Intergeneric Transfer of Chromosomal and Conjugative Plasmid Genes Between *Ralstonia solanacearum* and *Acinetobacter* sp. BD413

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Conjugative transfer of a broad-host range plasmid and transformation-mediated transfer of chromosomal genes were found to occur at significant frequencies between *Ralstonia solanacearum* and *Acinetobacter* sp. in planta. These intergeneric gene transfers are related to the conditions provided by the infected plant, including the extensive multiplication of these two bacteria in planta and the development of a competence state in *Acinetobacter* sp. Although interkingdom DNA transfer from nuclear transgenic plants to these bacteria was not detectable, plants infected by pathogens (e.g., *Ralstonia solanacearum*) and co-colonized by soil saprophyte bacteria (e.g., *Acinetobacter* sp.) can be considered as potential “hot spots” for gene transfer, even between phylogenetically remote organisms.

Phylogenetic studies and database analyses of completely sequenced microbial species indicate that microbial evolution has been driven by horizontal gene transfer (HGT) (Lawrence 1999; Ochman et al. 2000). Preliminary in situ experiments show that gene transfer can occur in diverse aquatic and terrestrial environments but at very low frequencies when transformation and transduction mechanisms are involved, whereas conjugation-mediated plasmid exchanges could be more frequent (Ashelford et al. 1997; Davison 1999; Dröge et al. 1999; Lorenz and Wackernagel 1994; Timms-Wilson et al. 2002).

Successful gene transfer from donor to recipient microorganisms depends on numerous and fluctuating biotic parameters (competition, predation, interactions with other organisms) and abiotic parameters (nutrients, humidity, pH, temperature, soil particles) in situ. For instance, competence development for naturally transformable bacteria rarely occurs under environmental conditions (Bertolla et al. 2000a). Transduction in natural habitats could be influenced by environmental factors affecting adsorption of bacteriophages to their specific host cell as well as phage propagation and specificity (Kidambi et al. 1994; Replicon et al. 1995; Vettori et al. 1999). Conjugation, which can mediate gene exchange in virtually all eubacterial species and even from bacteria to yeasts (Inomata et al. 1994; Nishikawa et al. 1992), requires physical contact between physiologically active donor and recipient cells.

Some terrestrial environments, such as those associated with plants, can provide bacteria with particularly favorable conditions for HGT. Conjugation-mediated gene exchange was detected in the rhizosphere (Smit et al. 1998; Troxler et al. 1997),

the phyllosphere (Bjorklof et al. 1995), and more generally in the phytosphere (Lilley and Bailey 1997), including within plant tissues (Manceau et al. 1986). In these “hot-spot” environments, bacteria might reach relatively high concentrations and achieve appropriate physiological conditions for HGT. Previous studies have shown that colonizing bacteria are able to reach high populations in planta and, in the case of plant colonization by *Ralstonia solanacearum* and *Acinetobacter* strain BD413, both bacteria were able to develop a competence state (Bertolla et al. 1999; Kay et al. 2002a).

This work investigated the ability of the plant pathogen *R. solanacearum* to exchange genes with an opportunistic microorganism, the naturally transformable bacterium *Acinetobacter* sp., during plant colonization. Intergeneric gene transfer was monitored in vitro and in planta and required the development of the appropriate molecular tools in order to demonstrate both conjugation and transformation transfer mechanisms. Finally, since the process of infection by *R. solanacearum* leads to the release of plant DNA during plant decay (Bertolla et al. 2000b), experiments were done to evaluate transkingdom gene transfer from infected transgenic tomato plants to the invading and metabolically active opportunistic *Acinetobacter* sp. cells.

RESULTS

Conjugation-based plasmid transfer in vitro.

The gentamicin-resistant strain *Ralstonia solanacearum* GM11000FB3 carrying the conjugative plasmid RP4 was the plasmid donor, and the nalidixic acid resistant *Acinetobacter* strain BD413 strain was the recipient. The plasmid RP4 confers resistance to ampicillin, tetracycline, and kanamycin; thus, transconjugants of *Acinetobacter* sp. BD413 could be detected on medium supplemented with nalidixic acid and ampicillin, tetracycline, and kanamycin.

Filter matings were performed on two different growth media as one (Boucher-Gelose [BG]) favored the growth of the donor and the other (Luria-Bertani [LB] medium) favored the growth of the recipient. A number of transconjugants ($5.7 \pm 3.3 \times 10^4$ per ml) were recovered with filters placed on BG medium and also with filters on LB medium ($1.3 \pm 0.8 \times 10^5$ transconjugants per ml). Plasmid transfer frequencies were 4.5×10^{-5} and 1.9×10^{-4} per recipient cells, respectively, and were not significantly different ($p \leq 0.05$). The addition of DNase I on filter matings did not modify the conjugation frequency. Control filters with only one of the two strains spotted on a filter did not produce isolated colonies on transconjugant-selective medium. These results demonstrated that the recipient strain, *Acinetobacter* sp., did not exhibit spontaneous resis-

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tance to the three antibiotics ampicillin, tetracycline, and kanamycin above the 1×10^{-10} detection limit.

Conjugation in planta.

