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Evaluation of possible horizontal gene transfer from transgenic plants to the soil bacterium *Acinetobacter calcoaceticus* BD413

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Abstract The use of genetically engineered crop plants has raised concerns about the transfer of their engineered DNA to indigenous microbes in soil. We have evaluated possible horizontal gene transfer from transgenic plants by natural transformation to the soil bacterium *Acinetobacter calcoaceticus* BD413. The transformation frequencies with DNA from two sources of transgenic plant DNA and different forms of plasmid DNA with an inserted kanamycin resistance gene, *nptII*, were measured. Clear effects of homology were seen on transformation frequencies, and no transformants were ever detected after using transgenic plant DNA. This implied a transformation frequency of less than 10^{-13} (transformants per recipient) under optimised conditions, which is expected to drop even further to a minimum of 10^{-16} due to soil conditions and a lowered concentration of DNA available to cells. Previous studies have shown that chromosomal DNA released to soil is only available to *A. calcoaceticus* for limited period of time and that *A. calcoaceticus* does not maintain detectable competence in soil. Taken together, these results suggest that *A. calcoaceticus* does not take up non-homologous plant DNA at appreciable frequencies under natural conditions.

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Introduction

The increasing exploitation of transgenic plants in agriculture has generated concerns over the ecological impact of engineered heterologous genes as pollutants, as these can be replicated and disseminated in the environment. The extensive use of antibiotic resistance genes as selectable markers in plants has further raised questions about the possible transfer of the resistance genes to indigenous microbes in soil. The transfer of genetic information between microbes has been widely demonstrated both in vitro and in natural systems (Fry and Day 1990; Wellington and van Elsas 1992). Of the known ways of gene transfer in bacteria, natural transformation (the uptake of naked DNA from the environment) is most likely to mediate horizontal gene transfer from plants. Natural transformation has been shown to occur in soil (Graham and Istock 1978; Lee and Stotzky 1990; Nielsen et al. 1997). It has been detected in more than 40 bacterial species, is a hereditary trait, and requires gene expression for competence development by the bacteria (Lorenz and Wackernagel 1994). Competence is usually developed in the late exponential or early stationary phase. Some bacteria preferentially take up homologous DNA of their own species based on specific interspersed sequences in their genome (*Neisseria* sp., *Haemophilus* sp.). Other bacteria, like *Bacillus subtilis* and *Acinetobacter calcoaceticus*, can take up DNA fragments independent of their sequence (Lorenz and Wackernagel 1994). As these bacteria can take up DNA aspecifically with respect to its sequence, DNA from non-homologous sources like plant DNA can be taken up in the cytoplasm of the bacteria. Most bacteria take up DNA in

a single-stranded form on which restriction enzymes are not functional. Restriction barriers can also be leaky (Bickle and Krüger 1993).

To be stably maintained in bacteria, the DNA in the cytoplasm needs to be linked to an origin of replication, such as via integration into the chromosome or into a plasmid. The integration of genes into the genome of the recipient bacteria has traditionally been regarded as being dependent on sequence homology, and the lack of homology between plant DNA and bacterial DNA has been assumed to contribute to the strong genetic barrier between these kingdoms. As outlined below, several modifications in transgenic plants challenge these requirements, and these should be given consideration when conducting risk assessment. Sequences with homology to bacterial DNA will be present in transgenic plants as the inserted DNA is cloned and maintained in vectors with flanking prokaryotic sequences. Regulatory and protein-encoding sequences prokaryotic in origin are also usually present in transgenic plants (Rissler and Mellon, anonymous 1996). Shen and Huang (1986) reported that the minimal length of homology required in *Escherichia coli* for homologous recombination is 20 base pairs, and it has been shown that short regions of homology can mediate recombination (Ikeda et al. 1982; Marvo et al. 1983) or recombination with the addition of non-homologous sequences (Duncan et al. 1978; Harris-Warrick and Lederberg 1978; Stuy and Walter, 1981). Furthermore, recombination mutants with less homology requirements (Rayssiguier et al. 1989) and illegitimate recombination events are known to occur (Ehrlich et al. 1993). If the engineered DNA is not transferred to the plant by *Agrobacterium*-mediated gene transfer, integration of DNA into the plant genome will be mediated by electroporation or particle gun systems, both of which usually use intact plasmids with an origin of replication (*oriV*). Stabilising sequences such as *oriV* inserted into the engineered plant DNA can by-pass the need for homology in the recipient bacteria by plasmid rescue (Andre et al. 1986). Thus, possible homology, plasmid rescue, illegitimate recombination events and recombination mutants can probably affect the fate of incoming plant DNA in recipient bacteria. Natural transformation can therefore in principle encompass DNA from any species, and engineered plant DNA as a source of transforming DNA in competent soil bacteria cannot be excluded.

