High frequency of conjugation versus plasmid segregation of RP1 in epiphytic *Pseudomonas syringae* populations

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The maintenance and transfer of the broad host-range plasmid RP1 in epiphytically growing populations of *Pseudomonas syringae* was monitored in the phyllosphere of bush bean (*Phaseolus vulgaris*). When foliage was inoculated with plasmid-containing bacteria, the plasmid was lost from the majority of the cells within 2 d but was stably maintained in 0.8% of the population. A high frequency of conjugation between added donors and recipients was observed under high humidity conditions. In 1 d, the number of transconjugants rose to 10^{-1} of the donors and the proportional level of transconjugants continued to increase until 3 d after inoculation. Under these conditions the proportion of plasmid-containing bacteria stabilized at about 0.8% of the total population. The conjugation rate appeared to be in equilibrium with plasmid loss and the slower growth of the plasmid-carrying cells. A factor that influenced the high conjugation frequency observed was the available nutrients provided by the leaf and also, to a lesser extent, the leaf surface itself. Transfer of the plasmid from added donors to indigenous bacteria was also studied, using a donor-specific bacteriophage for counterselection of the donor. Transfer was observed to 10 different species of Gram-negative epiphytically growing bacteria. The bean leaf surface appears to function as a hotspot at least for intraspecific transfer of plasmids in high humidity. The frequency of transfer was higher than in soil or in rhizosphere habitats. This is likely to be the result of an environment that is nutritionally rich in combination with a limited colonizable surface area which permits close contact between the bacterial cells.

Keywords: *Pseudomonas syringae*, leaf surface, conjugation, indigenous bacteria, leachates

INTRODUCTION

Potential applications of recombinant pseudomonads in agricultural production are increasing. In particular, pseudomonads have been proposed as biological control agents of disease-causing micro-organisms, insects and nematodes in addition to preventing frost damage (Lindow, 1992). However, before large-scale release of recombinant strains into the environment is envisaged,

questions related to survival and possible effects on indigenous organisms in the relevant habitat must be addressed (Tiedje *et al.*, 1989). In some of the proposed applications, the site of action is the leaf surface, which has many distinctive features when compared to other habitats. The prevalent conditions include high and rapid variations in humidity and temperature as well as higher exposure to UV radiation (Lindow, 1991). As the diurnal and seasonal changes are large, the microbial populations are likely to fluctuate more than in other habitats. Many of the bacteria are present only transiently on the leaves due to the high immigration and emigration levels (Hirano & Upper, 1991), and bacteria are also unevenly distributed (Leben, 1988). The true epiphytes are adapted for survival in conditions where bacteria typical of other habitats

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Abbreviation: MIS, Microbial Identification Systems.

decline rapidly. The nutrient status is generally limited on the leaf surface (Andrews, 1992) and the nutrients present diffuse out onto the surface. The leachates contain essential minerals as well as sugars, organic acids, amino acids and vitamins (Tukey, 1970). Variations in light intensity has also been shown to directly affect leaching of carbohydrates (Tukey *et al.*, 1957).

Gene transfer is one possible means by which introduced strains may interact with indigenous bacteria. The most readily monitored mode of transfer of genetic material is conjugation of self-transmissible plasmids. It has been estimated that about 18% of all epiphytes isolated from different geographical regions contain plasmids, and also phytopathogenic strains such as those of *Erwinia*, *Klebsiella* and *Xanthomonas*, and *Pseudomonas syringae* pv. *tomato* contain conjugative plasmids (Bender & Cooksey, 1986; Powell *et al.*, 1993). High frequencies of conjugation between added donors and recipients on the leaf surface and in the leaf tissue have been reported (Lacy & Leary, 1975; Lacy *et al.*, 1984; Manceau *et al.*, 1986).

In most of the other natural environments studied, conjugal transfer frequencies have been low, but increase has followed nutrient amendments (Top *et al.*, 1990; Klingmüller, 1991) or been due to the presence of roots when considering soil habitats (van Elsas *et al.*, 1988; Lilley *et al.*, 1994). Plasmid transfer to indigenous bacteria was shown to occur in activated sludge and soil, after addition of donors with a poor survival capacity (McClure *et al.*, 1990; Henschke & Schmidt, 1990). By using an auxotrophic *Escherichia coli* donor strain in nutrient-amended sea water, Sørensen (1993) showed transfer of the plasmid RP4 to *P. fluorescens*, *P. cepacia*, and *Aeromonas* and *Enterobacter* strains. Smit *et al.* (1991) used a donor-specific phage as a selection against the donor bacteria for detection of RP4 transfer to *Pseudomonas*, *Xanthomonas*, *Comamonas*, *Enterobacter* and *Alcaligenes* strains in soil.