To detect bacterial gene transfer via conjugation in plants infected by two bacteria, the donor *R. solanacearum* GMI1000FB3 (RP4) and the recipient *Acinetobacter* strain BD413, were inoculated into tomato plants (stem). Potential RP4 plasmid transfer was monitored for six days by measuring the number of donor, recipient, and transconjugant cells every day. The number of bacterial cells was determined by plating dilutions of the plant tissue suspensions on BG medium supplemented with gentamicin, ampicillin, tetracycline, and kanamycin for donor strain, LB medium with nalidixic acid for recipient strain, and LB medium supplemented with nalidixic acid, ampicillin, tetracycline, and kanamycin for *Acinetobacter* sp. transconjugants. Before plating on selective media, the plant suspensions were treated with DNase I to degrade released plasmid DNA and to prevent gene transfer via transformation. Immediately after inoculation ($t = 0$), one infected stem was crushed and 2.1×10^6 CFU of *R. solanacearum* and 5.31×10^5 CFU of *Acinetobacter* sp. per g of fresh plant tissue were counted, but no transconjugants were recovered (Fig. 1). The population dynamics for the pathogenic strain *R. solanacearum* GMI1000FB3 (RP4) showed that it multiplied extensively inside plant tissues inducing plant wilting symptoms, and then, six days after infection, it caused plant death. The

number of CFU increased from the inoculum level of 2.11×10^6 CFU to 7.31×10^9 CFU per g of fresh tissue over five days, corresponding to a $\geq 1,000$ -fold increase. In parallel, the concentration of the opportunistic bacterium *Acinetobacter* sp. increased by the same order of magnitude (1,000-fold) but did not cause any plant wilting symptoms, as previously described by Kay and associates (2002a).

Transconjugants appeared 24 h after coinfection of the plants, although without apparent selective pressure, and reached 1.8×10^7 CFU after six days (number of recipient cells was 2.2×10^8 after six days) (Fig. 1). The potential for gene transfer occurring on plates or during the inoculation procedure was tested, but no transconjugants were detected, confirming that transconjugants had resulted from conjugative transfer of RP4 in plant tissues. The presence of the plasmid RP4 in transconjugants was detected by polymerase chain reaction (PCR) with primers oriT1-oriT2 and traG1-TraG2 (discussed below), which are specific for the RP4 plasmid. All transconjugant clones showed positive amplification with these primers. In addition, a specific band corresponding to high molecular weight plasmid appeared on Eckhardt gels in the positive control *R. solanacearum* GMI1000FB3 (RP4) and in transconjugant clones, whereas there was no band of the same size for the native recipient *Acinetobacter* sp. Chromosomal sequences from the transconjugants were also amplified with primers PA and PH (discussed below), which are complementary to conserved regions of the 16S rDNA. Restriction fragment length poly-

Fig. 1. Conjugative plasmid transfer in plant tissues between plant-associated bacteria. Colonization of tomato plant by *Ralstonia solanacearum* GMI1000FB3 (RP4) and *Acinetobacter* strain BD413 followed concomitant injection into the stem. Infection kinetics were determined as the number of CFU after the plants were crushed and the suspension was plated onto selective media to monitor viable counts of each bacterium. Detection of conjugative gene transfer in situ was determined by CFU counts of transconjugants of *Acinetobacter* strain BD413 on agar plates supplemented with antibiotics. Error bars indicate standard deviation of triplicate experiments.

morphism (RFLP) on these PCR products digested by the enzyme *Hae*III showed a restriction pattern identical to that of the recipient strain; thus, confirming that the transconjugant clones originated from the *Acinetobacter* sp. recipient strain.

Natural transformation experiments in vitro.

To assess the potential of chromosomal gene transfer from *R. solanacearum* to *Acinetobacter* sp., specific donor and recipient strains were constructed as described below. The donor strain *R. solanacearum* GMI1000 Zpop1 contains the *aadA* and *acc3-IV* genes conferring resistance to spectinomycin and gentamicin, respectively. These antibiotic resistance genes were inserted inside the *popA* gene of the megaplasmid-borne *hrp* regulon. The bacterial strain in which this gene is disrupted displays a wild-type pathogenic phenotype (Arlat et al. 1994). The recipient strain *Acinetobacter* strain BD413, when carrying a *popA* DNA sequence from *R. solanacearum* integrated into a broad-host spectrum plasmid, was called *Acinetobacter* strain BD413 (pPopA) and was resistant to nalidixic acid and ampicillin. The presence of identical DNA sequences in the donor and the recipient genomes allows transforming DNA to be integrated into the recipient genome.

The recipient strain *Acinetobacter* strain BD413 (pPopA) was exposed to increasing concentrations of purified *R. solanacearum* GMI1000 Zpop1 genomic DNA on nitrocellulose filters (0.01 µg, 0.1 µg, 1 µg, and 10 µg per filter). Transformant clones of *Acinetobacter* strain BD413 pPopA were detected on LB medium supplemented with nalidixic acid, ampicillin, spectinomycin, and gentamicin. The number of transformants produced was roughly proportional to the quantity of DNA placed on the filter, from 0.001 µg per filter up to 10 µg per filter (Table 1).