Based on these evaluations regarding the fate of the incoming DNA in bacteria, we studied transformation patterns in *Acinetobacter calcoaceticus* using both highly homologous DNA sources and transgenic plant DNA. *A. calcoaceticus* strains with or without homology based on the *nptII* gene were used as recipients. The incubation of *A. calcoaceticus* directly on crushed transgenic plant material and on transgenic leaf material placed on selective agar plates was also investigated. This study was designed to improve the basis for risk assessment of transgenic plants with respect to the possibility for the dissemination of engineered plant genes in microbial populations.

For discussions and reviews of horizontal gene transfer see Kidwell (1993), Davies (1994), Redenbaugh et al. (1994), Syvanen (1994) and Schlüter and Potrykus (1996).

Materials and methods

Bacteria

Acinetobacter calcoaceticus BD413 Rp^R (Juni 1972; Nielsen et al. 1997), *A. calcoaceticus* BD413 Rp^R (*chrom*::*nptII*) and *A. calcoaceticus* BD413 Rp^R (pGSBNYC1) were cultured in LB medium (LB) (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 l H_2O, pH 7.5) with antibiotics as indicated: rifampicin (Rp: 50 µg/ml), kanamycin (Km: 50 µg/ml), streptomycin (Sm: 50 µg/ml) and ampicillin (Ap: 50 µg/ml). Liquid cultures were grown at 27°C (225 rpm). LB agar (LBA) plates (1.5% agar, Oxoid nr. 3), supplemented with antibiotics as indicated, were incubated at 28°C for 48 h.

Transforming DNA

Chromosomal DNA of *A. calcoaceticus* DSM586 (*chrom*::*nptII*) was isolated using the protocol of Wilson (1987). The plasmids pGSFR160 (Sm^R, Ap^R, Km^R) and pGSBNYC1 (Ap^R, Sm^R) (Plant Genetic Systems, Gent, Belgium) were isolated by the Midi plasmid isolation kit (Qiagen, Germany) from *Escherichia coli*. Linearised plasmid DNA fragments were generated by *Sac*I (Boehringer, Germany) treatment followed by ethanol precipitation. Fragments without *oriV* were generated by successive treatment with *Nde*I (New England Biolabs, USA) and *Sac*I (Boehringer) followed by Qiaex (Qiagen) purification of the fragments separated by agarose gel electrophoresis. Transgenic potato (*Solanum tuberosum*) DNA with 1–2 *nos-nptII* insertions (Bones et al. unpublished) and transgenic sugar beet (*Beta vulgaris*) DNA (PGS, Belgium) with the insertion as shown in Fig. 1 were used as transforming plant DNA. Sterile in vitro-grown potato plants and sugar beets obtained by field sampling from field trials performed by Kleinwanzlebener, Saatsucht GmbH at locations in Bavaria, Germany, were used for DNA isolation. Plant DNA was isolated as described by Draper and Scott (1988) and Tinker et al. (1993). Concentrated DNA of sugar beet was produced by *Bgl*II (Boehringer) treatment generating a 4936-bp fragment with the *nptII* insertion. The 4- to 6-kbp area of agarose gel electrophoresis-separated DNA was isolated using the Qiaex fragment isolation kit (Qiagen). The presence of the *nptII* sequence in the isolated fragments was confirmed by Southern blotting (data not shown).

