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Thematic Issue on Horizontal Gene Transfer

Assessment of transformability of bacteria associated with tomato and potato plants

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Transformation of plant-associated bacteria by plant DNA has never been demonstrated in agricultural fields. In total 552 bacterial isolates from stems of *Ralstonia solanacearum*-infected and healthy tomato plants and from stems and leaves of healthy potato plants were tested for natural genetic competence using plasmid pSKTG DNA and homologous DNA extracts. Control strain *Acinetobacter baylyi* ADP1 was transformable with both DNA extracts. No transformable isolates were observed after treatment with plasmid pSKTG DNA. Two isolates, P34, identified as *Pseudomonas trivialis* and A19, identified as *Pseudomonas fragi*, were selected on the basis of the consistently higher Rp-resistant CFU numbers after treatment with DNA from Rp-resistant cells than with that from wild-type cells. P34 showed 2.1-fold and A19 1.5-fold higher Rp-resistant CFU numbers after treatment with DNA from homologous Rp-resistant cells *versus* that from wild-type cells. It is concluded that bacteria capable of *in vitro* capture and integration of exogenous DNA into their genomes are relatively rare in culturable bacterial communities associated with tomato and potato plants, or that conditions conducive to transformation were not met in transformation assays.

Keywords: transformation / plant-associated bacteria / homologous recombination / bacterial endophytes

INTRODUCTION

Transformation of plant-associated bacteria by DNA originating from plant cells might occur (De Vries and Wackernagel, 2004; Nielsen et al., 2000b; Thomas and Nielsen, 2005). A factor that may spur the dissemination of inserts, *via* integration by homologous recombination, is the presence of DNA sequences in plant cell organelles of prokaryotic origin, such as chloroplast and mitochondrial genomes. The estimated potential frequency of transformation of plant-associated bacteria by DNA released from plants is extremely low, *i.e.* on the order of 10^{-13}–10^{-17} per cell (De Vries and Wackernagel, 2004; Nielsen et al., 1998). Transformation of plant-associated bacteria by plant DNA has never been demonstrated in open environments such as agricultural fields.

Transformation has been demonstrated in a wide range of other prokaryotic species exposed to environmental conditions (De Vries and Wackernagel, 2004). The state of competence leading to natural transformability is commonly related to the presence of competence genes, like the *com* genes present in *Thermus thermophilus* and *Acinetobacter baylyi* (Friedrich et al., 2001 and 2002). The naturally transformable strain *A. baylyi* ADP1 (ADP1; also known as *A. baylyi* BD413, see Vaneechoutte et al., 2006) has been demonstrated to develop genetic competence and to be transformable in soil (Ceccherini et al., 2003; Kay et al., 2003; Nielsen et al., 2000b). Transformants successfully occurred after treatment with DNA homologous to recipient genomes (Nielsen et al., 2000a). However, plant colonization is also an important factor for transformation (Kay et al., 2002; 2003). *A baylyi* ADP1 is not a natural colonist of the phytosphere, thereby making the importance of *in planta* transformation experiments with ADP1 difficult to interpret. Other species may be more important in natural transformation because they live in close association with plants. In order to fully assess the potential of these species to undergo natural transformation *in situ*, their potential for development of natural genetic competence first must be evaluated.

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The purpose of this study was to investigate a selected suite of bacteria, including plant-associated isolates from tomato plants infected by *Ralstonia solanacearum*, as well as endophytes from potato and tomato plants, for their ability to develop competence for natural transformation *in vitro*. This information will allow predictions of gene transfer potential between genetically modified plants and associated phytosphere bacteria in the field.

RESULTS

Isolation of bacteria from *R. solanacearum*-infected and healthy plants

Wilting symptoms were observed in *R. solanacearum*-infected tomato plants 7 days after inoculation, and complete wilting 24 days after inoculation. *R. solanacearum* 1609 CFU numbers in inoculated plants increased from around 10^2 g^{-1} plant tissue directly after inoculation to 10^{10} g^{-1} plant tissue after 7 days. Numbers remained stable at this level until the end of the experiment, after 24 days.

In total, 180 randomly picked isolates were obtained from wilted tomato plants. The total number of isolates tested for transformability was extended to 552 with 92 endophytic isolates obtained from stems of healthy tomato plants and 280 from healthy potato plants. Isolates were stored in the endophyte culture collection at Plant Research International, Wageningen, The Netherlands.

