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Conjugative transfer of a derivative of the IncP-1α plasmid RP4 and establishment of transconjugants in the indigenous bacterial community of poplar plants

Andreas Ulrich[1,*], Regina Becker[1], Kristina Ulrich[2] and Dietrich Ewald[2]

[1]Leibniz Centre for Agricultural Landscape Research (ZALF), Institute for Landscape Biogeochemistry, D-15374 Müncheberg, Germany and [2]Johann Heinrich von Thünen-Institute, Federal Research Institute for Rural Areas, Forestry and Fisheries, Institute of Forest Genetics, Waldsieversdorf D-15377, Germany

*Corresponding author: Leibniz Centre for Agricultural Landscape Research (ZALF), Institute for Landscape Biogeochemistry, Eberswalder Str. 84, D-15374 Müncheberg, Germany. Tel: +49-33432-82-345; Fax: +49-33432-82-344; E-mail: aulrich@zalf.de

One sentence summary: Conjugative transfer of an IncP-1α plasmid and its establishment in the indigenous bacterial community of poplar plants depend on the phenological stage.
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ABSTRACT

The persistence of traits introduced into the indigenous bacterial community of poplar plants was investigated using bioluminescence mediated by the *luc* gene. Three endophytic bacterial strains provided with the IncP-1α plasmid RP4-Tn-*luc* were used to inoculate poplar cuttings at different phenological stages. Screening of isolates by bioluminescence and real-time PCR detection of the *luc* gene revealed stable persistence for at least 10 weeks. Although the inoculated strains became established with a high population density after inoculation at leaf development (April) and senescence (October), the strains were suppressed by the indigenous bacteria at stem elongation (June). Transconjugants could be detected only at this phenological stage. Indigenous bacteria harbouring RP4-Tn-*luc* became established with densities ranging from 2×10^5 to 9×10^6 CFU g^{-1} fresh weight 3 and 10 weeks after inoculation. The increased colonization of the cuttings by indigenous bacteria at stem elongation seemed to strongly compete with the introduced strains. Otherwise, the phenological stage of the plants as well as the density of the indigenous recipients could serve as the driver for a more frequent conjugative plasmid transfer. A phylogenetic assignment of transconjugants indicated the transfer of RP4-Tn-*luc* into six genera of *Proteobacteria,* mainly *Sphingomonas, Stenotrophomonas* and *Xanthomonas*.

Keywords: horizontal gene transfer; plant-associated bacteria; bacterial community composition; RP4; IncP-1; *Populus*

INTRODUCTION

Plant-associated bacteria have numerous mechanisms by which they can promote plant growth and health. The synthesis of phytohormones and siderophores, or the ability to degrade pollutants, indicate that the employment of endophytic bacteria in techniques for improving plant production and in phytoremediation of contaminated soil is promising (Glick 2012; Sessitsch *et al.* 2013). For example, several studies have shown that the performance of trees and crops grown on polluted soils can be essentially improved by inoculation with bacteria that mediate the degradation of xenobiotic compounds or heavy metal resistance (Weyens *et al.* 2011; Hechmi *et al.* 2012). However, if these applications are to become practical, the long-term establishment of the introduced traits in the plant-associated microflora is necessary. Accessory bacterial metabolic functions, such as xenobiotic resistance, are known to be plasmid encoded (Schlüter

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Table 1. Inoculated strains used in this study.

Strain	Origin			16S rRNA gene accession number
	Poplar clone	Plant material	Location	
Pseudomonas sp. E9	Geneva ♀ (*Populus maximowiczii* × *P. berolinensis*)	Branch section	Tree nursery Waldsieversdorf, Germany 52.54 N, 14.06 E	HE652089
Pseudomonas sp. Q16				HE652090
Stenotrophomonas sp. 169-1	741 ♀ (*P. alba* × [*P. davidiana* + *P. simonii*] × *P. tomentosa*)			HE652088

