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RESEARCH PAPERS

A simple and stable method of tagging *Agrobacterium fabrum* C58 for environmental monitoring

Meriam BOURI[1,2], Haifa Ben GHARSA[1,2], Ludovic VIAL[3], Céline LAVIRE[3], Bernard R. GLICK[4] and Ali RHOUMA[1,5]

[1] Laboratory of Improvement and Protection of Olive genetic Resources - Olive tree Institute, Avenue H. Karray, 1002 Tunis Belvedere, Tunis, Tunisia
[2] Université Tunis El Manar (FST), Campus Universitaire, 2092, Tunis, Tunisia
[3] Université de Lyon, Université de Lyon 1, CNRS, INRA, UMR5557, UMR1418, Ecologie Microbienne, Villeurbanne, France
[4] Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, N2L3G1, Canada
[5] Institution of Agricultural Research and Higher Education, Tunis, Tunisia

Summary. *Agrobacterium fabrum* is one of the eleven *Agrobacterium* spp. complex species that has been observed to carry a Ti plasmid and induce crown gall, a disease causing significant damage to economically important perennial agricultural crops. Members of this species complex, including *A. fabrum*, are morphologically indistinguishable from one another on culture media and are known to grow together in soil and within host galls. Consequently, the tracking of this species in its natural environment requires a cautious approach to tagging strains without altering any of their ecologically important traits. A gentamicin resistant cassette (*aacC1*) was inserted, by homologous recombination, into a non-coding region of the *A. fabrum* C58 chromosome between the genes *atu1182* and *atu1183*. The resultant strain did not show any significant *in vitro* growth differences compared to the wild-type strain, and the marker was stable in rich medium, both with and without selective pressure. The mutant/marked strain was indistinguishable from the parental strain for ability to induce galls, grow in bulk soil and colonize the rhizosphere of tomato plants. Easy, precise, safe and stable tagging of the *A. fabrum* C58 genome facilitates environmental population surveys by either simple selection or direct detection by PCR. This methodology provides understanding of the ecology of this species complex as an integral part of managing the soil microbiota for improved crown gall management.

Key words: crown gall, gentamicin resistant cassette, chromosome, non-coding region, population survey.

Introduction

Crown gall is one of the most important bacterial diseases of stone fruit trees in nurseries of Mediterranean countries (Krimi *et al*., 2002). The disease is caused by pathogenic agrobacteria carrying a Ti plasmid that is responsible for tumour formation (Watson *et al*., 1975; Wood *et al*., 2001; Goodner *et al*., 2001). Significant practical and fundamental knowledge on the biology of crown gall pathogens has been gathered. Tumorigenic strains transfer a portion of their Ti plasmids (T-DNA) into host plant cells, that is incorporated into the host genomes (Chilton *et al*., 1977; Kerr *et al*., 1977). The DNA transmission capacities of *Agrobacterium* spp. have been widely studied as a means of inserting foreign genes into plants for beneficial uses (Gelvin, 2009). However, study of establishment of agrobacteria inoculated into natural soils, their rhizosphere colonization, gall occupancy, and exchange of genetic traits with other soil bacteria is not as well understood, and requires the use of appropriately tagged strains for their recognition in complex soil environments.

Antibiotic resistance is often used for studies on survival kinetics of introduced bacteria by plate counting. This method, although time consuming

Corresponding author: M. Bouri
E-mail: mariem_bouri@hotmail.fr

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and posing risks of antibiotic resistance transfer in the microbiota, when the resistance is coded by mobile genetic elements (Kloepper and Beauchamp 1992; Janson *et al*., 2000), is sensitive, cost-effective, reliable and easy to perform (Van Elsas *et al*., 1986; Glandorf *et al*., 1992; Prosser, 1994; Mirleau *et al*., 2001). Most studies of bacterial survival kinetics have been based on the use of spontaneously occurring antibiotic-resistant mutants (Smalla *et al*., 1993), or plasmids or transposons encoding antibiotic resistance.