Filter transformation

Transformation on the filters were done as described in Nielsen et al. (1997). Briefly, a bacterial culture (overnight) was washed and resuspended to a final density of 10^9 CFU/ml (CFU: colony forming units). To 100 µl of this suspension 10 µl DNA (1 µg/ml) was added, and the mixture was placed onto nitro-cellulose filters (Millipore, GS) on LBA plates with Rp. After incubation for 24 h at 28°C, filters were shaken in 0.85% NaCl (saline), and the suspensions were plated either concentrated, undiluted or serially diluted (tenfold) in saline onto LBA plates containing Rp and antibiotics as described (Table 1). The LBA plates were then incubated at 28°C for 48 h before enumeration of CFU. Concentrated recipient culture was plated onto LBA plates containing Rp and selective antibiotics as described for the transformant selective plates to check for spontaneous

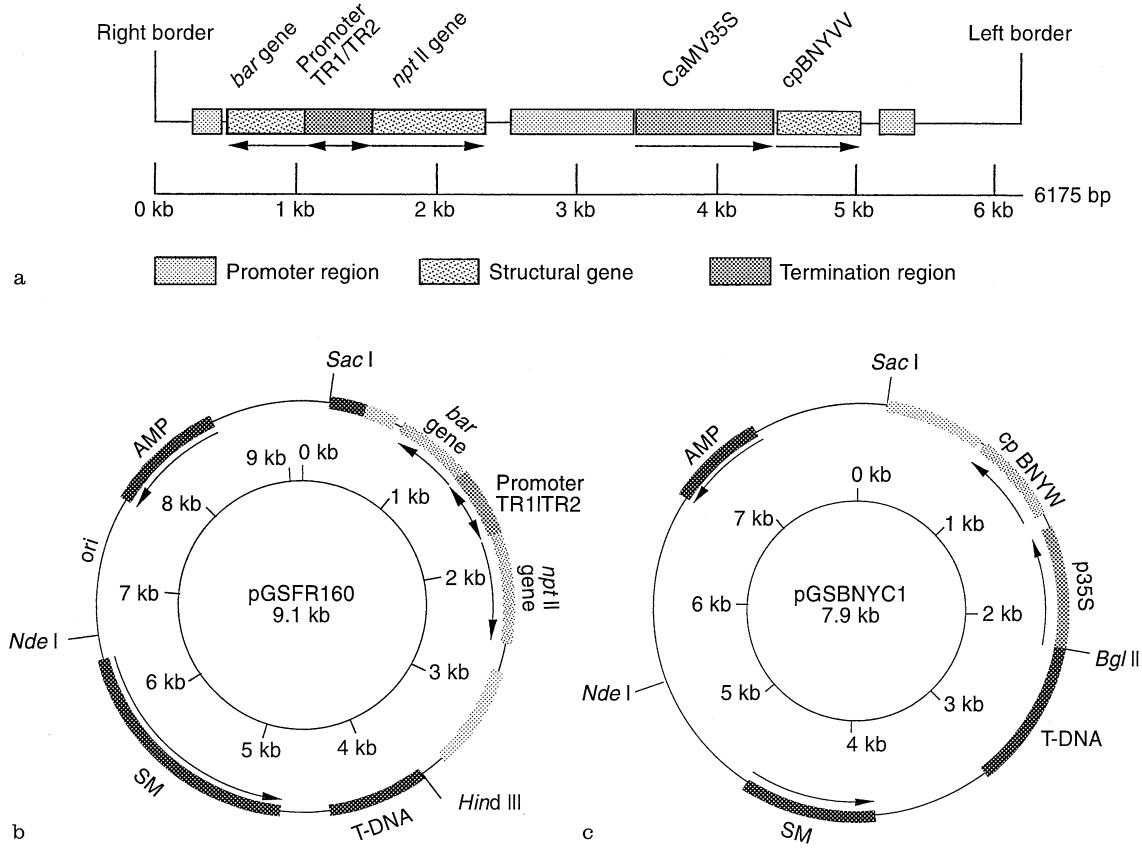

Fig. 1A–C Transforming DNA. **A** T-DNA insertion in the transgenic sugar beet, **B** plasmid pGSFR 160, **C** plasmid pGSBNYC1

Table 1 Natural transformation of *Acinetobacter calcoaceticus* BD413, BD413 (chr::*nptII*) and BD413 (pGSBNYC1) with different types of DNA