Other filter experiments, performed with crude cell lysates instead of purified DNA (1, 10, or 100 µl per 100 µl) of the same donor bacterial strain led to numbers of transformants that were lower than those obtained with purified DNA (Table 1). CFU of transformants increased with increasing amounts of cell lysates, but the maximum transformation frequency obtained was 8.8×10^{-8} per recipient compared to 8.3×10^{-6} per recipient with purified DNA (10 µg). When an estimated equivalent amount of DNA in a pure DNA solution or in the lysate was used, transformation frequencies remained systematically lower with the lysate DNA, thus suggesting a potential limiting effect of cell debris on transformation of *Acinetobacter* sp. (Table 1).

A living culture of *R. solanacearum* GMI1000 Zpop1 was also tested as a potential source of biologically active DNA capable of transforming *Acinetobacter* sp., since extracellular DNA can be released at the death of some cells or following an active secretion process. Filter experiments were conducted on two different growth media favoring growth of either the donor cells (on BG medium) or the recipient cells (on LB medium) as stated above. The transformation frequency obtained was 200 times higher on LB medium than on BG medium. When the number of donor cells was greater than or equal to that of the number of recipient cells on LB medium, the frequency reached up to 4×10^{-6} per viable recipient cell. When the number of the donor cells was lower than that of the recipient cells, the transformation frequency was below 10^{-7} (Table 1). In all cases, the highest frequencies were obtained for a DNA source composed of living bacterial cells and not with purified DNA or cell lysates. Moreover, the transformation frequencies obtained were not related to growth of the donor cells, since the LB medium used for filter matings inhibited the growth of *R. solanacearum*.

Transformation experiments gave negative results in the presence of DNase I (0.2 mg per filter) or when the recipient

strain did not harbor the plasmid pPopA; thus, confirming the involvement of a transformation mechanism, which requires homologous sequences to promote recombination.

Natural transformation of *Acinetobacter* sp. in planta.

The fact that *Acinetobacter* strain BD413 was found to develop natural competence in planta (Kay et al. 2002a) and was transformed efficiently in vitro with various sources of DNA led us to test whether or not *Acinetobacter* sp. could be transformed in planta with *R. solanacearum* DNA. *R. solanacearum* Zpop1 and *Acinetobacter* strain BD413 (pPopA) were inoculated into stems of tomato plants, and donors, recipients, and recombinants were monitored for six days. A natural transformation test, as described by Kay and associates (2002a), was performed on the inoculum suspension containing both donor and recipient bacteria. A volume of 100 µl of pAVA213-8 plasmid DNA solution (10 ng DNA per µl) (Palmen et al. 1992) was added to 900 µl of inoculum suspension before incubation at 30°C for 90 min in order to allow DNA uptake by competent cells (if present). The mixture was then treated with 0.2 mg of DNase I before being plated on LB transformant-selective medium supplemented with nalidixic acid, ampicillin, spectinomycin, and gentamicin. Absence of transformants confirmed that *Acinetobacter* sp. cells in the inoculum were not in a competent state and that competence development occurred after inoculation in plant tissues. When the bacterial suspension was injected into the plant stem, wilting symptoms leading to plant death were observed. Multiplication of both bacteria occurred within the vascular plant tissues (Fig. 2), as previously observed in the course of in planta conjugation experiments. Recombinant clones exhibiting resistance to nalidixic acid, ampicillin, spectinomycin, and gentamicin were detected three days after inoculation. A maximum of $2.6 (\pm 1.7) \times 10^1$ CFU of transformants per g of fresh plant tissue was detected five days after bacterial inoculation in plant tissues, corresponding to a transformation frequency of 6.6×10^{-8} . The infection of plants with a less concentrated inoculum yielded similar wilting symptoms of plants, but the bacterial cell densities reached in planta remained lower and did not lead to the isolation of *Acinetobacter* sp. transformants.

The presence of the marker genes in the genome of recombinants was confirmed by PCR, using specific primers FGPaad1172 and FGPaac2 (discussed below). All recombinant clones showed a positive amplification of a 1.6-kb DNA fragment, which is similar to that obtained with the GMI 1000FB3 Zpop1 donor strain. In addition, PCR-RFLP on 16S rDNA confirmed that recombinant clones originated from the inoculated *Acinetobacter* sp. strain.

Tests to detect in planta gene transfer from nuclear-transgenic plants to *Acinetobacter* sp. containing transgene homologous sequences.

Next, the potential for transkingdom gene transfer from transgenic plants to the recipient strain *Acinetobacter* sp. during plant colonization was investigated. Transgenic plants with spectinomycin and gentamicin resistance genes flanked by the *popA* gene sequences were used (Bertolla et al. 2000b). The transgenic tomato plants were inoculated by the *R. solanacearum* GMI1000 wild type, which caused plant tissue necrosis, plant cell lysis, and putative release of plant DNA close to bacterial cells. *Acinetobacter* strain (pPopA), which contains DNA sequences homologous to the *popA* gene in the transgenic tomato genome, was used as a potential recipient strain.

Infected stems were removed and crushed, and plant suspensions were plated on LB medium supplemented with acid nalidixic and ampicillin to detect recipient cells and plated on LB medium with acid nalidixic, ampicillin, spectinomycin, and

gentamicin to recover potential transformants. However, during the six-day experiment, no transformants could be detected.

To increase plant DNA availability to recipient bacteria, 5 µg of purified genomic plant DNA was injected into different parts of the infected stems. After 24 h, plant stems were crushed. However, transformants remained undetected in spite of these new conditions.