et al. 2007). Accordingly, bacterial functions essential for success can be lost if the plasmid is lost. On the other hand, the spread of plasmids by conjugal transfer to and between the indigenous bacteria can contribute considerably to the maintenance and enhancement of the desired traits (Taghavi *et al.* 2005; Weyens *et al.* 2009; Wang *et al.* 2010). In particular, the broad-host-range plasmids belonging to the IncP-1 group are known to promote gene transfer because they are stably replicated in almost all *Proteobacteria* and effectively transferred by conjugation in a wide range of bacterial taxa (Adamczyk and Jagura-Burdzy 2003; Popowska and Krawczyk-Balska 2013; Klümper *et al.* 2015). Horizontal gene transfer has been documented in diverse natural environments, such as bulk soil (Filonov *et al.* 2010; Heuer and Smalla 2012); aquatic habitats (Dahlberg, Bergstrom and Hermansson 1998); animal systems (Hoffmann *et al.* 1998); the rhizo-, phyllo- and endospheres of plants (Normander *et al.* 1998; van Elsas *et al.* 1998; van Elsas, Turner and Bailey 2003; Wang *et al.* 2010); and biogas plant digestates (Wolters *et al.* 2015).

In general, transfer events require an adequate bacterial density and metabolic activity. These conditions are specifically present in microbial biofilms that occur in many habitats. Nevertheless, even under favourable conditions, transfer events are not easy to detect because they depend on environmental factors, the plasmid type and the host strain (Sørensen *et al.* 2005; Król *et al.* 2011). Although much fundamental research has been conducted, studies on plasmid transfer in the phytosphere are, up to now, rare and the establishment of transconjugants is not fully understood.

In this study, we analysed the persistence of endophytic bacterial strains harbouring a recombinant IncP plasmid that was introduced back into poplar cuttings. Moreover, the conjugative transfer and the subsequent establishment of the plasmid in the indigenous bacterial community were studied. For this purpose, cuttings obtained from field-grown poplars were inoculated with endophytic isolates carrying an RP4 derivative. The survival of the introduced bacteria and the presence of the plasmid within the bacterial community were assessed at three phenological stages of the plants to gain insight into the factors influencing the persistence and transfer of genes in the poplar phytosphere.

MATERIALS AND METHODS

Bacterial cultivation

Escherichia coli strains were cultivated on Luria–Bertani medium (Sigma-Aldrich Co., St Louis, USA). *Rhizobium leguminosarum* and bacterial strains isolated from poplar cuttings were grown on R2A or TSA (Sigma-Aldrich). Media were supplemented with the following antibiotics (μg ml^{-1}): tetracycline 15, kanamycin 50, rifampicin 30, streptomycin 100 and cycloheximide 100 to inhibit growth of eukaryotic microorganisms.

Construction of the recombinant IncP plasmid RP4-Tn-*luc* and preparation of the bacterial strains used for inoculation

The plasmid RP4-4 (IncP-1α, Tc^r, Ap^r, Km^s; Hedges and Jacob 1974) was introduced into *R. leguminosarum* G122Sm Tn*163*-*luc* (Ulrich and Pühler 1994) using *E. coli* DSM 3880 RP4-4 as the donor strain. Transconjugants were selected on TSA supplemented with tetracycline and streptomycin. A mixture of transconjugants obtained directly from the selective agar plates was used as the donor for a second mating with the rifampicin resistant strain *E. coli* DH5α-2 (Ulrich and Pühler 1994). Transconjugants carrying RP4-4 with the transposon Tn*163*-*luc* inserted were selected with kanamycin and rifampicin. The resulting plasmid RP4-Tn-*luc* conferred bioluminescence and kanamycin resistance via a 3.2 kb *npt*II-*luc* cassette within Tn*163* (Ulrich and Lentzsch 1997). The strains were assessed for the bioluminescence phenotype and multiple antibiotic resistances. The insertion of the transposon did not affect the maintenance or transfer functions of the plasmid.

For the screening of endophytic isolates, four branch sections of 2 cm (Ø 5 mm) were sampled from the poplar clones Geneva and 741 (Table 1). Surface disinfection of the samples and screening of isolates were performed as described by Ulrich, Ulrich and Ewald (2008). Several isolates were subcultured on R2A and taxonomically classified by PCR and sequencing of the partial 16S rRNA gene (Ulrich, Klimke and Wirth 2008). Two *Pseudomonas* isolates (Q16 and E9) obtained from the poplar clone Geneva and a *Stenotrophomonas* strain (169-1) from the clone 741 were chosen as the strains for inoculation (Table 1). RP4-Tn-*luc* was transferred from DH5α-2 pBR322-*sacB* RP4-Tn-*luc* using filter matings. The *E. coli* donor possesses a pBR322 derivative with a 3.8 kb *sacB*-Km^r cassette (originated from pSUP104-sac; Simon *et al.* 1991), which does not allow growth on medium containing sucrose. Transconjugants were selected on TSA supplemented with kanamycin and 5% sucrose. The presence of *sacB* in the *E. coli* donor allowed selection against the donor without the presence of a selectable resistance marker or characteristic in the recipient strain.