Recipient bacteria and transforming DNA	Selection (μg/ml)	Transformants (CFU/plate)[a]	Transformation frequency[b]
Recipient *A. calcoaceticus BD413*			
DNA of *A. calcoaceticus* DSM586 (chr::*nptII*)	Km^{50}	$9.7 \pm 4.4 \times 10^{7}$	7.4×10^{-3}
Plasmid pGSBNYC1	Ap^{50}, Sm^{50}	$7.5 \pm 3.2 \times 10^{4}$	1.7×10^{-5}
Plasmid pGSFR160	Km^{50}, Ap^{50}	$2.7 \pm 0.7 \times 10^{5}$	1.9×10^{-5}
Linearised pGSFR160	Km^{50}	$1.0 \pm 0.4 \times 10^{2}$	2.0×10^{-8}
Linearised pGSFR160 without *oriV*	Km^{50}	b.d[c]	$< 10^{-11}$
Transgenic plant DNA	Km^{50}	b.d	$< 10^{-11}$
Concentrated transgenic plant DNA	Km^{50}	b.d	$< 10^{-11}$
Recipient *A. calcoaceticus BD413* (*chr::nptII*)			
Plasmid pGSFR160	Sm^{50}, Ap^{50}	$7.3 \pm 1.2 \times 10^{5}$	1.5×10^{-4}
Linearised pGSFR160	Sm^{50}, Ap^{50}	$7.9 \pm 1.3 \times 10^{3}$	1.6×10^{-6}
Linearised pGSFR160 without an *oriV*	Sm^{50}, Ap^{50}	$2.0 \pm 3.2 \times 10^{0}$	4.6×10^{-10}
Recipient *A. calcoaceticus BD413* (*pGSBNYC1*)			
Transgenic plant DNA	Km^{50}	b.d	$< 10^{-11}$
Concentrated transgenic plant DNA	Km^{50}	b.d	$< 10^{-11}$

[a] Average recipient count for *A. calcoaceticus* BD413 was $1.0 \pm 4.5 \times 10^{10}$ and for BD413 (*chr::nptII*) $5.5 \pm 0.9 \times 10^{9}$. Values presented as mean ± standard deviation, both given with the same order of magnitude
[b] Calculated as transformants (CFU) divided to recipients (CFU)
[c] Below detection

mutations. The DNA used in transformation was plated onto LBA plates with Rp for a control of sterility and absence of donor cells. Controls also consisted of transformations with non-transgenic plant DNA. Heat treatment experiments were done similar to the above except that the washed bacterial suspension was incubated for 15 min at 47°C in a waterbath before use. All transformations were done in triplicate and repeated. Transformation frequencies (t/r) are given as transformant CFU per recipient CFU.

Transformation assay of whole plant material

Washed and peeled sugar beet was crushed in a sterilised Waring blender. One gram of plant material was mixed with 1–5 ml sterile distilled water with 100 μg/ml cycloheximide (Sigma) and 10^3 or 10^8 CFU of competent *A. calcoaceticus* strain BD413. The solutions were incubated with shaking at 28°C for 3, 7 and 14 days. The solutions were then plated on LBA plates with 50 μg/ml kanamycin for selection. Whole leaf material dipped in washed *A. calcoaceticus* solution was placed directly onto selective LBA plates with 50 μg/ml kanamycin. After 4 weeks of incubation resuspended plant material was plated on selective LBA plates.