DISCUSSION

Over the last 20 years, numerous experiments have been designed and conducted to detect gene transfer occurring under natural conditions. Various environments have been investigated, including fresh water and marine environments, sediments, soils, the rhizosphere, plant leaf surfaces, and the gut of insects or other animals. HGT frequencies are relatively high in plant and insect gut environments and relatively low in soil and water.

Conjugative HGT has been observed under a range of environmental conditions. This type of HGT was found to occur in seawater between inoculated *Pseudomonas putida* and indigenous bacteria at frequencies ranging from 10^{-4} and 10^{-6} (Dahlberg et al. 1998). Experiments conducted in animal-related environments, such as the gut of soil microanimals, provide a habitat for high bacterial density (Hoffmann et al. 1998, 1999; Thimm et al. 2001). A wide range of donor cells have been tested, e.g., *Escherichia coli* (Adamo and Gealt, 1996), *Enterobacter clonacae*, *Erwinia herbicola* (Watanabe and Sato 1998; Watanabe et al. 1998), and *Bacillus thuringiensis* (Thomas et al. 2001), for conjugation with either specific bacterial recipient bacteria or the indigenous microflora (Hoffmann et al. 1999; Hoffmann et al. 1998; Thimm et al. 2001). In such experiments, frequencies ranged from 10^{-3} to 10^{-1} , depending on the plasmid used (Watanabe and Sato 1998; Watanabe et al. 1998).

The same approaches were also used to study conjugative HGT from donor strains introduced into soil, using sterile or nonsterile soil microcosms into which the recipient bacteria had been inoculated or in which indigenous microflora were targeted recipients. In nonsterile soil, HGT occurred at relatively high frequencies (10^{-3} to 10^{-4}) between donor and recipient *Pseudomonas* or *Bradyrhizobium* strains (Berg and Trevors 1990; Kinkle et al. 1993). Finally, conjugative transfer was also examined in the plant environment, including the phyllosphere (Normander et al. 1998), where frequencies reached

10^{-1} (Bjorklof et al. 1995, 2000), in the rhizosphere (Smit et al. 1998; Troxler et al. 1997; Van Elsas et al. 1988), and the entire phytosphere with frequencies up to 10^0 (Lilley and Bailey 1997).

When attacked by the pathogen *R. solanacearum*, plants are submitted to a mixture of lytic enzymes including endonucleases, polygalacturonases and pectin methylesterase (Spok et al. 1991; Roberts et al. 1988; Schell et al. 1988). These enzymes disorganize plant tissue, causing cell wall degradation and releasing large quantities of nutrients and metabolizable molecules into an aqueous environment. These conditions are favorable for bacterial cell movement and pathogen multiplication and might lead to plant co-colonization by soil microorganisms, including the soil bacterium *Acinetobacter* strain BD413. Previous work reported that this strain could co-colonize plants by their roots and multiply extensively in the rest of the pathogen-infected plant (Kay et al. 2002a).

The results presented here illustrate that the broad-host range conjugative plasmid RP4 was transferred efficiently from *R. solanacearum* to *Acinetobacter* strain BD413 throughout the colonization process. The fact that HGT was insensitive to DNase I in vitro and all the crushed plant suspensions were treated with DNase I supports the hypothesis that conjugation is the mechanism involved. Moreover, gene transfer was verified to have occurred in planta and not during the selection process on selective medium, which contained sufficient nalidixic acid concentrations to prevent plasmid transfer in vitro, as this antibiotic inhibits DNA replication. Transfer frequencies were already high in the first 24 h after inoculation (4.8×10^{-3}) and increased to reach a maximum of up to 1.1×10^{-2} when populations of donor and recipient bacteria increased. These in planta frequencies are two orders of magnitude higher than those obtained with filter mating, although the number of donor bacteria (about 1×10^8) was similar. In vitro experiments were conducted both with an optimized donor-recipient ratio (according to observations by Smit and Van Elsas [1990]) and on agar plates on which the rigid pili-like structure of the RP4 plasmid functions well (Bradley et al. 1980). In spite of these optimized in vitro conditions, our data indicate that conjugative transfer conditions can be more favorable in natural habitats than in vitro. Similar conclusions have been drawn by Thimm and associates (2001), who found higher transfer frequencies in the gut of an earthworm than in vitro, thus indicating that hot-spots for intra- and intergeneric genetic transfer exist in the environment.

Table 1. Natural transformation of *Acinetobacter* strain BD413 (pPopA) with purified genomic DNA and crude cell lysates of *Ralstonia solanacearum* Zpop1^a