In this work, we studied the spread and maintenance of a self-transmissible plasmid in an epiphytic bacterial population. The transfer of the broad host-range IncP plasmid RP1, from an added *P. syringae* strain to added intraspecific recipient bacteria and to the resident epiphytic bacterial flora was shown to occur readily in the absence of selection pressure for the plasmid.

METHODS

Bacterial strains, growth media and plant material. *Pseudomonas* strains (Table 1) used in conjugations and inoculations as well as indigenous bacteria isolated from plant surfaces (Tables 1 and 2), were grown in Kings B (KB) broth (King *et al.*, 1954) overnight at 28 °C or at room temperature on a shaker. KB agar plates used for enumeration of bacteria from leaf washes were incubated for 2–5 d. The *E. coli* strains K12 (used as donor), DH5 α (used as recipient) and HB101N (used in phage testing) were similarly grown in Luria Bertani (LB) broth (Miller, 1972) at 37 °C overnight. Antibiotics were added to KB and LB media when required at the following final concentrations ($\mu\text{g ml}^{-1}$): ampicillin (Ap), 150; kanamycin (Km), 25; tetracycline (Tc), 10; spectinomycin (Sp), 100; rifampicin (Rif), 75; nalidixic acid (Nal), 40. For measurements of bacterial

growth, a Klett–Summerson colorimeter with a 520–580 nm filter was used.

A derivative of the Rif^R, non-pathogenic leaf colonizer *P. syringae* Cit7 was used as the donor strain in all experiments (Table 1). The derivative used was constructed by introducing a mini-Tn5 spectinomycin unit to the chromosome of the strain by the method of de Lorenzo *et al.* (1990). The plasmid RP1, identical to RP4 (Burkardt *et al.*, 1979), was introduced to Cit7Sp by conjugation from *E. coli* K12(RP1). This 60 kb self-transmissible plasmid confers resistance to Ap, Km and Tc (Grinsted *et al.*, 1972), and sensitivity to the phage PRD1 (Olsen & Shipley, 1972). The resulting strain, Cit7Sp(RP1), was used as the plasmid donor in all experiments. The donor was isolated from leaf washes with Rif-Sp-Km selection.

All introduced recipient strains used were Nal^R (Table 1), as Nal used in the plates inhibits DNA replication and thus conjugation from Nal^S cells (Smit & van Elsas, 1990). A Nal^R derivative of the *xylE* gene-carrying strain *P. syringae* Cit7*xylE* (Lindow *et al.*, 1992) was used as the isogenic recipient. This strain (Cit7*xylE*N) has the *ice* gene replaced by the catechol-2,3-dioxygenase gene *xylE* which enables identification of the strain by formation of the yellow-coloured compound 2-hydroxymuconic semi-aldehyde when plates are sprayed with catechol (Worsey *et al.*, 1978). Recipients were isolated from leaf washes with Rif-Nal selection and transconjugants with Rif-Nal-Km selection.

Bean plants (*Phaseolus vulgaris* cultivar Red Mexican) were grown in a peat moss-vermiculite mixture until the primary leaves were fully extended (10–14 d) in a growth room at 24 $\pm$ 2 °C with a photoperiod of 18 h. A halogen light source giving a light intensity of 7000 lux was used. For experiments involving indigenous bacteria, the plants were grown in pots outdoors in a peafield for up to 6 weeks and then transferred to the growth room.

Counterselection of donors. Purified phage for counterselection of donors was obtained as described by Bamford & Palva (1980). For counterselection of the donor, a host-range mutant with high plating efficiency and clear plaques on Cit7Sp(RP1) was isolated from the *P. syringae* pv. *phaseolicola*-specific phage ϕ J1 (J. Hantula, personal communication) and designated ϕ J1.1. Sensitivity for this phage was tested by pipetting 5 μl drops of different dilutions to semi-solid agar plates containing 200 μl of various laboratory and indigenous bacteria. Clear plaques after incubation overnight indicated sensitivity to the phage. When selecting for transconjugants of indigenous recipients, counterselection of the donor was achieved by adding phage at 10⁷ particles per plate.

Molecular biology techniques. Standard molecular biology techniques (Sambrook *et al.*, 1989) were used for restriction endonuclease digestions, ligations and agarose gel electrophoresis of DNA. To detect plasmid transfer to indigenous bacteria, colony blots were made using nylon membranes (Amersham). For probing of RP1, DNA fragments were separated and labelled with [³²P]CTP in low-melting-point agarose. Colony blots were probed using a 7 kb *EcoRI*–*BamHI* fragment of RP1.