Data that depends upon plasmids or transposons that encode antibiotic resistance can be confounded by the frequent transfer of plasmids and transposons within bacterial populations (Bentjen *et al*., 1989; Zeph and Stotzky, 1989; De Lorenzo, 1994; Prosser, 1994; Van Overbeek *et al*., 1997). For the *A. tumefaciens* species complex, researchers have reported T-DNA genetic manipulation for engineering plant transformation (Hao *et al*., 2010; Gelvin, 2003), deletion mutant generation to study the bacterial pathogenicity and physiological pathways *in vitro* (Nair *et al*., 2003; Suksomtip and Tungpradabkul, 2005; Lassalle *et al*., 2011; Campillo *et al*., 2014) as well as the use of plasmid-borne gene tagged mutants (Farrand *et al*., 1989; Raio *et al*., 1997). *Agrobacterium fabrum* strain C58 (Lassalle *et al*., 2011) (previously called *A. tumefaciens* genomic species G8) has served as a model for the majority of these studies, because its complete genome sequence was available (Goodner *et al*., 2000). Nevertheless, no previously described techniques have reliably tagged this strain for use in complex biological environments including for epidemic and ecological studies. This is because the markers were not stable or/and some bacterial ecologically important traits were affected. We have constructed and characterized an *A. fabrum* C58 mutant strain that carries a single stable chromosomal copy of a gentamicin cassette that is neutral for several ecologically important traits. This strain can be readily detected by PCR and simple selective culture methods in biologically complex environments.

Material and methods

Bacterial strains, plasmids and growth conditions

Agobacterum fabrum strains were cultivated in liquid medium with 190 rpm of shaking or solid media at 28°C. Yeast extract-peptone-glycerol (YPG) (Campillo *et al*., 2012) and minimal salts medium AT (Petit *et al*., 1978) were used during Bioscreen analyses (Campillo *et al*., 2014), for growth kinetics study of the mutant in comparison with the wild-type. Mannitol-glutamic acid MG (Ophel and Kerr, 1990), YPG and Luria Bertani (LB) (Miller, 1972) agar media with 30 μg mL^{-1} gentamicin (Gm) were used for phenotypic evaluation of Gm resistance, and evaluation of strain stability and colony morphology. LB medium with ampicillin (Amp; 100 μg mL^{-1}) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (60 μg mL^{-1}) additions were used for *E.coli* carrying the modified vector pGMTeasy (Promega). LB medium with 30 μg $mL^{-1}$of Gm was used for *E. coli* carrying the modified vector pJQ200-SK (Quandt and Hynes, 1993). YPG medium with 5% of sucrose was used to identify mutants with double crossover events. The gentamicin cassette (aacC1) was obtained from the pMGm plasmid (Murillo *et al*., 1994).

PCR assays

Standard PCRs were used for amplification of different DNA fragments for insert construction as well as for the detection of successful recombinant formation. Standard PCR mixtures consist of 1 × Taq polymerase buffer, 2 μM of each dNTP, 1.5 mM $MgCl_2$, 10 μM primers, 2.5 U mL^{-1} Taq polymerase (Invitrogen) and 2 μL of fresh bacterial cell suspension. The cycling steps were: an initial denaturation step at 94°C for 3 min followed by 30 cycles each of 94°C for 30 s, annealing temperature of 55°C for 30 s, 72°C for 30 s and a final extension step at 72°C for 3 min. PCRs were conducted in a Biometra thermocycler Whatman (Biometra,).

A specific PCR mixture consists of a standard PCR mixture but without primers; it is used to link different fragments of the insert before they are amplified. The mixture was subjected to an initial denaturation step at 94°C for 3 min followed by four cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The mixture is maintained at 72°C for 5 min while 3′ and 5′ end primers are added. The PCR is then continued with 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The insert was added before a final extension step at 72°C for 3 min.

Construction of the gentamicin-resistant strain of Agrobacterium fabrum C58

The complete genome sequence of *A. fabrum* C58 was checked on AgrobacterScope (https://www.