Inoculation of poplar cuttings, plant growth, sampling

The bacterial strains used as inocula were grown in R2A broth to late exponential phase, concentrated by centrifugation (6,000 *g*)

for 2 min and resuspended in 5 ml Schenk–Hildebrand medium (Schenk and Hildebrandt 1972) to a density of approximately 10^8 CFU ml^{-1}. Cuttings (Ø 5 mm, length 15 cm) were taken from the field grown poplar clone Geneva at the phenological stages of leaf development (April), stem elongation (June) and senescence (October), and inoculated with 200 μl bacterial suspension by infiltration. In total, 10 cuttings per strain and stage were inoculated; three cuttings were used to determine the abundance of the indigenous bacteria before inoculation. The inoculated cuttings were placed immediately into a mini-greenhouse in sand saturated with water and cultivated under a light regime with 16 h of white light (photon flux of 30 μmol m^{-2} s^{-1}) at 23°C for 10 weeks. Three cuttings per inoculated strain were harvested at 3 and 10 weeks after inoculation.

Screening of isolates for inoculated strains and putative transconjugants

Branch sections of approximately 2 cm were sampled, washed three times with sterile tap water and ground in 4 ml of 0.3% NaCl with a mortar and pestle. Tissue extracts were serially diluted, plated on R2A supplemented with kanamycin and incubated for 3–6 days at 26°C. For each inoculation and sampling date, 300 representative isolates were screened for bioluminescence after transferring the colonies onto nylon membranes (Hybond-N). The membranes were merged with a few drops of luciferin (1 mg ml^{-1} in 100 mM sodium citrate pH 5.6) and exposed to an X-ray film (Kodak T-Mat G/RA film) for 2 h at 26°C. Bioluminescent colonies appeared as dark spots on the X-ray film.

Identification of isolated bacteria

To distinguish the inoculated strains from the transconjugants, bioluminescent isolates were grouped based on phenotypic characteristics and restriction analysis of the 16S rRNA gene using *MspI* (Ulrich, Klimke and Wirth 2008). Five to eight representative isolates from each group were identified by sequencing the partial 16S rRNA gene (Ulrich, Klimke and Wirth 2008). The assignment of the isolates at the genus level was based on NCBI-BLAST searches and on the taxonomic assignment by the CLASSIFIER program of the RDP. A phylogenetic tree was generated by the neighbor-joining algorithm using the original dataset as well as 1000 bootstrap datasets to evaluate its topology (PHYLIP vers. 3.6; Felsenstein (1993)).

Quantitative detection of RP4-Tn-*luc* in the indigenous community of poplar plants

For the isolation of total DNA from poplar, branch sections were washed three times with sterile tap water and cut into 1–2 mm pieces (200 mg in total). The plant material was mixed with 1 g silica beads (Ø 1 mm) and ground using a bead beater (FastPrep 24 Instrument, MP Biomedical) for 60 s at 6 m s^{-1}. The DNA was isolated using a modified CTAB-Protokoll (Dumolin, Demesure and Petit 1995). The presence of the plasmid was determined in community DNA by real-time PCR with the primers (5′-GGGCGCACCTCTTTCGA-3′; 5′-CCTTGTCGTATCCCTGGAAGAT-3′) and the probe (5′-TTGCAACCGCTTCCCCGACTTC-3′) specific for the *luc* gene (position 945–1016, AB261984). The probe was labelled at the 5′ end with the reporter dye FAM (6-carboxy-fluorescein) and at the 3′ end with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine). PCR was conducted in 20 μl reactions with each primer at 0.9 μM, 0.25 μM probe and TaqMan Universal PCR Master Mix (Life Technologies, Germany) in a 7500 Fast real-time PCR system (Life Technologies) using a standard protocol. The standard curve was generated using serial dilutions of genomic DNA from *Stenotrophomonas* 169-1 RP4-Tn-*luc* containing between 10^6 and 2 copies of the *luc* gene. The PCR efficiency was approximately 94%. All samples were run in duplicate. The inhibitory effects of the extracted DNA on PCR performance was tested by the quantification of serial dilutions.