Screening of plant-associated bacteria for transformability using pSKTG DNA and cell lysates

No measurable OD_{600} values were detected in wells after treatment of all 552 isolates with plasmid pSKTG DNA. The control transformation mixture containing *A. baylyi* ADP1 cells with plasmid pSKTG consistently developed OD_{600} values of 0.6 and higher, verifying the experimental transformation setup. PCR amplification with cell lysates from restreaked transformant ADP1 (pSKTG) colonies with pSKTG-specific primers confirmed the presence of the expected 410 bp fragment, indicating that successful transformation with plasmid pSKTG had occurred in strain ADP1.

In total, 61 of 552 (11%) tested isolates developed higher OD_{600} values after treatment with lysate from the homologous rifampicin (Rp)-resistant cells than after treatment with lysate from the wild-type cells or Salmon sperm DNA. Plating of lysates from Rp-resistant cells did not reveal any CFU, demonstrating that culturable cells were not present in these lysates. Isolates showing higher CFU numbers after treatments with lysates from Rp-resistant cells than with those from wild-type cells were considered as putative transformants. The majority of these putative transformants originated from surface-sterilized potato stems (Tab. 1).

Table 1. Initial selection of putative transformants from tomato and potato plants.

Origin of isolates	Number of isolates tested	Number of presumptive transformants[2]
Wilted tomato stem[1]	180	3
Surface-sterilized tomato stem	92	3
Surface-sterilized potato stem	200	50
Surface-sterilized potato leaves	80	5

[1] Isolates from *R. solanacearum*-infected plants sampled 9 and 24 days after infection.

[2] Presumptive transformants were selected on the basis of a higher OD_{600} value after treatment with cell lysate from homologous Rp-resistant derivative than with that of the wild-type strain and Salmon sperm DNA.

Confirmation of putative transformable isolates using homologous genomic DNA

The number of Rp-resistant *A. baylyi* ADP1 colonies after treatment with DNA from a homologous Rp-resistant derivative was 21 000-fold higher than after treatment with DNA from the wild-type strain. This indicates that the method used for screening transformants was suitable for highly transformable strains. For 59 of the 61 putative transformants, the number of Rp-resistant CFUs obtained after treatment with DNA from homologous Rp-resistant cells was similar to the number obtained after treatment with DNA from wild-type cells. Isolate A19 demonstrated a ratio of 1.5 and P34 of 2.1 of Rp-resistant CFU numbers after treatment with DNA from homologous Rp-resistant *versus* that of wild-type cells. The presented ratios in CFU numbers were derived from a single experiment showing the highest ratio, whereas statistics were performed on four experiments. For both isolates, P34 and A19, Rp-resistant CFU numbers after treatment with DNA from homologous Rp-resistant cells were consistently higher than those after treatment with DNA from wild-type cells (Tab. 2). These results suggest that both isolates expressed low-level genetic competence under the conditions tested.

Comparison of partial 16S ribosomal RNA genes of putative transformant P34 revealed nearest matches with *Pseudomonas trivialis* and of A19 with *Pseudomonas fragi* (Tab. 2). Sequences were submitted to the EMBL database (http://www.ebi.ac.uk/), available under accession numbers AM745097, for P34 and AM745098, for A19.

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Table 2. Putative transformable endophytic isolates from potato plants.

Isolate	Identity (% similarity)	Ratio[2] (standard deviation)
A19	*Pseudomonas fragi* (99%)[1]	1.5 (0.3)
P34	*Pseudomonas trivialis* (99%)[1]	2.1 (0.8)

[1] Nearest match with 16S rRNA sequences in public database (http://www.ncbi.nlm.nih.gov). Sequences were deposited to the EMBL database (http://www.ebi.ac.uk/) and are accessible under AM745097, P34, and AM745098, A19.
[2] Ratio of Rp-resistant colonies after treatment with DNA from the homologous Rp-resistant strain *versus* that from the wild-type strain.

DISCUSSION

An initial screen of all 552 isolates using plasmid pSKTG did not reveal any instance of natural genetic competence. However, it cannot be excluded that transformation occurred during these experiments, as pSKTG may have replicated poorly or was otherwise incompatible in these isolates. Low transformation frequency was observed in a *Vibrio* sp. isolate from marine sediment using plasmid DNA (Jeffrey et al., 1990). The observed frequency of transformation was, however, too low for detection in the environment.

Treatment of all 552 isolates with DNA from homologous Rp-resistant derivatives resulted in two putative transformants. The estimated frequency of transformation for both isolates was in the range between 10^{-8} and 10^{-9} transformants per μg of DNA, slightly above the frequency of occurrence of spontaneous mutation to Rp-resistance. Successful transformation with DNA carrying small mutations also has been reported in different species (De Vries and Wackernagel, 2004; Nielsen et al., 1998; Thomas and Nielsen, 2005). Our results suggest that highly competent naturally transformable isolates like *A. balylyi* ADP1 are not commonly present in the culturable fraction of potato- and tomato-associated communities.