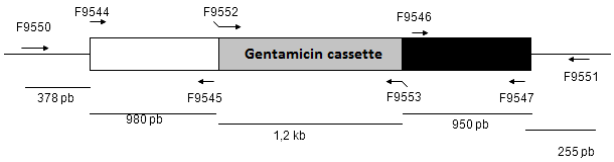

Figure 1. Descriptive schema of primer positions used for the generation of a gentamicin insert, with upstream (white fragment) and downstream (black fragment) regions of mutant strain indicated.

genoscope.cns.fr/agc/microscope) with the MaGe web interface (Vallenet *et al*., 2009), to choose a neutral chromosomal region where the Gm resistance cassette could be safely inserted. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) to generate the cassette and two fragments for the upstream and downstream portions of the 3′ and the 5′ ends of the required region (Figure 1). These fragments allow the integration of the cassette into the *A. fabrum* C58 genome. DNA extraction from *A. fabrum* C58 was performed according to the NucleoSpin Tissue kit protocol (Macherey-Nagel), from a 24 h culture of the bacterium. A standard PCR performed on the genomic DNA (50 ng) was used to amplify the 1 kb region of interest. The primers used are detailed in Table 1. After fragment amplification and purification with NucleoSpin ExtractII (Macherey-Nagel), another PCR was used to link these fragments because of their complementary sequences in a 3 kb insert. PCR fragments were then cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. The amplified fragment was digested with the restriction enzymes ApaI and SpeI (FastDigest, Fermentas). After digestion, fragments were ligated with a 3:1 ratio of insert:vector, in a mixture containing 1 µL of T4 DNA ligase (Promega) and 5 µL of ligation buffer (2×), in a 10 µL final total volume. The mixture was incubated for 1 h at room temperature then overnight at 4°C, then transformed into *E. coli* JM109 competent cells. The resulting plasmid was subcloned onto pJQ200-SK, a plasmid carrying the sac*B* gene conferring sucrose sensitivity (Quandt and Hynes, 1993), digested with the same enzymes, and ligated (as above) before transformation into *E. coli*.

The transformation of *E. coli* JM109 competent cells (Promega) was performed with thermal shock. Fifty µL of competent *E. coli* JM109 cells and 3 µL of the ligation mixture were incubated consecutively at 42°C for 40 s then on ice for 2 min. An aseptic addition of 900 µL of S.O.C medium (Invitrogen) proceeded before incubation at 37 °C for 1 h without agitation. The mixture was then plated on either LB medium with Amp and X-Gal additions for the *E.coli* carrying a modified vector pGMTeasy, or LB medium with a Gm addition (30 µg mL^{-1}) for the *E. coli* carrying a modified pJQ200-SK vector.

The generation of a gentamicin resistant mutant of *A. fabrum* C58 (*A. fabrum* C58Gm^r) was achieved with electroporation of the strain *A. fabrum* C58 and 2 µL of the pJQ200-SK ligation mixture. A Bio-Rad electroporator at 200 Ω and 2.4 kV was used. Cells were incubated in YPG for 2 h at 28°C under agitation. Single recombinants were selected on YPG agar

Table 1. Primers used for sequence analysis of the *Agrobacterium fabrum* mutant.

Primer	Sequence
F9550	3′GGCATCCGTCACCTTCTTTA5′
F9551	3′GATTGACCAAGCCGTGTTCT5′
F9552	3′GGCGCGGTAATGCGGACGTGGCGAAATCGGTAGACCAGACGGCCACAGTAACCAACAAAT5′
F9553	3′CTCAGTAGGACGTCAAATTCCCGCATTTACGCCACAAGTCCTGCGAACGCAGCGGTGGTAAC5′
F9544	3′TCCGCTGAAGGTTTATCCAC5′
F9545	3′GTCTGCTGCGTCTACCGATT5′
F9546	3′GCAGGACTTGTGGCGTAAAT5′
F9547	3′TCCAACGTTTCCTTGGTAGC5′

containing 20 μg mL^{-1} of gentamicin after 48 h at 28°C. Plasmid pJQ200-SK is a suicide vector that contains a *sacB* gene (from *Bacillus subtilis*) that is toxic for the clone in the presence of sucrose (Ried and Collmer, 1987). Double recombination was selected by streaking clones on YPG agar supplemented with 5% sucrose. Only clones that had succeeded in excising the gene by double recombination were able to grow. One hundred μL of the incubated bacterial suspension were re-streaked onto YPG agar and incubated for 48 h at 28°C. The presence of the insert with double crossover event was confirmed by PCR with the appropriate primers.