Statistics

Differences between the experimental variants were tested by a three-factorial ANOVA using a block design with phenological stage as block and inoculated strain and sampling date as factors. Subsequently, differences within the stage were proven with a two-factorial ANOVA and the post-hoc test Tukey (R software, R-3.1.3, package multcomp; R Core Team 2015).

RESULTS

Establishment of inoculated endophytic bacteria and RP4-4-Tn-*luc* transconjugants

Cuttings of the poplar clone Geneva obtained at three growth stages were inoculated with endophytic isolates harbouring the recombinant IncP plasmid RP4-Tn-*luc*. Two of the strains (*Pseudomons* E9 and Q16) were previously isolated from the clone Geneva but the other strain (*Stenotrophomonas* 169-1) was obtained from the distinctly related poplar clone 741.

As a result of inoculation at leaf development in April and senescence in October, all three strains became established within the poplar twigs for at least 10 weeks (Table 2). The number of culturable inoculated strains and quantification of the *luc* gene showed high and seasonal stable population densities with significant differences between the two developmental stages ($P < 0.01$) but a rather marginal variance of the single strains and the two sampling dates. A transfer of the recombinant plasmid to the indigenous bacteria could not be detected. Depending on the abundance of the inoculated strains, the limit of detection for transconjugants ranged from 8×10^3 to 1×10^2 g^{-1} fresh weight. The population densities of indigenous bacteria were similarly low in both spring and autumn (Table 2).

Different results were obtained by inoculating the cuttings at the stem elongation stage in June. Three weeks after inoculation, only the two clone-specific *Pseudomonas* strains persisted. After 10 weeks, none of the inoculated strains was detectable. However, transconjugants were found at both sampling dates. Indigenous bacteria harbouring the recombinant plasmid were detected with population densities ranging from 2×10^5 to 9×10^6 CFU g^{-1} fresh weight (Table 2). Except for inoculation with *Pseudomonas* E9, the numbers of culturable bacteria possessing RP4-Tn-*luc* were similarly high at both sampling dates. This indicates the stable presence of bacteria containing the plasmid. Corresponding to the observed conjugation events, the density of the indigenous bacteria was considerably higher than at the two other growth stages (Table 2).

Real-time PCR verified the presence of bacteria carrying RP4-Tn-*luc* and indicated an increasing trend from 3 to 10 weeks which was significant for the *Stenotrophomonas* inoculation. The PCR detection of the recombinant plasmid was consistent with the abundance of the inoculated strains and the indigenous bacteria harbouring the plasmid, except for strain E9 10 weeks after

Table 2. Establishment of the recombinant plasmid RP4-Tn-*luc* in the indigenous microflora of poplar cuttings inoculated with *Stenotrophomonas* sp. 169-1, *Pseudomonas* sp. E9 and Q16, each harbouring RP4-Tn-*luc*.