Molecular analysis

The genomic localisation of the plasmid (pGSFR160) after transformation was analysed in the *A. calcoaceticus* (*chrom*::*nptII*) transformation assays. Bacterial DNA obtained from putative transformants was separated by agarose (0.7%) gel electrophoresis, blotted to Hybond N (Amersham) uncharged nylon membranes and probed with a 769-bp digoxigenin-labelled polymerase chain reaction (PCR)-generated fragment from plasmid pGSFR160 (hybridising to parts of the *TR2* promoter and *nptII* gene) as described by the manufacturer (Boehringer). For PCR amplifications, a PCR reaction mix consisting of 27 μl water, 5 μl 10 × PCR buffer II (Perkin Elmer), 5 μl 25 m*M* $MgCl_2$, 10 μl 4 m*M* dNTP mix (Perkin Elmer) and 1 μl of each 10 μ*M* primer was overlaid with mineral oil and heated at 98°C for 10 min. A 0.25-μl aliquot of AmpliTaq DNA polymerase (Perkin Elmer) was added to the tubes at 92°C. Amplification was done by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C. The primers used were selected to specifically amplify a 769-bp product by annealing to part of the *TR2* promoter and *nptII* gene. Primer sequences were 5′-GCA TTC CGT TCT TGC TGT A-3′ and 5′-GAT GTT TCG CTT GGT AAT C-3′.

Results

The transformation experiments were done with a plasmid vector containing the construct *nptII-TR1/TR2-bar* which, in contrast to the insertion in the transgenic sugar beet DNA, conferred a selectable kanamycin resistance in *E. coli* and *A. calcoaceticus*. Due to an observed altered restriction enzyme banding pattern, the *TR1/TR2* promoter (Velten et al. 1984) had undergone genetic rearrangement before expression occurred in the bacteria. The construct was, however, present as it was detected by PCR amplification with *nptII/TR2*-specific primers and DNA hybridisation. Low levels of expression of the *nptII* gene in *A. calcoaceticus* by the *nos* promoter was shown by the transformation of *A. calcoaceticus* with the plasmid pHTT27, which contained the selectable *nptII* gene under control of the *nos* promoter inserted into the plants (Teeri et al. 1989).

To evaluate the possibilities for transformation of *A. calcoaceticus* with transgenic plant DNA, we transformed *A. calcoaceticus* Rp^R with homologous bacterial DNA, plasmid DNA, linearised plasmid DNA, linearised plasmid DNA without the origin of vegetative replication (*oriV*), total transgenic plant DNA of potato or sugar beet and concentrated transgenic sugar beet DNA with the inserted *nptII* gene. Selection was based on acquired kanamycin resistance.

As seen from Table 1, the high transformation frequency obtained with homologous chromosomal DNA (7.4×10^{-3}) was reduced over a 100 fold (1.7×10^{-5}) when plasmid DNA (pGSFR160) was used as transforming DNA. The reduction in transformation frequency that we observed with plasmid DNA compared to chromosomal DNA has also been reported by Palmen et al. (1993). A further 1000-fold reduction in transformation frequency (2.0×10^{-8}) was observed when linearised plasmid instead of circularised plasmid was used as transforming DNA. This was probably due to lowered recircularisation efficiency within the bacteria. Transformation with linearised plasmid DNA without *oriV* did not give any detectable transformants nor did transgenic plant DNA from six independently transformed clones of in vitro-grown potato or field-sampled sugar beet. The detection limit was lower than 10^{-11} (t/r).

An *nptII*-tagged derivative (*chrom*::*nptII*) of *A. calcoaceticus* BD413 was used as the recipient strain to clarify if sequences of homology between the transforming DNA and the bacterial genome would increase transformation frequencies. Selection was based on acquired streptomycin and/or ampicillin resistance from the transforming plasmid vector sequences. From Table 1 it can be seen that this *nptII*-based homology improved the transformation frequencies for the plasmid tenfold, the linearised plasmid 100-fold and the linearised plasmid without *oriV* from below detection to 4.6×10^{-10}. To clarify if the enhanced transformation frequencies obtained after using the recipient with integrated *nptII* was caused by homology-based integration (of pGSFR160 or its fragments) into the bacterial chromosome, we conducted Southern blot analysis of selected transformants using a *bar* gene (Thompson et al. 1987)-specific probe. No integration could be seen when *oriV* were present on the fragments; however, the enhanced transformation frequency obtained with linearised plasmid (pGSFR160) without *oriV* in the *A. calcoaceticus* (*chrom*::*nptII*) recipient was due to integration.

In this approach no antibiotic resistance markers for selection of transgenic plant DNA were available. An alternative approach based on homology of the virus coat protein was therefore utilised as described below.