Marker stability and strain growth kinetics

Stability of the marker

Agrobacterium fabrum C58 Gm^r was grown overnight in YPG + Gm (30 μg mL^{-1}) medium and then diluted the next day into YPG medium without the antibiotic. The culture was allowed to grow to saturation and then diluted. This was done five consecutive times over the course of the experiment. The ratio of Gm resistant bacterial colony-forming units (cfu) was assessed by plating on YPG and YPG + Gm (30 μg mL^{-1}) after 24 h at 28°C.

Growth kinetics

The mutant and parental strains were grown in YPG broth for 20 h at 28°C, and their growth kinetics were evaluated using a Bioscreen microbiology reader (Bioscreen C Labsystems). A 20 μL aliquot of cell suspension was inoculated into each well containing 200 μL of fresh medium. All cell suspensions were adjusted to an OD_{600} of 0.1 before the inoculation. Cultures were incubated in the dark for 5 d at 28°C with shaking at medium amplitude. The Bioscreen reader was set to automatically read the optical density at OD_{600nm} every 5 min. Each sample was assayed in triplicate, and wells without bacterial inoculant were used as blank controls.

Inoculation essays

Autoclaved soil (50% peat and 50% sand) was equally distributed in aseptic tubes (BD Falcon™ 50 mL capacity). The field capacity of the soil was determined and the required water volume was used to prepare the bacterial suspension for soil inoculation. The soil was then inoculated with 10^6 cfu of C58 or C58 Gm^r per gram of soil. The *Agrobacterium* strains were obtained from exponential-phase YPG cultures. *Solanum lycopersicum* cv. Rio Grande seeds (Petoseed) were surface-sterilized with 12% sodium hypochlorite solution for 5 min followed by 70% alcohol for 7 min, and then washed five times with distilled water. After seed germination in water agar (1%, w/v), 4-day-old seedlings were co-inoculated with 10^8 cfu mL^{-1} of bacterial suspension according to the method of Pistorio *et al*. (2002). The plants were cultivated in the tubes containing soil at field capacity, in a growth chamber set at 22°C and a 16 h photoperiod. The soil was inoculated at field capacity because greater root colonization occurs at soil moisture levels near field capacity than at drier levels (Howie *et al*., 1987). The bacterial populations were estimated by plate counts done every week for one month. Plants were removed from tubes and gently shaken to remove soil particles. Roots were cut, weighed and then soaked in water for 10 min with 300 rpm of agitation. AT minimal medium was used for bacterial isolation whereas YPG + Gm (30 μg mL^{-1}) was used to detect mutant colonies. Ability of the bacterial strains to cause galls on host tissues was assessed to cause galls on host tissues on carrot taproot discs (*Daucus carota* subsp. *sativus*) and stems of one-month-old tomato plants (*Solanum lycopersicum* cv. Rio Grande) 3-4 weeks after inoculation with 10^8 cfu mL^{-1} of bacterial suspensions, or distilled water as negative control.

All assays were each performed three times.

Data analyses

Data were subjected to analysis of variance (ANOVA) using SPSS software (version 20). Significance of mean differences was determined using the Duncan's test, and responses were judged significant at the 5% level (P=0.05).

Results

Generation of the gentamicin resistant mutant

The cassette was inserted into a neutral chromosomal region between the *atu1182* (coding for a hypothetical protein) and *atu1183* (coding for putative phage polymerase) genes, by homologous recombination. First, upstream (980 bp) and downstream (950 bp) regions were amplified with, respectively, F9544-F9545 and F9546-F9547 primers, and linked

to the Gm cassette using complementary sequences of the primers F9552 and F9553 (respectively, 40 bp and 44 bp). The homologous recombination target sequences of *A. fabrum* C58 were then cloned in *E. coli* JM109 using the pGEM-T Easy vector. This was then sub-cloned in the pJQ200-SK vector before being inserted in *A. fabrum* C58 by electroporation. The presence of the Gm cassette in clones was verified by PCR with outside and inside primers (Figure 2). Colonies of the generated mutant had the typical circular glistening morphologies of agrobacteria on nutrient agar media (Figure 3).