	Population densities (CFU g^{-1} fresh weight)[c]		
	Inoculated strain	Transconjugants	Real-time PCR detection of RP4-Tn-*luc* (genomes g^{-1} fresh weight)[c,d]
Inoculation at leaf development in April[a]			
Indigenous bacteria[b]	$7.0 \times 10^4 \pm 5.3 \times 10^3$ A		
3 weeks after inoculation			
Stenotrophomonas sp. 169-1	$2.3 \times 10^6 \pm 5.7 \times 10^5$ C	n.d.	$1.2 \times 10^7 \pm 6.5 \times 10^6$ A
Pseudomonas sp. E9	$1.7 \times 10^6 \pm 2.2 \times 10^5$ B,C	n.d.	$3.7 \times 10^7 \pm 3.4 \times 10^6$ B
Pseudomonas sp. Q16	$1.5 \times 10^6 \pm 2.7 \times 10^5$ B	n.d.	$9.6 \times 10^7 \pm 1.3 \times 10^7$ C
10 weeks after inoculation			
Stenotrophomonas sp. 169-1	$1.6 \times 10^6 \pm 7.1 \times 10^4$ B,C	n.d.	$2.0 \times 10^7 \pm 1.2 \times 10^7$ A,B
Pseudomonas sp. E9	$1.2 \times 10^6 \pm 1.0 \times 10^5$ A,B	n.d.	$9.8 \times 10^6 \pm 1.1 \times 10^6$ A
Pseudomonas sp. Q16	$6.4 \times 10^5 \pm 2.2 \times 10^5$ A	n.d.	$2.1 \times 10^7 \pm 3.6 \times 10^6$ A,B
Inoculation at stem elongation in June			
Indigenous bacteria	$3.1 \times 10^6 \pm 1.5 \times 10^5$ B		
3 weeks after inoculation			
Stenotrophomonas sp. 169-1	n.d.	$1.6 \times 10^5 \pm 6.3 \times 10^3$ A	$4.4 \times 10^6 \pm 8.5 \times 10^5$ A
Pseudomonas sp. E9	$3.8 \times 10^5 \pm 3.2 \times 10^5$	$5.3 \times 10^5 \pm 4.5 \times 10^5$ A	$1.0 \times 10^7 \pm 1.0 \times 10^6$ A
Pseudomonas sp. Q16	$4.1 \times 10^6 \pm 3.3 \times 10^6$	$2.5 \times 10^6 \pm 2.0 \times 10^6$ A	$5.1 \times 10^6 \pm 1.8 \times 10^6$ A
10 weeks after inoculation			
Stenotrophomonas sp. 169-1[e]	n.d.	$9.3 \times 10^6 \pm 8.8 \times 10^6$ A	$5.2 \times 10^7 \pm 2.4 \times 10^7$ B
Pseudomonas sp. E9	n.d.	n.d.	$9.2 \times 10^5 \pm 5.5 \times 10^5$ A
Pseudomonas sp. Q16[e]	n.d.	$6.8 \times 10^5 \pm 7.5 \times 10^5$ A	$8.7 \times 10^6 \pm 7.3 \times 10^5$ A
Inoculation at senescence in October			
Indigenous bacteria	$8.6 \times 10^3 \pm 1.2 \times 10^3$ A		
3 weeks after inoculation			
Stenotrophomonas sp. 169-1	$3.1 \times 10^5 \pm 8.2 \times 10^4$ C	n.d.	$3.8 \times 10^6 \pm 4.6 \times 10^5$ A
Pseudomonas sp. E9	$2.3 \times 10^5 \pm 5.3 \times 10^4$ B,C	n.d.	$2.3 \times 10^6 \pm 7.9 \times 10^4$ A
Pseudomonas sp. Q16	$5.9 \times 10^5 \pm 1.2 \times 10^5$ D	n.d.	$9.8 \times 10^6 \pm 4.3 \times 10^6$ B
10 weeks after inoculation			
Stenotrophomonas sp. 169-1	$7.5 \times 10^4 \pm 1.4 \times 10^4$ A,B	n.d.	$1.2 \times 10^5 \pm 3.9 \times 10^4$ A
Pseudomonas sp. E9	$1.2 \times 10^4 \pm 3.9 \times 10^3$ A	n.d.	$3.8 \times 10^5 \pm 4.8 \times 10^4$ A
Pseudomonas sp. Q16	$5.0 \times 10^4 \pm 8.7 \times 10^2$ A	n.d.	$6.5 \times 10^5 \pm 5.9 \times 10^4$ A

[a]Principal growth stages. Leaf development: green leaf tips 10 mm above the bud scales, first leafs unfolded; stem elongation: stem approximately 80% of final length; senescence: leaf discoloration (Finn, Straszewski and Peterson 2007). Inoculation of the freshly harvested cuttings was performed at these three dates.
[b]Culturable indigenous bacteria were determined directly before inoculation by plating on R2A. The abundance of the inoculated strains and transconjugants was estimated by plating on R2A supplemented with kanamycin and subsequently testing for bioluminescence and phylogenetic characterisation 3 and 10 weeks after inoculation.
[c]Numbers are means and standard deviations of triplicates. Different capital letters indicate significant differences ($P < 0.05$). Limit of detection was approximately 1×10^2 CFU g^{-1} fresh weight for the indigenous bacteria and the inoculated strains. Due to screening of 300 kanamycin resistant isolates per sample, limit of the detection of transconjugants was possibly increased to 1/300 of the population density of the inoculated strains of the respective sample. Limit of detection for the real-time PCR quantification was approximately 5×10^2 genomes g^{-1} fresh weight.
[d]The values were calculated based on the whole-genome size of *Stenotrophomonas maltophilia* (5.2 fg).
[e]Transconjugants were detected in two of the replicates.
n.d. = not detectable

inoculation. Similar to the other phenological stages, quantification of the *luc* gene resulted mostly in 10-fold higher population densities compared with the cultivation approach.