<i>Ralstonia solanacearum</i>		<i>Acinetobacter</i> sp. BD413 pPopA		
GMI1000 Zpop1 DNA source	Recipient cells (CFU) spotted on filter	Recipient cells (CFU) after 48 h of incubation	Transformants (CFU) ^b	Transformation frequency (Transformants/Recipient cells)
Purified genomic DNA				
0.01 µg	...	$1.8 (\pm 0.1) \times 10^9$	$2.1 (\pm 0.2) \times 10^2$	1.1×10^{-7}
0.1 µg	5×10^6	$1.7 (\pm 1.2) \times 10^9$	$9.8 (\pm 1.9) \times 10^2$	5.7×10^{-7}
1 µg	...	$8.9 (\pm 0.2) \times 10^8$	$3.2 (\pm 0.9) \times 10^3$	3.6×10^{-6}
10 µg	...	$8.1 (\pm 3.4) \times 10^8$	$6.8 (\pm 2.7) \times 10^3$	8.3×10^{-6}
Crude cell lysates				
10^6 cells $\approx$ 6 ng DNA	...	$2.1 (\pm 0.4) \times 10^9$	7 ($\pm$ 5.2)	3.3×10^{-9}
10^7 cells $\approx$ 60 ng DNA	5×10^6	$1.8 (\pm 0.2) \times 10^9$	$3.7 (\pm 2.1) \times 10^1$	2.1×10^{-8}
10^8 cells $\approx$ 600 ngDNA	...	$1.8 (\pm 0.4) \times 10^9$	$1.6 (\pm 0.5) \times 10^2$	8.8×10^{-8}
Living cells				
10^8 cells	5×10^5	$2.6 (\pm 0.8) \times 10^9$	$2.3 (\pm 1.7) \times 10^4$	8.8×10^{-6}
10^8 cells	5×10^6	$2.8 (\pm 0.9) \times 10^9$	$1.0 (\pm 0.6) \times 10^4$	3.5×10^{-6}
10^8 cells	5×10^7	$3.1 (\pm 0.4) \times 10^9$	$8.0 (\pm 2.3) \times 10^3$	2.5×10^{-6}
10^6 cells	5×10^6	$1.9 (\pm 0.9) \times 10^9$	$1.8 (\pm 0.4) \times 10^2$	9.4×10^{-8}
10^4 cells	5×10^6	$1.6 (\pm 0.9) \times 10^9$	5 ($\pm$ 3.2)	3.1×10^{-9}

^a Incubated on nitrocellulose filters for 48 h.

^b Significantly different (Student *t* test) *p* = 0.05.

Transformation-mediated gene transfer was also studied in various environments by using an approach similar to that for conjugation, including inoculation of the donor organism or donor DNA and recipient bacterial strains. Soil environments rarely provide favorable conditions for competence development of naturally transformable bacteria. For example, *Pseudomonas stutzeri* (Stewart and Sinigalliano 1990) and *Acinetobacter* strain BD413 (Nielsen et al. 1997) develop competence in situ only in the presence of added nutrients. In the case of *Acinetobacter* strain BD413, transformation frequencies in soil ranges from 10^{-7} to 10^{-4} . However, one bacterium, *Pseudomonas fluorescens*, was transformed naturally in soil in the absence of added nutrients (Demanèche et al. 2001a). Physical parameters, such as those related to lightning discharges, could potentially increase the diversity of transformable bacteria in soil environments (Demanèche et al. 2001b). Yet, soil probably continues to represent an environment in which transformation frequencies are relatively low.

The work presented here examined whether *Acinetobacter* strain BD413 could participate in transformation-mediated gene transfer in planta, since it developed a competence state in planta (Kay et al. 2002a). The donor strain was the *R. solanacearum* Zpop1, which is a GMI1000 derivative containing spectinomycin and gentamicin resistance genes. The *Acinetobacter* strain BD413 was constructed to act as a recipient by providing it with the *R. solanacearum* *popA* sequences flanking the marker genes in the donor strain. Transfer frequencies were first determined in vitro to test efficiency of the *popA* sequences to promote homologous recombination in *Acinetobacter* sp. and to confirm that the marker genes could be expressed in this new host. Various sources of donor DNA were tested, including pure DNA, inactivated cells, and living bacterial cells, in order to simulate what could occur in plant tissues. In planta extracellular DNA can be released passively out of bacterial cells at their death or from active secretion, providing unknown amounts and purity of transforming DNA molecules. Heat-treated cells provided transformants on filters at frequencies that were one order of magnitude lower than those obtained with pure DNA (on a DNA-equivalent basis). However, this same recipient bacterium was transformed at similar frequencies with pure DNA and heat-inactivated treated cells from *Burkholderia cepacia*, *P. fluorescens*, or *Acinetobacter* spp. (Nielsen et al. 2000). Transformation frequencies rose by a factor of 15 when the DNA source was living donor cells, suggesting that *R. solanacearum* might actively secrete DNA, as already described for *Acinetobacter* sp. (Lorenz et al. 1991; Palmen et al. 1995), *Burkholderia*, *P. stutzeri*, and *Bacillus* spp. (Lorenz et al. 1991; Paul and David 1989).

The use of constructed strains in this work was necessary in order to clearly demonstrate the conjugation and transformation potential at measurable frequencies. In addition, the definition of strict control conditions was only possible by the presence of multiple antibiotic resistances in these strains. Arguably, these strains, as constructed, have not been reported to occur in nature. However, the possibility of other homologous sequences existing between these bacteria is real and is simulated reasonably by the antibiotic resistant marker system used here.

In planta experimental conditions were used to see whether intergeneric transformation-mediated gene transfer between *R. solanacearum* strain GMI1000 Zpop and *Acinetobacter* strain (pPopA) could occur. Some recombinant clones were detected three days after plant infection, confirming that chromosomal gene transfer could happen between these two bacteria. Considering both the in planta and in vitro results presented here, as well as those concerning intraspecific transformation of *R. solanacearum* in planta (Bertolla et al. 1999), the plant envi-

ronment could be considered as a hot-spot for gene transfer. While numerous physical, physiological, and molecular barriers, such as those related to restriction and modification mechanisms or the mismatch repair system, might make intergeneric chromosomal gene transfer a rare event, the 10^{-8} frequency observed here is relatively high. Thus, the plant infected by a pathogen could be one of these environmental hot-spots for gene transfer, at least between bacteria.