Plate matings. Donor and recipient strains were grown overnight, diluted 1/10 and cultivated for a further 3 h. Cells were pelleted and resuspended in 1/5 vol. of growth medium without antibiotics. Donor and recipient strains were mixed and drops were dried onto agar plates in a laminar hood. Cultures of donor and recipient bacteria were plated separately as controls. The plates were incubated at room temperature overnight. Cells were then resuspended in growth media and plated with appropriate antibiotic selection.

Table 1. Bacteria used in this study and their sensitivity to the phage ϕ J1.1

Bacterium	Origin/reference	ϕ J1.1*
Laboratory strains:		
<i>Pseudomonas syringae</i> :		
Cit7Sp	Orser <i>et al.</i> (1985)*	+++
Cit7XylEN	Lindow <i>et al.</i> (1992)†	+++
pv. <i>phaseolicola</i> HB10Y	Vidaver <i>et al.</i> (1973)	+++
pv. <i>syringae</i> B728N	Loper & Lindow (1987)†	+++
pv. <i>syringae</i> 3556N	ICMP†‡	++
pv. <i>syringae</i> J900N	Mukopadhyay <i>et al.</i> (1990)†	—
<i>P. fluorescens</i> A506N	Wilson & Lindow (1993)†	—
<i>Acinetobacter calcoaceticus</i> ADP-6N	N. Norton, Yale University†	—
<i>Escherichia coli</i> :		
HB101N	Sambrook <i>et al.</i> (1989)†	—
K12(RP1)	Datta <i>et al.</i> (1971)	ND
DH5 α	Sambrook <i>et al.</i> (1989)	ND
Epiphytic isolates:		
<i>P. cichorii</i>	This study	++
<i>P. syringae</i>	This study	—
<i>P. aureofaciens</i>	This study	—
<i>Enterobacter agglomerans</i>	This study	—
<i>Sphingobacterium spiritivorum</i>	This study	—
<i>Rhodococcus fascians</i>	This study	—
<i>Arthrobacter oxydans</i>	This study	—
<i>Aureobacterium liquefaciens</i>	This study	—

* Reference for the Sp^S parental strain made Sp^R in this study.

† Reference for the NaI^S parental strains made NaI^R in this study.

‡ ICMP, International Collection of Micro-organisms from Plants, Auckland, New Zealand.

ND, not determined.

Table 2. Indigenous bacteria to which RP1 transferred *in planta*

Isolate*	RP1-specific characteristics		No. of isolates	
	PRD1 ^S	Restriction pattern†	1993	1994
<i>Alcaligenes faecalis</i>	+	+		1
<i>Alcaligenes piechaudii</i>	+	+		2
<i>Alcaligenes xylosoxydans</i> subsp. <i>denitrificans</i>	+	+		10
<i>Enterobacter agglomerans</i>	+	+		1
<i>Enterobacter intermedius</i>	—	+	1	
<i>Pseudomonas fluorescens</i>	+	+	1	
<i>Pseudomonas putida</i>	+	+		1
<i>Sphingobacterium spiritivorum</i>	+	+	2	3
<i>Xanthomonas campestris</i>	+	+		3
<i>Xanthomonas maltophilia</i>	+	+	12	4

* Indigenous strains were identified by MIS and API tests. All isolates were identified as plasmid carriers by hybridization with the 7 kb *Eco*RI–*Bam*HI fragment of RP1.

† Plasmids isolated and restricted after conjugation to *E. coli*.

Matings in liquid. Overnight cultures of donor and recipient bacteria, washed twice in 10 mM potassium phosphate buffer (pH 7.2), were resuspended to a concentration of 10^5 c.f.u. ml⁻¹ in 250 ml Erlenmeyer flasks containing 25 ml buffer as a shallow layer. To study the effect of surfaces and nutrients, a bean leaf was added. To study the direct effect of nutrients, peptone was added to a concentration of 0.1% to parallel samples. To study the effect of plant leachates, leaf wash was used as medium for conjugation. This was prepared by washing a leaf in buffer for 2 h and removing the leaf before addition of donors and recipients. Flasks were incubated in the light of the growth room or in the dark without shaking. Each experiment was performed in triplicate. Donors or recipients were incubated alone as controls in similar conditions in duplicate.

Inoculation of plants and quantification of bacteria *in planta*.