Community composition of indigenous bacteria harbouring the plasmid RP4-Tn-*luc*

Taxonomic assignment of RP4-Tn-luc-harbouring isolates based on phenotypic characteristics and sequencing of the 16S rRNA gene revealed 10 16S rRNA genotypes (Table 3). At the first sampling date, transconjugants of eight taxa of the α- and γ-*Proteobacteria* were detected. In cuttings inoculated with *Stenotrophomonas* 169-1 RP4-Tn-*luc* and *Pseudomonas* E9 RP4-Tn-*luc*, the plasmid had been transferred to members of the genera *Sphingomonas, Xanthomonas*, *Rhizobium, Methylobacterium* and *Stenotrophomonas*. In contrast, conjugation with the donor *Pseudomonas* Q16 RP4-Tn-*luc* predominantly resulted in the occurrence of transconjugants of the genus *Stenotrophomonas*. Four weeks later, only two genera—*Achromobacter* (β-*Proteobacteria*) and *Stenotrophomonas*—were found in the indigenous bacteria carrying the plasmid (Table 3). Thus, for the entire observation period, gene transfer in the genus *Stenotrophomonas* contributed obviously to the maintenance of the plasmid in the bacterial community of the poplar cuttings.

As shown in Fig. 1, two of the *Rhizobium* genotypes were phylogenetically related to *R. radiobacter* (formerly *Agrobacterium tumefaciens*), whereas the third genotype showed a high similarity to *R. soli*. A phylogenetic differentiation was also found within

Table 3. Taxonomic classification of isolates carrying RP4-Tn-*luc*.

	3 weeks after inoculation			10 weeks after inoculation		
16S rRNA genotype	169-1	E9	Q16	169-1	E9	Q16
Donor strains (RP4-Tn-*luc*)						
Stenotrophomonmas 169-1	–			–		
Pseudomonas E9		42			–	
Pseudomonas Q16			78			–
Transconjugants						
α-Proteobacteria						
Rhizobium 1		1				
Rhizobium 2[a]	11	8				
Methylobacterium		2				
Sphingomonas 1		2				
Sphingomonas 2	42	11	3			
β-Proteobacteria						
Achromobacter				3		
γ-Proteobacteria						
Stenotrophomonas 1			43	1		3
Stenotrophomonas 2		1				
Xanthomonas	19	32				
Sum of isolates	72	99	124	4	–	3

[a]This group of isolates is represented by two similar 16S rRNA genotypes. Because the isolates of the group were not distinguishable based on phenotypic characteristics, both groups were considered as one group.

the genus *Sphingomonas*. Most isolates of this genus showed homology to *S. yanoikuyae,* and two isolates were related to *S. faenia*. The genus *Methylobacterium* was represented by two isolates with a high similarity to *M. mesophilicum*. *Betaproteobacteria* represented by the genus *Achromobacter* were detected based on three isolates with nearest relationship to *A. piechaudii*. The remaining 16S rRNA genotypes were *γ-Protebacteria* of the genera *Stenotrophomonas* and *Xanthomonas*. Isolates identified as members of *Xanthomonas* and *Stenotrophomonas* 1 displayed identical RFLP patterns, but clearly differed in colony colour. *Xanthomonas* transconjugants detected after two different inoculations had identical 16S rRNA gene sequences and were most nearly related to *X. campestris* und *X. populi*. By contrast, transconjugants of the genus *Stenotrophomonas* represented two genotypes that were similar to *S. rhizophilia* or *S. chelatiphaga*, and each was distinguishable from the donor stain *Stenotrophomonas* 169-1 RP4-4-Tn-*luc*.

The donor strains *Pseudomonas* Q16 and E9 differed noticeably in colony morphology and were found to be related to *P. graminis* or *P. rhizosphaerae*.

DISCUSSION

We introduced the broad-host-range plasmid RP4-Tn-*luc* into the indigenous microflora of poplars by inoculating cuttings with three endophytic bacterial strains as donors. The *luc* gene served as a model for genes conferring growth promoting traits and was used to trace the plasmid by bioluminescence and real-time quantification. Inoculation at all three phenological stages revealed the nearly stable establishment of the plasmid and the corresponding bioluminescence trait. The culture-independent approach always gave higher population densities of recombinant bacteria in comparison to cultivation, which is well known from studying bacterial communities in different habitats (Yang *et al*. 2001; Kent and Triplett 2002). This discrepancy can be explained by limitations of bacterial culture and by the detection of dormant and dead cells as well as free bacterial DNA with PCR (Blagodatskaya and Kuzyakov 2013). Furthermore, these factors can vary during plant growth (Saito *et al*. 2007). To assess viable and metabolically active bacteria, data from the cultivation-based approach should be taken into account.