The transformation of *A. calcoaceticus* (*pGSBNYC1*) facilitating homology between pGSBNYC1 and the virus coat protein gene inserted in the transgenic sugar beet did not give any detectable transformants (Table 1). Selection here was based on the kanamycin resistance gene in the transgenic sugar beet. The detection limit was lower than 10^{-11} (t/r). We attempted to avoid any possible restriction of the plant DNA in the bacteria by applying a heat shock treatment of the recipient bacteria before transformation. This reduced the CFU counts by a factor of 10 but did not result in any detectable transformants.

Different numbers of competent *A. calcoaceticus* cells were introduced into liquid suspensions of crushed transgenic sugar beet to investigate if transformation was enhanced by incubating *A. calcoaceticus* directly with transgenic plant material. The *A. calcoaceticus* growth rate was dilution-dependent, and the highest growth rate was obtained with 10^3 bacteria in a 1:1 water/crushed plant material dilution. This approach did not result in any detectable transformants. To see if selection pressure was necessary for obtaining transformants we inoculated transgenic leaf material of sugar beet with competent *A. calcoaceticus* and placed the leafs directly on selective media. Following up to 4 weeks of incubation no transformants could be detected after selective plating. The competence of the *A. calcoaceticus* cells was not confirmed in these studies utilising intact plant cells as a substrate for their growth.

Discussion

Both bacterial DNA (Recorbet et al. 1993; Romanowski et al. 1993) and plant DNA (Paget and Simonet 1994; Smalla et al. 1994; Smalla 1995; Nielsen et al. unpublished) have been shown to persist in soil over time. Mineral particles in soil can stabilise free DNA (Gallori et al. 1994; Ogram et al. 1994). Released DNA from decaying plants will probably contribute to the transient reservoir of genetic information available to microbial populations in soil.

Possible horizontal gene transfer of plant DNA to bacteria in soil can be evaluated by three different approaches: (1) a long-term retrospective approach with a comparison of sequences from characterised bacterial and plant genes (Smith et al. 1992); (2) a short-term retrospective approach based on initial phenotypic screening of putative transformants from field trials/soils with transgenic plants harbouring selectable marker genes (Becker et al. 1994; Smalla et al. 1994); (3) an experimental approach based on optimised laboratory conditions for gene transfer into a culturable soil bacterium. To our knowledge, only horizontal gene transfer from plants to bacteria by the transformation (natural/artificial) of culturable soil bacteria has been investigated (Broer et al. 1996; Schlüter et al. 1995).

We have chosen the latter approach and selected the Gram-negative soil bacterium *A. calcoaceticus* strain BD413. It has been shown to be naturally transformable in vitro, in water, in soil extract and in soil microcosms (Juni and Janik 1969; Bruns et al. 1992; Lorenz et al. 1992; Palmen et al. 1993; Williams et al. 1996; Nielsen et al. 1997). The bacterium is transformable with both chromosomal and plasmid DNA and, most importantly, it takes up both homologous and heterologous DNA (Chamier et al. 1993; Palmen et al. 1993). Competence development by *A. calcoaceticus* has been well-described (Cruze et al. 1979; Chamier et al. 1993; Palmen et al. 1993). In previous work, we have optimised the transformation conditions for strain BD413 reaching transformation frequencies of up to 10^{-2} (t/r) with homologous chromosomal DNA (Nielsen et al. 1997). In this study we have stepwise exchanged the homologous DNA to transgenic plant DNA and measured how this affected on the transformation frequencies. The lack of transformants of *A. calcoaceticus* Rp^R after transformation with linearised plasmid without *oriV* or transgenic plant DNA indicates that homology, or a stabilising sequence like an origin of replication, is needed for stable maintenance, as integration into the chromosome was not observed. This observation is relevant to the question of possible integration of plant DNA-harboured antibiotic resistance genes in bacteria as it demonstrated that even after transformation with high numbers of the (non-homologous) marker gene *nptII*, no expression of the gene was evident in the bacteria.