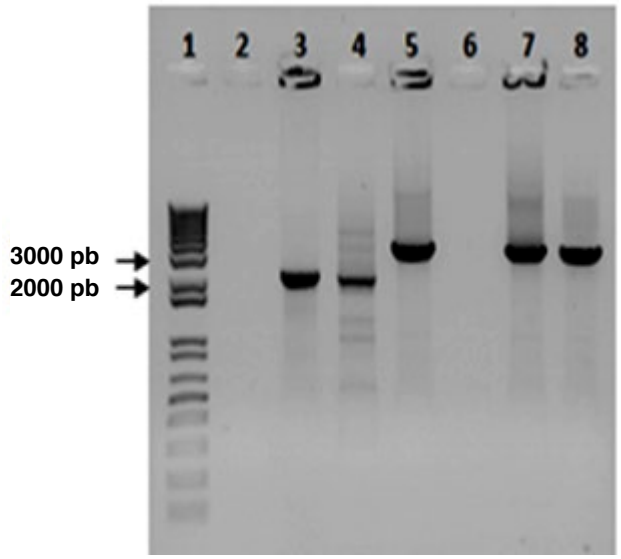

Figure 2. PCR analysis for the verification of the insert in *Agrobacterium fabrum* C58Gm^r. Lanes 1 and 2 correspond,respectively, to the 1 kb+ marker and negative control (DSW). The DNA band in lane 3 corresponds to PCR product when using primers F9550-F9551 on DNAg of *A. fabrum*. DNA bands in lanes 5, 7 and 8 correspond, respectively, to successful insertion in *A. fabrum* recovered from YPG agar after the double recombination when using primers F9550-F9551 and F9550-F9547 and F9544-F9551. Lanes 4 and 6 correspond, respectively, to a failed insertion in *A. fabrum* C58 recovered from YPG agar when using primers F9550-F9551 and F9550-F9547.

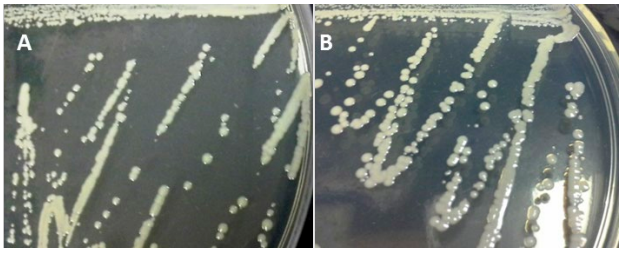

Figure 3. Identical colony morphologies of the parental stain *Agrobacterium fabrum* C58 (A) and the mutant (B) after 48 h of growth in nutrient agar medium.

Stability of the gentamicin resistant phenotype and growth fitness *in vitro*

Stability

The stability of the marker was analyzed after extensive cultivation of the mutant C58Gm^r in YPG medium without antibiotic addition. As shown in Figure 4, the ratio of the Gm^r cfu and total cfu was maintained at 1 during the experimental period.

Growth fitness

In vitro assays: A comparison of growth curves of the mutant and the parental strains in YPG and AT minimal medium showed similarity in growth rates (data not shown for AT minimal). Variances analyses of DO values during 20 h were not statistically significant (P<0.05) (Figure 5A).

In vivo assays: Statistical analysis of the bacterial colony-forming units formed showed no differences (P<0.05) between *A. fabrum* C58 and its mutant, under controlled conditions, in bulk soil (Figure 5B).

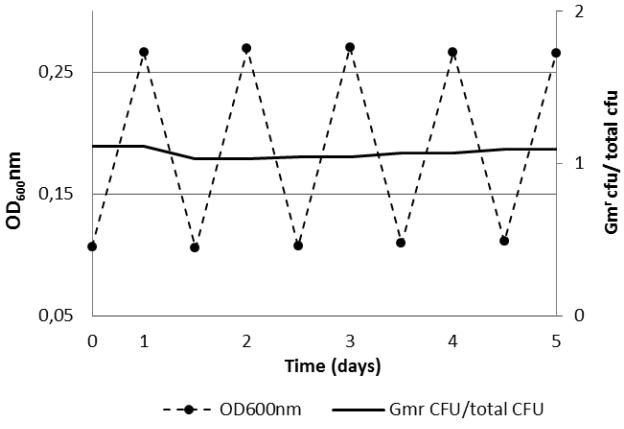

Figure 4. Optical density measurements indication stability of the Gm^r marker in the mutant strain, in the absence of selection, after several generations during 5 d of serial batch cultures.