Against this background, the results obtained by both approaches are consistent with other studies demonstrating the plant colonisation by endophytic bacteria or the persistence of their plasmids (Taghavi *et al*. 2005; Compant *et al*. 2008; Sun *et al*. 2014). We showed that the survival of the introduced strains and the transfer of the plasmid into the indigenous community differed at the various phenological stages of the plants. After inoculation during leaf development and senescence, all strains survived by maintaining nearly stable densities. Both at the beginning and the end of vegetation, the plants were only weakly colonized, which obviously favoured the establishment of the strains. In contrast, the abundance of the introduced bacteria finally decreased below the detection limit when inoculating cuttings at the stem elongation stage. During this period, bacterial colonization was about two orders of magnitude higher than in the other two stages. Adapted indigenous bacteria living in complex interactions with their hosts (van der Lelie *et al*. 2009; Hibbing *et al*. 2010) and known as plant genotype specific (Ulrich, Ulrich and Ewald 2008) seemed to be competing with the inoculated strains. Accordingly, 3 weeks after inoculation, the *Stenotrophomonas* donor strain—originating from a distantly related poplar clone—could not be recovered. The two *Pseudomonas* strains—once isolated from the clone used for inoculation—were still present at a similarly high level at least 3 weeks after inoculation and therefore demonstrated greater competitiveness. In general, a decrease in the inoculated strains at the stem elongation stage was accompanied by the occurrence of transconjugants. Similar results were reported by Taghavi *et al*. (2005), who inoculated poplar cuttings with two *Burkholderia* strains containing a plasmid conferring toluene degradation. After 10 weeks, none of the strains became established, but the plasmid was shown to be transferred into the indigenous community.