The apparent absence of highly transformable isolates in plant-associated communities of tomato and potato might indicate that the incidence of natural transformation of bacteria by DNA released from plants is rare. Absence of any transformant in *R. solanacearum*-treated tomato plants indicates that release of nutrients in decayed tissue of wilted plants did not increase the number of transformable species. However, the transformation experiments described in this study were performed under standardized laboratory conditions, including elevated nutrient levels and temperature. As it is unknown to what extent these conditions will induce competence for DNA uptake in the isolates, *in situ* transformation rates might actually be higher than those measured in this study. It has, for instance, been shown that *Pseudomonas fluorescens* becomes competent for natural transformation in soil but not *in vitro* (Demanèche et al., 2001). Induction of competence might be regulated under specific conditions inside plants or on plant surfaces. Potentially important triggers like carbon limitation or other stressful conditions may be required to induce competence and stimulate transformation at a higher level (Thomas and Nielsen, 2005). These tests were not included in this study, but could provide better insight into the potential for transformation of plant-associated bacterial communities by plant DNA. Transformation studies mimicking conditions present in the plant or studies in living plants, are thus important to further evaluate the possibility of transformation of plant-associated bacteria by plant DNA.

MATERIALS AND METHODS

Potato and tomato plant growth

Plant-associated bacteria were isolated from *R. solanacearum* biovar 2-infected and non-infected tomato (*Solanum lycopersicum* Mill.) plants grown under a light/temperature regime of 8 h light/26 °C and 16 h darkness/20 °C in the greenhouse. For treatment with *R. solanacearum* strain 1609, young tomato plants at the 4- to 6-leaf stage were injected with 10^3 cells. Potato (*S. tuberosum* L.) plants were grown in fields.

Isolation of bacteria from tomato and potato plants

Potato and tomato stems from 0–3 cm above soil level and leaves of *R. solanacearum*-treated tomato and non-treated potato plants were surface sterilized by successive immersion in 1.5% hypochlorite solution, 70% ethanol and sterilized tap water; 1 min for each step. The outer layers of all stem parts were then aseptically removed using a sterile scalpel, whereas surface-sterilized leaves were kept intact. Leaves and peeled stems in sterile plastic bags containing 3 mL 0.1 M phosphate buffer (pH 8.0) were homogenized by striking with a hammer.

Leaf homogenates of *R. solanacearum*-infected tomato plants were treated with an *R. solanacearum*-specific phage, θ PRI-1, in order to reduce the number of *R. solancearum* cells present in this sample. Therefore, 90 μL leaf homogenate was mixed with 10 μL θ PRI-1 (10^8 pfu.mL^{-1}) suspension and incubated for 6 h. Stem, leaf and phage θ PRI-1-treated leaf homogenates were dilution-plated onto 0.1 X TSBA (10 X diluted BD Trypticase Soy broth, Becton, Dickinson

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and Company, Sparks, USA; sucrose, 1 g.L^{-1}; technical agar no. 3, Oxoid, Basingstoke, UK, 12 g.L^{-1}; pH 7.2). Plates were incubated at 27 °C for 5 days. Colonies from leaves of *R. solanacearum*-infected plants were first tested for absence of a reaction with a *R. solanacearum*-specific antiserum by immunofluorescence colony staining (Van Vuurde and Van der Wolf, 1995). Colonies from all homogenates were selected on the basis of differences in morphology and color and were streaked to purity on the same medium. Cells from pure cultures were grown overnight in 0.1 X TSB broth at 27 °C, after which sterile glycerol was added to a final concentration of 20% for storage at −70 °C in the endophyte culture collection of Plant Research International, Wageningen.

Selection of Rp-resistant derivatives

Spontaneous Rp-resistant mutants were obtained from all isolates and *A. baylyi* strain ADP1. Cells were grown in 0.1 X TSB broth for 16 h at 27 °C until late log phase, concentrated by centrifugation at 7000× *g* and resuspended in 0.1 X TSB, reaching a final concentration of about 5×10^9 cells.mL^{-1}. Concentrated cell suspensions were plated onto 0.1 X TSBA amended with 50 μg.mL^{-1} Rp Plates were incubated for 5 days at 27 °C, after which single colonies were isolated and streaked to purity on the same medium.