The negative results obtained in the present study involving nuclear transgenic plants and bacteria could be related to a lack of DNA availability of plant nuclear DNA or to an overwhelming dilution of the transgene-borne marker gene in the plant genome, as previously proposed for *R. solanacearum* (Bertolla et al. 2000b). This latter hypothesis seems to be confirmed, since recent results demonstrate that *Acinetobacter* sp. can be transformed in situ by transplastomic plant DNA (genetic engineering of the chloroplast genome), in which the number of transgene copies may reach 5,000 to 10,000 (Kay et al. 2002b). Together, these results suggest that bacteria can acquire and integrate available homologous DNA and that plants infected by a pathogen could be considered a favorable environment for intraspecific, intergeneric, and even transkingdom gene transfer.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

The donor strains of *R. solanacearum* were grown at 30°C on BG medium (Boucher et al. 1985), and the recipient strains of *Acinetobacter* strain BD413 were grown on LB growth medium. Strain broth cultures were grown with vigorous shaking for at least 15 h, and plates were incubated at 30°C for 48 h before enumeration.

For conjugation experiments, the broad-host range plasmid RP4 was transferred from *E. coli* K12 to *R. solanacearum* GMI1000FB3, a gentamicin-resistant strain (Bertolla et al. 2000a). Nitrocellulose filters (Millipore SAS, San Quentin en Yvelines, France) placed on BG plate with 1×10^8 donor cells and 5×10^6 recipient cells, gave 3.3×10^4 transconjugants per ml after 48 h of incubation. The resulting *R. solanacearum* GMI1000FB3 (RP4), used as the donor strain in conjugation experiments, was grown in liquid or on solid BG medium supplemented with 10 µg of gentamicin per ml, 30 µg of kanamycin per ml, 50 µg of ampicillin per ml, and 10 µg of tetracycline per ml. *Acinetobacter* strain BD413, a nalidixic acid-resistant strain (20 µg per ml), was used as the recipient.

For the transformation experiments, a specific *R. solanacearum* donor strain and an *Acinetobacter* sp. recipient strain were constructed. The wild-type strain *R. solanacearum* GMI1000 was transformed with the recombinant plasmid pZpop1 (Bertolla et al. 2000b), which contains the *aadA* and *acc3-IV* genes conferring resistance to spectinomycin and gentamicin, respectively. Homologous recombination on *popA* sequences led to the integration of the antibiotic cassette into the *R. solanacearum* genome. The resulting strain, called *R. solanacearum* Zpop1, was cultured on BG medium supplemented with 10 µg of gentamicin per ml and 40 µg of spectinomycin per ml. The recipient *Acinetobacter* strain BD413 carries the *popA* DNA sequence from *R. solanacearum* integrated in the pMMB190 vector (Morales et al. 1991). This *popA* sequence was taken from the plasmid pFB1 (Bertolla et al. 2000b) and digested by *RsaI* and *BglII* restriction enzymes. This 1,279-bp fragment of *popA* was cloned into the vector pMMB190, which was previously digested with *SmaI* and *BamHI*. The construction of the plasmid was confirmed by restriction patterns and PCR amplification. The resulting recipient strain called *Acinetobacter* sp. strain BD413 (pPopA) is resistant to nalidixic acid (20 µg per ml) and ampicillin (50 µg per ml).

Plant material.

Wild-type and transgenic tomato seeds of *Lycopersicon esculentum* cultivar UC82B were sown in potting compost (Ets Grassot, Brignais, France), and seedlings were transferred to 10-cm pots and were grown in a greenhouse at $20 \pm 2^\circ\text{C}$. Transgenic tomato plants pZpop1 described by Bertolla and associates (2000b) contained the *aadA* and *acc3-IV* genes flanked by the *popA* DNA sequence, similar to the arrangement in the *R. solanacearum* Zpop1 genome (described below).

Infection of plants by bacterial strains was performed on 30-cm-high plants. Then, the infected plants were incubated in a growth chamber at $28 \pm 2^\circ\text{C}$ with a light regime consisting of 16 h of light and 8 h of darkness at 70% relative humidity.

Recombinant DNA techniques.

Plasmids and DNA fragments were purified with the appropriate kits (Qiagen, Mannheim, Germany). Restriction enzymes and T4 DNA ligase were obtained from Roche Diagnostics (Meylan, France). Purification of DNA and the ligation of DNA fragments were done according to standard methods (Sambrook et al. 1989). Ligation mix was used to transform *E. coli* DH10B electrocompetent cells (Gibco-BRL Life Technologies SARL, Cergy Pontoise, France) and electroporation was performed with a Bio-Rad gene pulser (Hercules, CA, U.S.A.). Introduction of recombinant plasmids into *Acinetobacter* sp. or *R. solanacearum* was achieved by natural transformation according to the protocols developed by Palmen and associates (1993) and Bertolla and associates (1997).

Filter mating.

