microb. 84 (1974) 188–198.
[28] T. Eckhardt, A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria, Plasmid 1 (1978) 584–588.