Preparation of cell lysates and DNA

For preparation of lysates, pellets from 1-mL overnight-grown cell cultures were suspended in 100 μL TE buffer (Tris-Cl, 10 mM; EDTA, 1 mM; pH 8), mixed with SDS to a final concentration of 0.1%, heated to 100 °C for 10 min, and then immediately chilled on ice. The presence of living cells in the lysates obtained was checked by plating 50-μL volumes onto 0.1 X TSBA, followed by incubation at 27 °C for 7 days.

Plasmid pSKTG DNA was extracted using standard methods (Sambrook et al., 1989). Construction of plasmid pSKTG has been described in Smit and Van Elsas (1992). Shortly, plasmid pSKTG was constructed from plasmid pSUP104, carrying the origin of replication (*rep*), mobilization (*mob*) and Tc resistance genes from broad-host range plasmid RP4. Gm (*aad*B) and Km (*npt*II) resistance genes and the structural crystal protein gene from *Bacillus thuringiensis* (*cry*IVB) were cloned into pSUP104, resulting in pSKTG. Chromosomal DNA, made from cells of isolates P34, A19 and *A. baylyi* strain ADP1 and Rp-resistant homologous derivatives was prepared according to standard procedures (Ausubel et al., 1988).

Transformation

All obtained isolates were tested for transformability in non-amended 0.1 X TSB in a 96-well microtiter setup. For each isolate, four treatments were applied in duplicate: Salmon sperm DNA, lysates from wild-type cells, lysates from Rp-resistant cells and pSKTG DNA. Aliquots of 150-μL cell suspensions (10^8 CFU.mL^{-1}) were mixed with either 1 μg of Salmon sperm DNA, or lysate (approximately 1 μg DNA) of the wild-type strain or corresponding Rp-resistant cells or 1 μg pSKTG DNA. Suspensions were incubated for 3 days at 27 °C, after which 100-μL subsamples were aseptically transferred to individual wells in 24-well microtiter plates containing 200 μL 0.1 X TSB with the appropriate antibiotics: Rp for treatments with cell lysates and Salmon sperm DNA, Km and Gm for treatment with pSKTG DNA. The 24-well microtiter plates were incubated for 48 h at 27 °C, with shaking. Turbidity was measured in each well by optical density at 600 nm (OD_{600}).

Transformation in isolates P34 and A19 and *A. baylyi* strain ADP1 as control was confirmed in a membrane filter setup. Washed and concentrated cells, 5×10^9 mL^{-1}, were mixed with 1 μg DNA from wild-type or corresponding Rp-resistant cells. Mixtures then were transferred to 0.22-μm-pore-sized nitrocellulose filters (Millipore, Bedford, MA, USA), three filters per DNA extract, and filters were placed on top of a layer of 0.1 X TSBA supplemented with 20 mM of both $CaCl_2$ and $MgSO_4$ in a Petri dish. Plates with mixtures on filters were incubated for 16 h at 27 °C. Then cells were washed from the filters, concentrated and plated onto 0.1 X TSBA with 50 μg.mL^{-1} Rp, and plates were incubated for 5 days at 27 °C. This procedure was repeated four times with independent DNA extracts in order to determine consistence in the ratio of Rp-resistant CFU numbers after treatment with DNA from Rp-resistant cells *versus* wild-type cells.

PCR amplifications

PCR amplifications with pSKTG primers pSKTG-L and pSKTG-R, covering a 410 bp stretch of pSKTG (Smit and Van Elsas, 1992) were performed on DNA from Km- and Gm-resistant *A. baylyi* ADP1 colonies after treatment with pSKTG DNA. PCR amplifications with bacterial primers B968F and B1378R, covering a 410 bp stretch of 16S ribosomal RNA genes (Heuer and Smalla, 1999; Heuer et al., 1997), were performed to elucidate the taxonomical identity of selected putative transformants. PCR reactions were carried out in a PTC-100 (MJ Research Inc., Ma, USA) thermocycler according to protocols described for pSKTG DNA (Smit and Van Elsas, 1992) and 16S ribosomal RNA genes (Heuer and Smalla, 1999; Heuer et al., 1997).

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Statistical analysis

Statistical analysis was performed on the number of CFUs obtained after treatments with DNA from wild-type and corresponding Rp-resistant cells with four independent replicates for isolates A19 and P34. Probabilities were calculated using a paired two-sample Students T-test (GenStat, release 8.11, Lawes Agricultural Trust, Rothamsted Experimental Station, UK) and differences were considered to be significant at the 95% confidence level.

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