Conjugative transfer between *E. coli*, *R. solanacearum*, and *Acinetobacter* sp. were performed on nitrocellulose filters on two different growth media such as BG medium or LB medium agar plates, favoring the growth of donor or recipient cells, respectively. Overnight cultures of strains were harvested at 7,500 rpm for 15 min, and pellets were resuspended in sterile water in order to remove growth medium and antibiotics. Cell density was adjusted to obtain an optical density at 600 nm (OD_{600}) equal to 1, which corresponds to a concentration of 1×10^9 cells per ml for *R. solanacearum*, 5×10^8 cells per ml for *E. coli*, and 5×10^7 cells per ml for *Acinetobacter* sp. (determined by CFU counts). Filters were placed on LB or BG plates with 100 μl of each bacterial suspension at $\text{OD}_{600} = 1$, and plates were incubated at 30°C for 24 h. DNase I (0.2 mg) (Roche Diagnostics) was added to filters to prevent gene transfer of pRP4 via natural transformation. Then, filters were transferred into test tubes with 2 ml of sterile water, and cells were dislodged by vigorous vortex mixing for a few seconds. The resulting suspension was serially diluted and was plated on appropriate selective media for enumeration of donors, recipients, and transconjugants.

R. solanacearum GMI1000 Zpop genomic

DNA purification.

A 15-h culture (10 ml) was centrifuged at 7,500 rpm for 15 min, and the pellet was resuspended in 10 ml of TE buffer at pH 8 (50 mM Tris and 20 mM EDTA). After 10 min of room temperature incubation, the suspension was centrifuged once again. Cell lysis was performed for 8 h by adding 10 ml of TE,

Fig. 2. In planta gene transfer via natural transformation between the donor bacterium *Ralstonia solanacearum* GMI1000Zpop1 and the recipient *Acinetobacter* strain BD413 (pPopA) concomitantly injected into the stem. Error bars indicate standard deviation of triplicate experiments.

pH 8, sarkosyl 0.5%, and proteinase K (0.3 mg per ml). Then, the standard protocol of the QIAamp tissue kit (Qiagen) was used to obtain purified DNA.

Preparation

of *R. solanacearum* GMI1000 Zpop cell lysates.

A 15-h culture of *R. solanacearum* GMI1000 Zpop was harvested at 7,500 rpm for 15 min at room temperature. The pellet was resuspended in sterile water to a final concentration of $OD_{600} = 1$ (1×10^9 cells per ml). One ml of this suspension was heat-treated at 80°C for 15 min. Heat-treated cells (100 μ l) were plated on LB medium to confirm cell mortality. Different aliquots (1 μ l, 10 μ l, or 100 μ l) of this crude cell lysate were then used for filter transformation experiments. The amount of chromosomal DNA has been roughly quantified at about 6×10^{-15} g of DNA per bacterial cell (Torsvik and Goksoyr 1978). Given this data, the DNA concentration in the cell lysate aliquots spotted on the filter ranged from 6 ng to 600 ng.

Filter transformation.

Filter transformation of the recipient *Acinetobacter* sp. strain BD413 (pPopA) was conducted with purified DNA, cell lysate, or living cells of *R. solanacearum* strain GMI1000 Zpop. Recipient cells were washed in sterile water and resuspended to a final concentration of 5×10^7 cells per ml ($OD_{600} = 1$). This recipient strain suspension (100 μ l) was mixed with 100 μ l of one of the DNA sources (purified donor DNA or cell lysate solution) and then placed on a nitrocellulose filter, which was placed on LB medium in petri plates.

When living donor cells were the source of DNA, donor cells were harvested and resuspended in sterile water before spotting on the filter. Serial dilutions of both donor and recipient cells were done to test different concentration ratios between donor and recipient cells in order to determine the highest transformation frequency. Each appropriate dilution (100 μ l) was spotted on the filter. All the experiments concerning filter transformation with living donor cells were performed on two different agar medium plates (BG and LB).

Filters were incubated for 48 h at 30°C and then were transferred into test tubes with 2 ml of sterile water, and cells were dislodged from the filter by vigorous vortexing. The resulting suspension was serially diluted and plated on appropriate selective media for enumeration of donors, recipients, and putative transformants. Control experiments were done to control purity and to determine the spontaneous mutation frequency for antibiotic resistance development of each strain. A sample of each donor and recipient strain, as well as genomic DNA solution or cell lysates of *R. solanacearum*, were spotted individually on filters. Cells or DNA were then resuspended and were plated on selective media to confirm the absence of contaminants.

Conditions of bacterial inoculation in plant stems.

Ten ml of 15-h cultures of each donor and recipient strain were harvested and resuspended in sterile water to remove growth medium and antibiotics. Each bacterial suspension was adjusted to $OD_{600} = 1$ (*R. solanacearum* strains to 1×10^9 cells per ml, and *Acinetobacter* sp. to 5×10^7 cells per ml). Inoculum was prepared by mixing an appropriate subsample of donor and recipient cell suspensions, depending on the experiment.