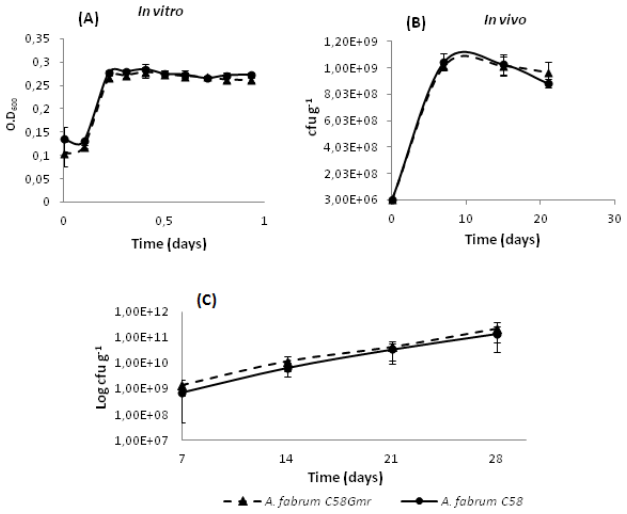

Figure 5. Growth curves (mean optical density measures) of *A. fabrum* C58 and its mutant: (A) *in vitro* (YPG medium) during 20 h; (B) *in vivo* (soil) during 21 d; and (C) in the rhizospheres of co-inoculated roots of seedling tomato plants.

Competitiveness

The competitiveness of the mutant for the colonization of tomato rhizospheres compared to the parental strain *A. fabrum* C58 was analyzed in co-inoculated soil (50:50 inoculation ratio of strains C58 andC58Gm^r. Plating was used to quantify rhizosphere colonization by the introduced bacteria. Statistical analysis of the bacterial cfu that formed for both strains showed no differences between *A. fabrum* C58 and its mutant (P<0.05) (Figure 5C).

Capacity to induce galls

The ability of the mutant to induce galls was successfully proven on carrot discs and stems of tomato plants. Compared to the parental strain *A. fabrum* C58, gall formation and tumours generated by the mutant were morphologically identical (Figure 6). The two strains also developed galls on 100% of inoculated sites, and the formation of tumours was recorded in the same period after inoculation for both strains (7 d on carrot discs and 12 d on stems of tomato).

Discussion

Monitoring of *Agrobacterium* spp., and especially *A. tumefaciens* complex species in soil, is currently

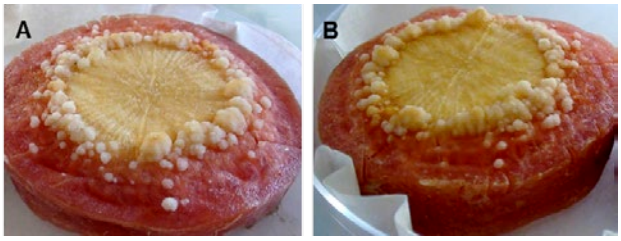

Figure 6. Carrot discs showing morphologically indistinguishable galls induced by *Agrobacterium fabrum* C58 (A) and its mutant (B).

difficult because the colonies are not always distinguishable from those of other bacteria, and highly selective media for detection of different *Agrobacterium* members are not yet available (Raio *et al*., 1997). In addition, the confirmation of strain identity is laborious with molecular techniques such as PCR. To bypass these difficulties, *A. fabrum* C58 was genetically tagged by the insertion of a Gm cassette into its chromosome, thereby allowing for selection for gentamicin resistance on isolation media.

The marker location on the chromosome, rather than on a plasmid or transposon, is expected to improve its stability and reduce its transfer in the environment (Van Elsas and Trevors, 1993). The choice of resistance gene markers depends on the environmental use of the marked bacterium and is influenced by the background resistance of the indigenous bacteria in a given habitat. For example, kanamycin resistance is widespread in soil bacteria due to the use of antibiotics in agricultural systems and medicine (Egan and Wellington, 2000). Kanamycin has been especially used for medical purposes, animal husbandry, aquaculture and in genetically modified plants (Conner, 1997). Wilson (1994) also reported that the background population of naturally kanamycin-resistant bacteria in a Dutch soil was 2×10^4 cfu g^{-1}. The level of indigenous resistant bacteria has been reported to range from 10^3 to 10^4 cfu g^{-1} of dry soil for tetracycline and ampicillin resistance; and from 17 to 10^8 cfu g^{-1} for tobramycin resistance (Henschke and Schmidt, 1993). However, little is known about the importance of soil as a source of gentamicin resistant bacteria. Heuer *et al.* (2002) could detect gentamicin resistance genes in different non-clinical environments. Walsh and Duffy (2013) reported that more than 56% of 412 antibiotic resistant bacteria, isolated from ten agricultural, urban, and pristine soils of Switzerland,