The bacteria in 10 mM potassium phosphate buffer (pH 7.2) were mixed immediately before inoculation of the plant foliage. The total inoculum level varied depending on the experiment (10^5 – 10^8 c.f.u. ml⁻¹). Bacteria were inoculated by a brief submersion using method I of Suoniemi *et al.* (1995). After inoculation, the pots were enclosed in 10 l plastic bags (high humidity, > 90%). Control plants were treated with sterile buffer. Primary leaves were sampled and bacteria were quantified as described by Suoniemi *et al.* (1995). Serial dilutions were grown on plates containing cycloheximide (200 µg ml⁻¹) and benomyl (50 µg ml⁻¹) to exclude fungal growth. The detection limit was ca. 100 c.f.u. (g leaf fresh wt)⁻¹. For calculation of the mean from parallel experiments containing some samples with populations below the detection limit, a value of log 0.4 less than the log value of the c.f.u. detection limit was used for the undetectable populations. Mean values of log[c.f.u. (g leaf fresh wt)⁻¹] from data from six determinations were compared using Student's *t* test in SPSS FOR WINDOWS.

In studies involving indigenous bacteria, the inoculation of two parallel pots with Cit7Sp(RP1) donor bacteria (10^8 c.f.u. ml⁻¹) was achieved by spraying until run off (30 ml per pot) and plants were incubated in high humidity.

Isolation and identification of indigenous epiphytes and transconjugants. To isolate indigenous bacteria from the leaf surface, the leaves submerged in 10 ml of 100 mM potassium phosphate buffer (pH 7.2) containing 0.1% peptone were sonicated in an ultrasonic bath for 7 min (Lindow, 1993). From buffer-inoculated plants, colonies were isolated from KB plates. From donor-inoculated plants, putative transconjugants were picked randomly from KB plates amended with Km, Tc, Ap and ϕ J1.1 and the colonies were plated against an RP1 fragment as described above. The presence of RP1 in the indigenous transconjugants was confirmed by testing for sensitivity to the phage PRD1 by cross-streaking bacterial suspensions across PRD1 streaks on plates, and by conjugation to *E. coli* DH5 α and visualization of the *EcoRI*- and *PstI*- or *SphI*-digested plasmid in agarose gel electrophoresis. Comparison of populations for occurrence of conjugation in various conditions was made using the chi-square test.

Indigenous bacteria were identified by standard methods including biochemical characteristics (API20E, API20NE) in combination with the whole-cell fatty acids based on Microbial Identification Systems (MIS-Microbial ID). Whole-cell fatty acids were saponified and methylated according to Miller & Berger (1985). Fatty acid methyl esters were analysed using a Hewlett Packard 5890A gas chromatograph with a 25 m $\times$ 0.2 mm 5% methylphenyl silicone fused silica capillary column. The isolates were classified by the MIS library software TSBA (revised version 3.80).

RESULTS

Characterization of donor and recipient strains

When the Cit7 derivatives, used in this study as plasmid donors (Cit7Sp) and recipients (Cit7Xyl/EN), were tested *in vitro* and *in planta* for changes in growth rate, no differences were observed as compared to parental strains Cit7 and Cit7Xyl/E. Plasmid RP1-carrying strains, both donors and transconjugants, grew slower than the plasmid-less strains. They were observed as smaller colonies after the standard incubation period and the count of culturable bacteria was 100-fold lower in overnight liquid cultures with antibiotic selection. Since equal volumes of overnight cultures were used, the initial density of recipients in plate matings and on the leaf surfaces was higher than that of the donor. All the cells in the donor inoculum contained the plasmid at the beginning of *in planta* experiments (Fig. 1). When strain Cit7Sp(RP1) was grown in the absence of selection, the generation time was estimated at 2 h 40 min, and in an overnight culture the number of plasmid-containing bacteria was 60% of the total. The corresponding generation time for the parental strain Cit7Sp was 2 h 15 min. Apparently, RP1 both slows the growth of *P. syringae* and is unstable in the absence of plasmid-maintaining selection pressure. A high frequency of RP1 transfer *in vitro* was achieved in plate matings with strain Cit7Sp as the donor and strain Cit7Xyl/EN as the recipient. After a typical overnight mating incubation followed by resuspension of the bacteria, the mean number of transconjugants (3.9×10^7 c.f.u. ml⁻¹) was higher than the number of donors (1.6×10^6 c.f.u. ml⁻¹) when the recipients were in excess (2.9×10^9 c.f.u. ml⁻¹). This was apparently a result of multiple consecutive conjugation events where newly formed transconjugants were also likely to function as donors.