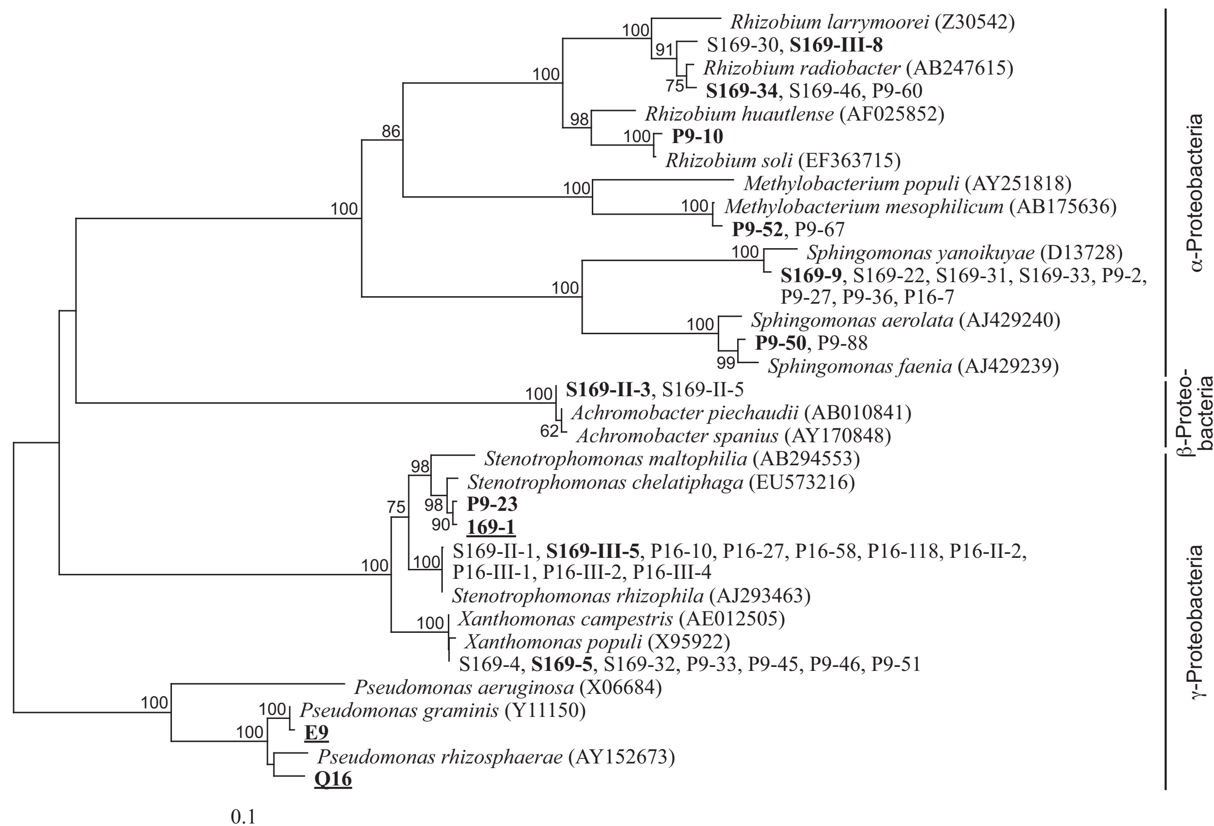

Figure 1. Dendrogram showing the similarity between the transconjugants and related bacterial species. The phylogenetic tree was generated using the neighbor-joining algorithm. The 16S rRNA gene sequence of *E. coli* (J01695) was used as an outgroup. Type strains of the bacterial species were used for the comparison; the sequence accession numbers are provided in brackets. Numbers at nodes indicate levels of bootstrap support >75%. Strains used for inoculation are underlined. 16S rRNA genotypes *Rhizobium* 1 were represented by S169-III-8 and S169-34, *Rhizobium* 2 by P9-10, *Sphingomonas* 1 by P9-50, *Sphingomonas* 2 by S169-9, *Stenotrophomonas* 1 by S169-III-5 and *Stenotrophomonas* 2 by P9-23. The 16S rRNA gene sequences of the transconjugants written in bold letters were deposited in the EMBL database (HE652088–HE652100).

In our study, gene transfer was associated with an increased abundance of potential plasmid recipients. However, it is safe to assume that factors related to the plant development and metabolism as well as the plant areas used as microhabitats for the bacteria represent a stronger driver for transfer events than a suitable density of recipients (Normander *et al.* 1998; Mølbak *et al.* 2003). For example, Lilley and Bailey (1997) detected transconjugants only within a narrow temporal window coincident with the midseason maturation of sugar beets. Moreover, Mølbak *et al.* (2003) revealed that the hypocotyl and the root areas were metabolically active regions that enabled plasmid transfer frequencies at detectable levels. In general, a correlation between the numbers of transconjugants and the density of potential plasmid recipients was not observed (Björklöf *et al.* 1995; Lilley and Bailey 1997). In this study, synergetic effects of an optimal plant growth stage providing conditions as known for protected biofilms (Sørensen *et al.* 2005) and a strong physical contact due to the high abundance of donor cells and putative recipients might contribute to the frequent plasmid transfer and establishment of the transconjugants after inoculation in June.

The plasmid RP4-Tn-*luc* was transferred into six genera of *Proteobacteria*. All these genera were previously described as endophytes of poplar (Ulrich, Ulrich and Ewald 2008; van der Lelie *et al.* 2009). Two of the genera belong to *γ-Proteobacteria* that were identified as the most frequent recipients for IncP plasmids in natural environments (Sørensen *et al.* 2005). Plasmid transfer events observed in the phytosphere are rare. Taghavi *et al.* (2005) showed a predominant transfer of the TOM plasmid (IncP-1) in different genera of *γ-Proteobacteria* as well.

Interestingly, plasmid transfer to the genus *Pseudomonas* was not observed in this study, although two of the donor strains belong to this genus and *Pseudomonas* is known to be a predominant genus of endophytic bacteria in poplar (Ulrich, Ulrich and Ewald 2008). Altogether, the detection of transconjugants within the three classes of *Proteobacteria* demonstrated the wide host range of the plasmid within the indigenous bacteria of poplar. A transfer beyond the *Proteobacteria,* e.g. to the phyla *Actinobacteria* and *Firmicutes*, could only be shown in more complex habitats such as the rhizosphere or bulk soil, and has only been demonstrated by a culture-independent approach; this is likely restricted to the plasmid transfer and did not include a stable replication (Musovic *et al.* 2006; Shintani *et al.* 2014; Klümper *et al.* 2015). Against this background, we found a widespread horizontal gene transfer in the poplar phytosphere.

The conjugative transfer of plasmids allows the possibility of introducing desired traits regardless of the competitiveness of the inoculated strains. This could change our understanding of the use of plasmids to improve the plant-promoting bacterial community, from the risk of plasmid loss to possible advantages of the establishment of desired traits.

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