In conjugation experiments, 1 ml of *R. solanacearum* GMI1000FB3 (RP4) ($OD_{600} = 1$) and 1 ml *Acinetobacter* strain BD413 were mixed into a volume of 10 ml of ultrapure water. This final suspension (1 ml) was used to inoculate the stem of a tomato plant. In control experiments, 1 ml of this inoculum suspension plated on selective medium did not generate antibiotic resistant colonies, and transconjugants were always absent

from time zero samples. The possibility of gene transfer occurring on plates was tested by plating 100 μ l of each bacterial suspension together on selective media containing nalidixic acid, ampicillin, tetracycline, and kanamycin. No transformant was detected (nalidixic acid concentration in selective media was sufficient to prevent donors from transferring the plasmid after plating).

In transformation experiments, inoculum was prepared in 10 ml of sterile water containing 1 ml of *R. solanacearum* GMI1000 Zpop1 ($OD_{600} = 1$) and 1 ml *Acinetobacter* strain BD413 (popA). A second set of experiments was conducted with a more concentrated inoculum containing 1.5 ml of the diluted *R. solanacearum* GMI1000 Zpop ($OD_{600} = 1$) and 3 ml of a *Acinetobacter* strain (pPopA) cell suspension ($OD_{600} = 1$). In both cases, 1.5 ml was injected into the stem of a tomato plant. After inoculation, plants were transferred into a growth chamber maintained at 30°C to favor *R. solanacearum* development.

Bacterial growth in plant tissue necrosis.

In all experiments, growth kinetics were determined by crushing an infected plant at different times (0, 1, 2, 3, 4, 5, and 6 days). The seven plants used per experiment were infected by the same bacterial inoculum at the same time. The protocol to extract bacterial cells from plant tissues was identical for all experiments. The infected stems were removed, crushed, and homogenized in sterile distilled water with an Ultra-Turrax T25 homogenizer operating at 25,000 rpm (IKA-Werke; GmbH and Co., Staufen, Germany). Plant tissue suspensions were treated with 200 μ l of DNase I at 20 mg per ml, according to Bertolla and associates (1999), for 15 min at 37°C before plating on LB and BG medium. Then, 100 μ l of appropriate dilutions of plant tissue suspensions were plated on BG medium and LB medium with appropriate antibiotics in order to enumerate donor bacteria, recipient bacteria, and the putative transconjugant or recombinant clones depending on the experiment. To limit fungal contamination, media were supplemented with cycloheximide and amphotericin (each at 200 μ g per ml). Transconjugants were selected on LB medium supplemented with 20 μ g of nalidixic acid per ml, 30 μ g of kanamycin per ml, 50 μ g of ampicillin per ml, and 10 μ g of tetracycline per ml. In transformation experiments, putative recombinant clones of *Acinetobacter* sp. were detected by plating plant tissue suspensions on LB medium supplemented with 20 μ g of nalidixic acid per ml, 50 μ g of ampicillin per ml, 10 μ g of gentamicin per ml, and 40 μ g of spectinomycin per ml.

Characterization of clones.

PCR amplifications were performed directly on colonies picked up from selective-media plates and were resuspended in 10 μ l of sterile distilled water. Experimental conditions consisted of transferring 29 μ l of sterile water, 5 μ l of 10 $\times$ PCR buffer (Gibco-BRL Life Technologies SARL), 2.5 μ l of the 1% detergent W-1 solution (Gibco-BRL), 2 μ l of 50 mM magnesium chloride, 5 μ l of 10 mM deoxynucleoside triphosphate mix (Pharmacia Amersham, Orsay, France), and 2.5 μ l each of 10 μ M primers into a PCR tube. Then, 0.25 μ l of Taq DNA polymerase (Gibco-BRL) and 1 μ l of cell suspensions were added. Amplifications were carried out in a Perkin-Elmer 7200 thermocycler for 35 cycles, each consisting of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 1 min.

PCR were conducted with primers complementary to part of the 16S rDNA gene (pA 5'-AGAGTTTGATCCTGGCTCAG-3' and pH 5'-AAGGAGGTGATCCAGCGCCA) (Edwards et al. 1989). The 1,600-bp fragment obtained was then digested with *AluI* or *HaeIII* restriction enzymes (Gibco-BRL), and the resulting patterns compared to those of the recipient strain.

Other primers, FGPaad1172 5'-ATTCCGTGGCGTTAT-3' (Bertolla et al. 1999) and FGpacc2 5'-GCAATACGAATGGC-GAAAAG-3' (Bertolla et al. 2000b), were complementary to part of the *aadA* and *acc3-IV* genes, respectively. Amplification of the 1,600-bp DNA fragment was expected from recombinant clone DNA as template.

Detection of plasmid RP4 in transconjugants.

The presence of plasmid RP4 in the transconjugants was examined on agarose gel using the procedure of Eckhardt (1978) as modified by Wheatcroft and associates (1990). This technique separates high molecular weight plasmids on gels. The gels were stained with ethidium bromide at 0.5 µg per ml.

In addition, PCR were performed as described below with primers sets oriT1 and oriT2 and traG1 and traG2, which are specific for IncP plasmids like RP4, as described by Götz and associates (1996).

Data analysis.

Each filter experiment was done at least in triplicate, and all calculated transformation or conjugation frequencies are given as the mean value. A statistical student *t* test was performed to evaluate significance of differing DNA source used.

Plant colonization by donor and recipient strains was given as the mean value of five or six experiments. Frequencies of chromosomal recombinants or conjugative transfer were expressed per recipient CFU.

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