showed gentamicin resistance, while 83% were resistant to kanamicin and 90% to streptomycin. The Gentamicin-resistant bacteria were identified as Bacillales, Pseudomonadales and Aeromonadales. To the best of our knowledge, gentamicin resistance has not been found in native agrobacteria, which makes it a suitable marker for this bacterium.

Most of *Agrobacterium* chromosome manipulations have been made for plant genetic engineering purposes. Thus, it is important that the chromosomal insertion site of the genetic element must not affect bacterial virulence and growth rate only, but other bacterial functions have been ignored. For instance, an *A. tumefaciens recA* mutant was used for plant genetic engineering purposes. Although the mutation was stable and had no effect on other genetic properties (including transformability and proficiency as a conjugal donor or recipient), the mutant was unable to carry out homologous recombination functions (Farrand *et al*., 1989). Rong *et al.* (1991) identified and characterized a plant-inducible locus (*picA*) on the *Agrobacterium* chromosome that is not required either for bacterial growth or for T-DNA transfer. This locus was later retained by Lee *et al*. (2001) for gene integration into the *A. fabrum* chromosome for plant engineering purposes.

In the present study, the insertion of a marker into the chromosome has been performed by homologous recombination in a neutral region between the *atu1182* and *atu1183* genes. This region is specific for *A. fabrum* which makes it very adequate for safe insertion. In the case of homologous recombination, it is necessary to clone the target sequences of the strain to be marked, before the marker cassette can be inserted into the gene. This requires knowledge of the genetics of the strain. Since, prior to this study, the *A. fabrum* C58 genome had been completely sequenced (Goodner *et al*., 2000), it was easy to choose a non-coding region where the marker could be inserted and where it was relatively straight forward to design primers for homologous recombination. Given that the insert can lack suitable cloning sites, the construct had to be cloned into a suicide vector (Waalwijk *et al*., 1991). Thus, the insert was first cloned in to pGEM-T Easy then sub-cloned into the suicide vector pJQ200-SK which can be introduced into *A. fabrum* C58, but not replicated. Thanks to the homology of the flanking sequences between the chromosomal DNA of the strain which is to be marked, exchange of the Gm cassette with the chromosomal DNA, and double recombination, will occur with an acceptable frequency at the defined region.

Antibiotic resistance may affect several ecologically important traits (Orvos *et al*., 1990; Prosser, 1994; Van Overbeek *et al*., 1997; Mirleau *et al*., 2000). The instability of the marker and changes in competitive ability or effects on the metabolism of very specific compounds may not be detectable *in vitro* studies. Therefore, there is no standard protocol to assess the ecological functioning of a strain, since this depends on the characteristics of the strain and the purpose of the introduction (Smit *et al*., 1996). Thus, we examined the survival of the mutant *in vitro* and *in vivo* in addition to his competitiveness for root colonization and its ability to induce galls. Comparison of growth fitness of *A. fabrum* Gm^r in soil and its competitiveness in the rhizosphere with the parental strain was performed using plate counts with a level of detection between 10-100 cfu g^{-1}. According to Colwell *et al*. (1985), some bacteria do not grow on nutritionally rich media after a period of starvation. Therefore, isolation of bacteria from soil was first performed in AT minimal medium to minimize the soil disadvantages. Colonies were then streaked one by one onto YPG medium + Gm for mutant detection. The results showed that the insert was stable and did not affect the fitness of the mutant. The neutral region used here for the insertion of the Gm cassette could be utilized for different mutations and marker systems, since it had no apparent side effects for the fitness and characteristics of the mutant. Furthermore, the mutant that was constructed shows a stable marker, without affecting important ecological traits, which is different to other published mutants. The new mutant therefore is ideal for studies of agrobacteria populations in various natural environments.

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