Effect of surfaces and nutrients on conjugation in suspension culture

Two factors that may affect conjugation are the availability of nutrients and the local concentrations of bacteria. The presence of a leaf surface may affect both the nutrient status and the distribution of bacteria in a suspension. To test how this affects the formation of transconjugants, donors and recipients were incubated as a shallow liquid layer in flasks. In unamended buffer, the donor concentration decreased to about 1/10 of the original, whereas the recipient concentration remained constant. No transconjugants were observed after a 2-d incubation period (Table 3). In nutrient-amended flasks, the recipients increased almost 100-fold, whereas the increase in the donor concentration was smaller. The ratio of transconjugants to total bacteria after 2 d was 8×10^{-4} . Addition of a bean leaf to unamended buffer caused no significant growth of either donors or recipients, but transconjugants were still recovered at about 2×10^{-3} (Table 3) after a 2-d incubation. Addition of peptone to the leaf-containing mating mixtures did not improve the yield of transconjugants despite growth of donor and recipient cells. The growth of donors and recipients in

Fig. 1. Plasmid loss from the donor strain *P. syringae* Cit7Sp(RP1) colonizing the leaf surface with high inoculum (▲, ●) or low inoculum (△, ○). c.f.u. density measured on plasmid selection (Rif-Sp-Km, △, ▲) and on host cell selection (Rif-Sp, ○, ●). The SD of six replicates is indicated by vertical bars.

mating mixtures of leaf washes was similar to growth in mating mixtures with nutrient amendments, indicating that the leaf washing procedure had released nutrients from the leaf in comparable amounts to the peptone-amended buffer. The ratio of transconjugants to total bacteria was, however, slightly lower than in mating mixtures with leaf and nutrient amendments, and considerably lower than mating mixtures incubated with the leaf alone. This indicates that although the nutrients of the leachates mainly contribute to the positive effect, the leaf surface itself stimulates conjugation further.

Plasmid stability in planta

When Cit7Sp(RP1) donor cells were incubated on leaves in the absence of plasmid-maintaining selection pressure, plasmid-less segregants appeared soon after inoculation. A low inoculum, giving an initial population of 250 c.f.u.

(g leaf fresh wt)⁻¹, resulted in rapid growth. After 1 d, over 60 % of the bacterial cells were plasmid-less (Fig. 1). An apparent equilibrium with plasmid-containing (Km^R) and plasmid-less (Km^S) bacteria was achieved when about 0.8 % of the cells carried the plasmid. With an inoculum level of 10⁸ c.f.u. (g leaf fresh wt)⁻¹, where no net bacterial growth occurred, the plasmid loss was slower and the proportion of plasmid-containing cells had reached 4 % by day 13 (Fig. 1). The final total bacterial numbers in these cases seem to represent the carrying capacity of the leaf under the conditions used. Also, *in planta* both plasmid segregation and more rapid growth of the plasmid-less segregants seemed to contribute to the rapid increase in numbers of plasmid-less cells. Plasmid loss from 150 Km^S strains was confirmed by colony hybridization.

Intraspecific conjugation in planta

Using a low bacterial inoculum level, incubation at high humidity levels stimulated rapid growth of the bacteria during the first 24 h, where the generation times were found to be two times longer for the recipient and five times longer for the donor compared to liquid cultures (Fig. 2). Conjugation was also efficient; after the first day the transconjugants represented 1:10 of the donor level. Between the first and the third day after inoculation, the donor population continued to increase, after which it declined to a level of about 10⁴ c.f.u. (g leaf fresh wt)⁻¹. The level of transconjugants had surpassed the donor level by the third day. After this, the total level of plasmid-carrying cells declined to about 0.8 % of the plasmid-less recipients after day nine, much in the same way as in the experiment measuring plasmid segregation.

Samples taken from the inoculum mixture, when plated as controls on selective plates, did not generate colonies with transconjugant characteristics, and transconjugants were always absent from time zero samples. We concluded that conjugation did not take place during the inoculation procedure and that the Nal concentration in the plates was

Table 3. Influence of nutrient and surface addition for appearance of transconjugants in buffer during a 2-d incubation

The inoculation densities were log(5.4 ± 0.2 c.f.u. ml⁻¹) for the donor and log(5.7 ± 0.2 c.f.u. ml⁻¹) for the recipient. Values are expressed as log(c.f.u. ml⁻¹) and are the mean values of three replicates ± SD.