To demonstrate homology requirements for obtaining detectable transformation frequencies, an *nptII*-tagged derivative of *A. calcoaceticus* was used as a recipient strain. The introduced sequence homology enhanced transformation frequencies significantly, between 10 and 1000-fold, for all plasmid-derived constructs. Enhanced transformation frequencies of plasmid DNA with homologous sequences to chromosomal DNA of the recipient has also been observed for *Bacillus subtilis* (Canosi et al. 1981). A possible role in stabilising plasmid intermediates was suggested. Our results support this observation and suggest that homology is indeed needed for the detectable integration of DNA into the bacterial chromosome or for enhanced stabilisation of extra-chromosomal elements. Due to a lack of selection markers no attempt was made to transform plant DNA into this *nptII*-tagged recipient. Another strategy was developed to utilise possible homology between plant DNA and bacterial DNA, but despite the virus coat protein-based homology between *A. calcoaceticus* (pGSBNYC1) and transgenic sugar beet DNA, no transformants were detected in these experiments. This observation suggested that the number of transformants was below detection, or that *A. calcoaceticus* failed to express the integrated kanamycin resistance gene. Natural transformation in *A. calcoaceticus* is effectively blocked by competing DNA (Palmen et al. 1993; Nielsen and Gebhard unpublished results), and a reduced transformation frequency due to the inhibitory effect of the excess plant DNA will lower the frequency below detection limit. Transformations with concentrated transgenic sugar beet DNA containing the *nptII* gene was tried in an attempt to reduce the effect of competing DNA. No transformants were detected in these studies either, suggesting that the potential frequency was below the detection limit of 10^{-11} or that the adequate experimental conditions were not met.

As the present transformations were conducted under optimised in vitro conditions, a significant decrease in the transformation frequencies is to be expected when soil conditions are applied. Our results suggest that chromosomal DNA from plants is practically unavailable for transformation of *A. calcoaceticus* under optimised laboratory conditions. Taken in conjunction with data on competence and DNA availability in soil (Nielsen et al. 1997), we conclude that *A. calcoaceticus* is not likely to be stably transformed in soil at detectable frequencies by the non-homologous plant DNA-inserted antibiotic resistance genes used. If homology is present, horizontal gene transfer could be expected at extremely low frequencies. From a risk assessment point of view, the frequency is, however, marginally important compared to the potential strength of selection acting on the transformants. Selection pressure in soil is unknown for such events or of the genes transferred. Kanamycin has not been found to be a strong selector in soil (Oliveira et al. 1995).

Other studies on possible gene transfer from plants to bacteria have focused on plant pathogenic bacteria. Schlüter and co-workers (1995) investigated the transformation of *Erwinia chrysanthemi* with transgenic potato DNA harbouring a selectable ampicillin resistance gene and *oriV*. Inoculation of the pathogenic bacteria directly on tuber tissue facilitated plant cell lysis, with released DNA enhancing the possible uptake of DNA by bacteria. Neither of these approaches resulted in any detectable transformants, and the potential transformation frequency was calculated to be below 10^{-17} (t/r) under natural conditions. Broer et al. (1996) used *Agrobacterium tumefaciens* as a recipient for T-DNA-transformed plant DNA, exploiting the advantages of homology based on the Ti-plasmid and the integrated T-DNA. Naturally selective conditions for *A. tumefaciens* in the induced plant tumours were used. This study also did not give any detectable transformants and the frequency was found to be below 6×10^{-12}.

So far, horizontal gene transfer from transgenic plants to naturally occurring bacteria has not been proven under laboratory or field conditions. The only reported experimentally determined horizontal gene transfer from plants to unrelated organisms has been from plants to plant-associated fungi. Uptake of host DNA has been claimed for *Plasmodiophora brassicae* (Bryngelsson et al. 1988; Buhariwalla and Mithen 1995) and *Aspergillus niger* (Hoffmann et al. 1994). A stable integration of this DNA into the genome of the fungi has not been shown. Horizontal gene transfer in soil microbial communities is, therefore, as judged from today's knowledge, mainly based on interspecies transfer of plasmid (Van Elsas et al. 1988; Smit et al. 1991) chromosomal DNA (Singh et al. 1987; Stewart and Sinigallino 1990) and possibly conjugative transposons (Salyers et al. 1995). To our awareness no experimental evidence exists today demonstrating horizontal gene transfer from transgenic plants to naturally occurring soil bacteria.

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