Addition to buffer	Donors	Recipients	Trans-conjugants	Ratio of trans-conjugants to total
No addition	4.0 ± 0.7	5.5 ± 0.3	< 1*	
0.1 % peptone	6.0 ± 0.5	7.6 ± 0.4	4.5 ± 0.6	8 × 10 ⁻⁴
Leaf	5.4 ± 0.1	6.1 ± 0.6	3.4 ± 0.4	2 × 10 ⁻³
Leaf + 0.1 % peptone	6.7 ± 0.5	7.0 ± 0.3	3.9 ± 0.6	5 × 10 ⁻⁴
Leaf wash	6.1 ± 0.1	7.1 ± 0.2	3.2 ± 0.1	1 × 10 ⁻⁴

* The detection limit was 10 c.f.u. ml⁻¹.

Fig. 2. Presence of *P. syringae* Cit7Sp(RP1) (on donor selection, Rif-Sp-Km, ▲), *P. syringae* Cit7xylEN (on recipient selection, Rif-Nal, ○) and transconjugants (Rif-Nal-Km, ●) on the surface of bean leaves in high humidity (> 90%) during a 2-week incubation. The SD of six replicates is indicated by vertical bars.

Fig. 3. Comparison of presence of donors [*P. syringae* Cit7Sp(RP1)], recipients (*P. syringae* Cit7xylEN) and transconjugants after 1 d of incubation in the growth room (open bars) or in the dark (shaded bars). The SD of six replicates is indicated by vertical bars.

sufficient to prevent donors from transferring the plasmid after plating.

To test the effect of absence of photosynthesis by the leaves, plants were incubated in darkness in high humidity 1 d before inoculation of donors and recipients. This was performed to increase the consumption of nutrients by resident bacteria on the leaf surface that were activated by the high humidity, while preventing production of new nutrients through photosynthesis. Before inoculation, the plants were further sprayed with buffer to remove residual nutrients. Inoculated plants were incubated in the growth room or in darkness. Surprisingly after 1 d, no significant differences in the amount of donors and recipients in the two treatments were detected, but the number of trans-

conjugants was significantly lower ($P < 0.01$) on plants that had been incubated in the dark (Fig. 3).

Indigenous epiphytes isolated from bean leaves and phage specificity

To observe plasmid transfer to the indigenous bacterial flora that did not contain any selectable markers, counter-selection of the added donor was required. Pre-seeding the plates with the phage ϕ J1.1 reduced the level of sensitive c.f.u. to 2×10^{-4} of the plated donors, apparently representing the phage-resistant spontaneous mutants of the host strain. To test the infectivity of the phage used for counterselection in other strains on the phylloplane, eight strains from field-grown beans that differed in colony morphology on KB plates were isolated and identified by MIS (Table 1). The MIS identification indices were relatively low, indicating that these strains were not closely related to the library strains. The host range of ϕ J1.1 was tested on these indigenous strains and on some laboratory strains (Table 1). The phage ϕ J1.1 infected most laboratory *P. syringae* strains tested but not *P. fluorescens* nor the other Gram-negative strains tested. It also infected one of the natural *Pseudomonas* isolates. Preseeding plates with ϕ J1.1 did not affect the total count of culturable bacteria in samples derived from field-grown plants, suggesting that bacteria sensitive to ϕ J1.1, although present (Table 1), did not form a large proportion of the bacterial flora of the plants used. The fact that phage counterselection did not affect the total viable count and exhibited a fairly narrow host-range enabled us to use it in monitoring plasmid transfer to indigenous bacteria other than those most closely related to the donor.

Conjugal transfer to indigenous bacteria in planta

For studying *in planta* transfer of RP1 to indigenous bacteria, plants were inoculated with 3×10^6 c.f.u. (g leaf fresh wt) $^{-1}$ of donors. The count of the indigenous bacteria increased rapidly from 2×10^6 c.f.u. (g leaf fresh wt) $^{-1}$ to 2×10^7 c.f.u. (g leaf fresh wt) $^{-1}$ when the donor- or buffer-inoculated plants were incubated at high humidity. After 4 d incubation, the total phage-resistant population on donor-inoculated and buffer-inoculated leaves was identical. The number of donor cells had decreased to 3×10^5 c.f.u. (g leaf fresh wt) $^{-1}$. Also the buffer-inoculated leaves were inhabited by about 4×10^4 c.f.u. (g leaf fresh wt) $^{-1}$ of bacteria with multiple antibiotic resistance (Ap^R Km^R Tc^R). Potential transconjugants represented only a part of the total c.f.u. resistant to the antibiotics used. During the experiment in the summer 1993, seven of the clones picked from the transconjugant selection on day 0 were identified as donor bacteria. At day four, no donor bacteria were found among the 500 strains studied. During experiments in 1994, no donors were found among the randomly picked potential transconjugants.

The presence of RP1 in the bacteria of a total of 1400 colonies from the Ap-Km-Tc selective plates from five separately treated donor-inoculated plants and 1250

colonies from the buffer-inoculated leaves was studied. From the donor-inoculated plants, 41 ϕ J1.1^R, RP1 hybridizing strains were found, of which all but one isolate was also PRD1^S. The presence of RP1 in hybridization-positive clones was confirmed by conjugation to *E. coli* DH5 α and isolation and visualization of the restricted plasmid (Table 2). During the experiment in 1993, three strains from the buffer-sprayed plants hybridized to RP1. All of these strains were identified as *Xanthomonas maltophilia*. In the later experiments (1994) no RP1-like plasmids were observed in controls that did not contain donors. Since putative transconjugants of *X. maltophilia* were obtained in both experiments, we also conclude that this bacterium had functioned as a recipient *in planta*.

As a control for plate mating, a buffer-sprayed plant was incubated for 4 d generating a total population of 10^6 c.f.u. (g leaf fresh wt)⁻¹ of which 10% were resistant to the antibiotics. Dilutions of this leaf wash and of overnight cultures of donors suspended in buffer (10^9 c.f.u. ml⁻¹) were mixed and plated on phage-treated plates. From the Tc^R Ap^R Km^R colonies, 140 were picked and probed against RP1. No RP1-containing strains were found, indicating that transconjugants had not arisen on the phage-seeded plates. The observed control frequency differs significantly from the *in planta* conjugation frequency ($P < 0.05$). Also, no transconjugants were observed on day 0 in the *in planta* conjugations, despite the presence of donors and recipients (see above) in the plated samples. We concluded that the transconjugants observed had arisen *in planta* and not on the plates.

In conclusion, although a proportion of the indigenous transconjugants isolated from the same plant may have been siblings, transfer had occurred in two separate experiments to 10 different bacterial species.

DISCUSSION

Studies of Lacy *et al.* (1984) indicated that the leaf surface could act as a hotspot region for conjugation of the plasmid RP1. A high frequency of transfer of RP1 from *P. syringae* pv. *glycinea* to *P. syringae* pv. *phaseolicola* in the phylloplane of bean was reported during the first 4 h after inoculation with an excess of donors (Lacy & Leary, 1975). Likewise, during the first hours after inoculation, *P. syringae* pv. *syringae* and *Erwinia herbicola* were shown to transfer the same plasmid to *E. amylovora* on pear blossoms (Lacy *et al.*, 1984). In another plant-associated environment, the rhizosphere, van Elsas *et al.* (1988) observed a high frequency of conjugation after inoculation with a high number of donor and recipient bacteria.

In this study, a high frequency of conjugation was observed in the phyllosphere at a high humidity level. The transfer frequencies were also high with a low inoculum level where rapid increase of the transconjugant level continued until 3 d after inoculation. This shows that these bacteria, actively colonizing the leaf surface, can function both as donors and recipients. As the transconjugants increased beyond the donors when recipients were in excess on the leaf surface (Fig. 2), it was evident

that the transconjugants formed on the leaf surface could also function as donors. Since plasmid RP1 slows the growth of the *P. syringae* donors and transconjugants, this relative increase in the transconjugant level could not have been due to the increased growth rate of these bacteria. Efficient conjugation also occurred in the leaf-amended buffer despite the lack of bacterial growth. Presumably, conjugation can also occur between viable, metabolizing but non-dividing cells.

High humidity permitting vigorous bacterial replication and, as shown here, high frequency of conjugation occurs on the phylloplane as a result of dew deposition at night and following rain showers (Lindow, 1991; Juniper, 1991). The leaf surface, exposed to high humidity conditions, was able to support a population density of up to 10^7 c.f.u. (g leaf fresh wt)⁻¹. The high humidity provides an aqueous environment for the bacterial cells and causes a leaching of nutrients from the leaf, making these nutrients accessible for the microbes on the surface. Free water on the surface of leaves also enables movement of the bacteria and possibly clustering in certain locations via selective leaf surface attachment as described for *P. syringae* pv. *phaseolicola* (Romantschuk *et al.*, 1993). It is also likely that photosynthesizing leaves induce chemotactical behaviour in motile *P. syringae* strains, such as Cit7.

RP1 encodes the production of a rigid conjugation pilus that has been reported to require a surface for high frequency of conjugation (Bradley *et al.*, 1980). Addition of a leaf to mating mixtures gave rise to transconjugants in this otherwise nutrient-free environment without permitting extensive bacterial growth (Table 3). This suggests that the microhabitat on the leaf surface supports conjugation by increasing the local concentrations of bacteria and shows that efficient conjugation apparently also took place in conditions where bacterial growth was limited. As conjugation also occurred in leaf washes where the effect of a surface was not present, it appears that both the nutrients supplied by the leaf and the presence of a surface contribute towards efficient conjugation. The positive effect of leachates can also be deduced from the fact that conjugation occurred more efficiently on plants incubated in the light than plants incubated in the dark, although a difference in the chemical composition of the leachates affecting the bacterial behaviour cannot be excluded. Under certain conditions, the phylloplane may form a similar nutrient-rich surface as the rhizoplane, where a sufficiently dense bacterial population can be supported to promote efficient transfer of conjugative plasmids between bacterial cells (van Elsas *et al.*, 1988).

The fact that plasmid segregation also took place where no net bacterial growth occurred (Fig. 1, high inoculum) indicates that the bacteria metabolize and divide also under these conditions, but that the bacteria die or become unculturable at a rate faster than the growth rate. This is always likely to happen when the cell density is higher than the carrying capacity of the habitat. The growth, and thereby also the segregation of the viable bacteria, is probably determined by the nutrients pro-

duced by the leaf and released from dying bacterial cells under the conditions used. Plasmid segregation and a somewhat slower growth of transconjugants contributes towards a decreasing concentration of plasmids in the epiphytic population, whereas conjugation works in the opposite direction. The fact that both plasmid loss and conjugation measurements resulted in the same relative plasmid content, suggests that these factors are in equilibrium within 10 d of incubation under the conditions used. Changes in RP4-carrying strains of *X. campestris* making the plasmid more stable have been reported (Tu *et al.*, 1989). Such mutations, which apparently may occur also in *P. syringae* given sufficient time, would be likely to increase the level of plasmid-carrying cells in the population. Whether such mutations have taken place during the *in planta* incubations reported here is not known. Thus, despite the additional metabolic burden in hosting RP1, such highly conjugative plasmids will not be diluted out from a population even in the absence of selection pressure and therefore behave as selfish, parasitic DNA (Gordon, 1992). Similar observations have been reported for the original TOL plasmid (117 kb) in *P. putida* batch cultures (Smets *et al.*, 1993). The presence of a plasmid in a subpopulation may be sufficient to give the population the adaptability provided by a plasmid whose genes are required only in certain conditions. In fact, conjugation might have evolved as a means not only for the dissemination of parasitic plasmids, but also to enable their survival despite a tendency to segregate. In this experimental set-up it was not possible to measure the relative importance of conjugation, segregation and growth retardation for the maintenance of the plasmid at the approximately 1% level.

It was evident from this study that the indigenous epiphytic bacterial flora of field-grown plants contain a high number of bacteria with multiple resistance markers ($\text{Ap}^R \text{Tc}^R \text{Km}^R$). Bacterial resistance to these antibiotics in soils has also been reported at a level of 10^3 – 10^4 c.f.u. (g dry wt) $^{-1}$ (Schmidt *et al.*, 1990). Whether or not this is a general feature of the phylloplane is not known.

RP1 was also shown to transfer to indigenous bacteria. The observed selection of 10 bacterial species that functioned as recipients is likely to be an underestimate of the total diversity of recipients. As RP1 replicates in a wide variety of Gram-negative bacteria, it is likely that not all of the indigenous transconjugants grew in the conditions and on the media used. Although most of the isolated transconjugants probably received the plasmid directly from the introduced donor on the leaf surface, some of the transconjugants may have arisen as a result of transfer via an intermediate plasmid host. Furthermore, despite the low effect of phage ϕ J1.1 on the total indigenous population, the counterselection method is expected to be also effective on putative indigenous transconjugants that are closely related to the donor. It may be assumed that strains that would most readily function as recipients to conjugation from Cit7 are the same strains that are sensitive to ϕ J1.1. Plasmid transfer to these strains can therefore not be observed using this form of counterselection. It seems likely that a self-transmissible

plasmid, at least via intermediate hosts, may reach any putative recipient where the plasmid is able to replicate. Spread to the rhizosphere and soil population via bacteria transiently present in the phyllosphere also seems likely. The results presented here show that large differences in gene transfer between habitats may occur. This aspect has to be considered in the context of risk assessment when using genetically modified micro-organisms.

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